RESEARCH PAPER

Novel Analytical Method for Simultaneous quantification of Letrozole and Clomiphene citrate in combined therapy

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ABSTRACT
A robust, selective, and efficient analytical approach was established for the simultaneous quantification of Letrozole and Clomiphene Citrate in a laboratory-prepared synthetic mixture using UV-spectrophotometry and RP-HPLC techniques. Methanol was employed as the common solvent throughout the analysis. The UV method utilized first-order derivative spectroscopy, enabling precise measurement at 222 nm for Letrozole and 315 nm for Clomiphene Citrate, with corresponding zero-crossing points at 315 nm and 222 nm. Both methods exhibited excellent linearity over concentration ranges of 2.5-12.5 µg/mL for Letrozole and 50-250 µg/mL for Clomiphene Citrate, with correlation coefficients approaching unity. Assay results demonstrated high accuracy for both techniques. Complementarily, an isocratic RP-HPLC method was optimized using a Kromstar C_{18} column (250 × 4.6 mm, 5 µm) with a mobile phase comprising acetonitrile and phosphate buffer (pH 3.2) in a 60:40 (% v/v) ratio. Detection was carried out at 239 nm, achieving well-resolved peaks with retention times of 4.0 min for Letrozole and 6.5 min for Clomiphene Citrate at a flow rate of 1 mL/min. Both analytical strategies were rigorously validated in accordance with ICH Q2 (R2) guideline, confirming their reliability in terms of linearity, precision, accuracy, sensitivity, and reproducibility. The developed methods are therefore suitable for routine quality control analysis of these drugs in bulk and synthetic blend.

KEYWORDS: Letrozole (LETRO); Clomiphene Citrate (CLOMI); First Order Derivative UV spectrophotometry, Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

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1. INTRODUCTION

The combination of Letrozole and Clomiphene Citrate for ovulation induction in women with Polycystic Ovary Syndrome (PCOS) has gained considerable attention due to its superior efficacy compared to monotherapy. This combination therapy has been reported to yield higher ovulation and pregnancy rates than either drug used alone [1-7]. The enhanced therapeutic outcome is attributed to a synergistic effect, wherein both agents act through complementary mechanisms to stimulate the hypothalamic–pituitary–ovarian axis more effectively [2, 3, 7].

Letrozole, chemically known as 4-[(4-cyanophenyl) (1H-1,2,4-triazol-1-yl) methyl] benzonitrile (Figure 1 A), is a non-steroidal type II aromatase inhibitor. It inhibits the aromatase enzyme (CYP19A1) by blocking its active site and interfering with the electron transfer chain, thereby preventing the conversion of androgens into oestrogens. The resulting decrease in circulating estrogen levels reduces negative feedback on the hypothalamus and pituitary gland, leading to increased secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which promotes ovulation [8, 9].

Clomiphene Citrate, chemically described as 2-[4-(2-chloro-1,2-diphenylethenyl) phenoxy]-N, N-diethylethanamine; 2-hydroxypropane-1,2,3-tricarboxylic acid (Figure 1 B), is a selective estrogen receptor modulator (SERM). It exerts its pharmacological action by binding to estrogen receptors, primarily in the hypothalamus, thereby blocking estrogen-mediated negative feedback. This stimulates the release of gonadotropins (FSH and LH), which in turn induce follicular development and ovulation [10, 11].

The synergistic effect of the Letrozole-Clomiphene combination arises from their dual and complementary mechanisms: letrozole reduces estrogen synthesis, while clomiphene blocks estrogen receptor signaling. This dual action amplifies endogenous gonadotropin release more effectively than either agent alone, resulting in improved follicular recruitment and

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ovulation rates. Clinical studies, including phase IV trial, have demonstrated that this combination is both safe and more effective, particularly in patients resistant to single-drug therapy [1-7].

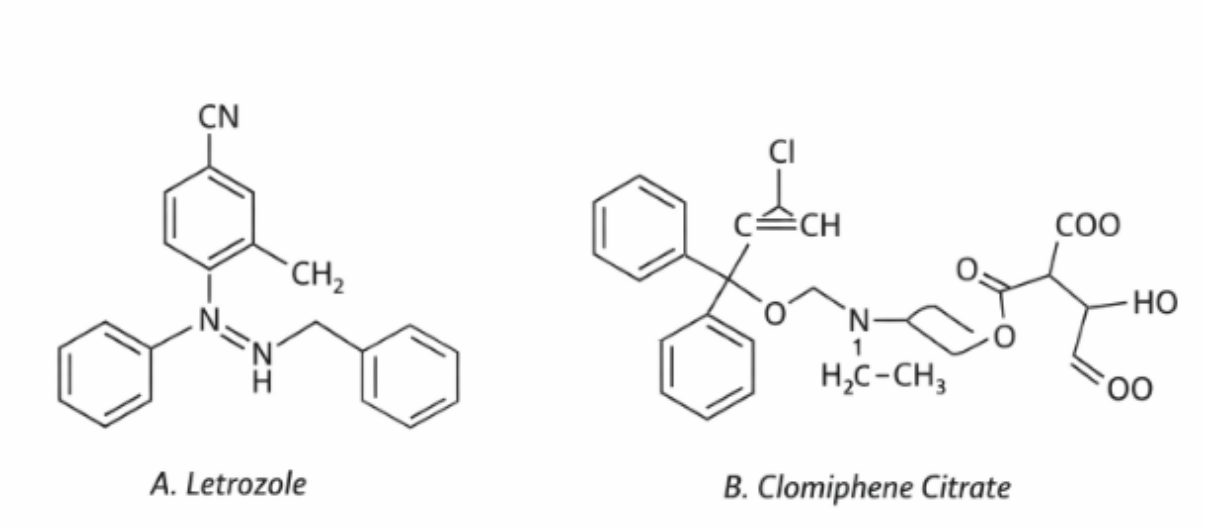

Figure 1: Chemical Structure: (A) Letrozole and (B) Clomiphene Citrate

Letrozole is official in the Indian Pharmacopoeia (IP) [9], and several analytical methods have been reported, including UV spectrophotometric methods for bulk and dosage forms [12], high-performance liquid chromatography (HPLC) methods for pharmaceutical formulations [13, 14], and stability-indicating ultra-fast LC methods [15]. Clomiphene Citrate is official in major pharmacopoeias, including the British Pharmacopoeia (BP) [16], Indian Pharmacopoeia (IP) [9], United States Pharmacopeia (USP) [17], and European Pharmacopoeia (EP) [18], primarily employing liquid chromatographic methods. Reported analytical techniques include UV spectrophotometry [19, 20], RP-HPLC [21, 22], LC–MS/MS [23], and other chromatographic methods [24]. A comprehensive literature survey reveals that no analytical method has been reported for the simultaneous estimation of Letrozole and Clomiphene Citrate in a synthetic mixture. Therefore, this combination was selected for method development.

The objective of the present study is to develop and validate simple, accurate, precise, and sensitive analytical methods, including first-order derivative UV spectrophotometry and RP-HPLC, for the simultaneous estimation of Letrozole and Clomiphene Citrate in a synthetic mixture. The proposed methods were validated in accordance with ICH Q2 (R2) guideline [25], covering all essential validation parameters such as specificity, linearity, accuracy, precision, LOD, LOQ, Assay and robustness.

2. EXPERIMENTAL MATERIALS AND ANALYTICAL CONDITIONS

All chemicals and reagents used in the present study were HPLC grade and analytical grade to ensure accuracy and reproducibility of the results. Acetonitrile, methanol, and water (HPLC grade) were procured from Finar Chemicals Pvt. Ltd., India, used for the preparation of mobile phases, diluents, and standard solutions. Ortho phosphoric acid of analytical reagent (AR) grade, obtained from Astron Chemical India, was employed for pH adjustment of the phosphate buffer. Letrozole and Clomiphene Citrate used as the reference standard, was kindly supplied by Yarrow Chem Products., Mumbai.

2.1 Instruments & Software

The spectrophotometric measurements were performed using a UV-Visible spectrophotometer (Shimadzu-1900, UV Probe 2.7 version software) with a spectral bandwidth of 1 nm was employed for all spectroscopic measurements, using a pair of 1.0 cm matched quartz cells over the range of 200-400 nm. For chromatographic information acquisition and analysis, High-Performance Liquid Chromatography Systronic RP-HPLC (LC-20-AD) (SPD-20 A) Instrument [Clarify] with UV Detector was utilized together. The Scale-Tec analytical balance was utilized to weigh the samples. The HPLC mobile phase was subjected to sonication using an Sonicator- Digital Pro^{+}, PS-10A, (Broleo).

2.2 Analytical conditions

In accordance with ICH Q2 (R2) requirements [18], the analytical conditions for a simultaneous technique for the measurement of Letrozole and Clomiphene Citrate in UV and HPLC were optimized and validated. For UV Spectroscopy Methanol was used as a Solvent. Detection wavelength (λmax) of LETRO and CLOMI were 240 nm and 240 nm, 296 nm, respectively. The first-order derivative UV spectra were derived from the zero-order spectra using methanol as the solvent. Quantitative analysis was performed at the zero-crossing point (ZCP) of Letrozole at 315 nm for the estimation of Clomiphene Citrate, and at the ZCP of Clomiphene Citrate at 222 nm for the estimation of

Letrozole. For RP-HPLC, Kromstar C_{18} (250 × 4.6 mm, 5 µm) was used in the procedure. The mobile phase consisted of acetonitrile and phosphate buffer (pH 3.2) in a 60:40 (% v/v) ratio at 239 nm wavelength was selected for RP-HPLC, with 1 mL/min flow rate.

2.3 Preparation of Solutions

2.3.1 Preparation of Stock Solution

Accurately weighed 10 mg of Letrozole was transferred into a 100 ml volumetric flask, dissolved with Methanol and diluted to the mark to obtain a standard stock solution (100 µg/ml). Accurately weighed 100 mg of Clomiphene Citrate was transferred into a 100 ml volumetric flask, dissolved with Methanol and diluted to the mark to obtain a standard stock solution (1000 µg/ml).

2.3.2 Preparation standard solution

Pipetted out 0.5 ml solution from stock solution of Letrozole (100 µg/ml) and 1 ml Clomiphene Citrate (1000 µg/ml) into different 10 ml volumetric flask and diluted up to mark with Methanol to get the 5 µg/ml of Letrozole and 100 µg/ml Clomiphene Citrate. Each solution was scanned in the range of 200-400 nm.

2.3.3 Preparation of standard working solution

To produce concentration ranges of 2.5-12.5 µg/mL of LETRO and 50-250 µg/mL of CLOMI, From each stock solution, LETRO (0.25, 0.5, 0.75, 1 and 1.25 ml) and CLOMI (0.5, 1, 1.5, 2.0 and 2.5 ml) were pipetted out in ten different 10 ml volumetric flasks and made up to mark with Methanol to obtained 2.5,5,7.5,10 and 12.5 µg/mL of CLOMI and 50, 100, 150, 200 and 250 µg/mL for CLOMI, respectively. Under the optimized spectrophotometric conditions, the samples were analyzed using a 1 cm quartz cuvette in the UV spectrophotometer. Similarly, the optimized chromatographic conditions, 20 µL of each standard working solution was injected into the RP-HPLC system.

3. METHODOLOGY

3.1 Method I: UV-spectrophotometric method

First Order Derivative Method was selected for simultaneous estimation of Letrozole and Clomiphene Citrate in Synthetic Mixture. Each working standard solution was scanned individually over the wavelength range of 200-400 nm. In zero order UV spectra, Letrozole exhibited an absorption maximum at 240 nm (Figure 2), close to the reported value of 240 nm, while Clomiphene Citrate showed an absorption maximum at 240 nm, 296 nm (Figure 2) close to the reported value of 235 nm 292 nm. LETRO and CLOMI standard stock solutions were prepared in Methanol at concentrations of 100 µg/mL and 1000 µg/mL, respectively. A small amount of each stock solution was taken and placed into 10 mL volumetric flasks. Methanol was used to adjust the volumes to the mark, resulting in final concentrations of LETRO ranging from 2.5 to 12.5 µg/mL and CLOMI ranging from 50 to 250 µg/mL. All zero-order absorption UV spectra were converted to first-order derivative UV spectra. Calibration functions were established by plotting first-order derivative absorbance against corresponding concentrations for each analyte. Appropriate volume, 0.50 mL of Letrozole and 1.0 ml Clomiphene Citrate standard stock solution was transferred to two separate 10 mL volumetric flasks and the volume was adjusted to mark with methanol to get concentration 5.0 and 100 µg/mL, respectively. The solutions were scanned separately in the UV-region i.e., 400-200 nm. The zero-order UV absorption spectra of LETRO and CLOMI in Methanol shown in Figure 2. The zero-order spectrum was processed to obtain first-derivative spectrum. The two first derivative spectra were overlaid which shows that Letrozole showed zero crossing at 315 nm, while Clomiphene Citrate showed zero crossing at 222 nm which showed in Figure 3. The determinations were made at 222 nm for Letrozole (ZCP of Clomiphene Citrate) and 315 nm for Clomiphene Citrate (ZCP of Letrozole). The first order overlay for linearity UV spectra of Letrozole and Clomiphene Citrate are presented in Figure 4 and 5, respectively.

3.2 Method II: Reverse Phase High Performance Liquid Chromatography Method:

For RP-HPLC, the analysis was carried out using an isocratic elution technique using a mobile phase comprised of different mobile phases such as Acetonitrile: ACN: Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid) (60:40 % v/v) at a flow rate of 1 mL/min found better separation of both the drug peaks. Prior to usage, the solvents were filtered through a 0.45 µm filter and sonicated for 30 min. The stationary phase was a Kromstar C_{18} (250 mm × 4.6 mm, 5 µm), and the eluent was observed by a U.V Detector from 200 to 400 nm, alongside chromatograms extracted at 239 nm. The calibration curves were prepared by measuring the peak areas of LETRO and CLOMI and plotted their values against the pertinent concentrations. In accordance, the equations for linear regression were calculated.

3.3 Method Validation

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), *ICH Q2(R2): Validation of Analytical Procedures* [25] established standards for the validation of the analytical procedures utilized in this investigation.

3.3.1 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

3.3.2 Linearity and Range: (n=6)

The linearity of Letrozole and Clomiphene Citrate was

found to be in the range of 2.50-12.50 µg/mL and 50-250 µg/mL, respectively. Plot the calibration curve of peak area vs. concentration (µg/mL). Linearity of both the drugs were checked in term of slope, intercept and correlation coefficient.

***3.3.3 Precision**:*
The Intraday and Interday precisions also referred to as repeatability and intermediate accuracy, respectively were used to assess the precision of Methods I and II. The experiment was conducted on the same day and for the next three days for both Intraday and Interday precision, analysing freshly made solutions at concentrations of 2.50, 5.0, and 7.50 µg/mL of LETRO and 50, 100, and 150 µg/mL of CLOMI. To assess intermediate precision, the mean absorbance (UV) and peak area (HPLC) were recorded for each set of experiments. For repeatability, 5.0 µg/mL of LETRO and 100 µg/mL of CLOMI were used. The results were represented as a percentage Relative Standard Deviation (RSD), with a value of less than two considered acceptable. This meticulous approach ensures a comprehensive evaluation of the precision of the analytical methods, providing confidence in the reliability and consistency of the results obtained for the concentrations of LETRO & CLOMI in the tested solutions.

3.3.4 Limit of Detection (LOD):
Limit of detection can be calculated using following equation as per ICH guidelines.

$$\mathrm{LOD} = 3.3 * \frac{\sigma}{S}$$

Where, σ = standard deviation of the calibration curve
S = slope of the calibration curve

3.3.5 Limit of Quantification (LOQ):
Limit of quantification can be calculated using following equation using the standard deviation of the Y-intercept (σ) and the mean slope (S) of the calibration curve according to ICH Q2 (R2) guideline.

$$\mathrm{LOQ} = 10 * \frac{\sigma}{S}$$

3.3.6 Accuracy (Recovery study) (n=3):
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50 %, 100 %, 150 % and the values were measured for Letrozole (5.0 µg/mL) and Clomiphene Citrate (100 µg/mL). This performance was done in triplicate. The accuracy of the method was determined by calculating recovery of Letrozole and Clomiphene Citrate by the standard addition method.

3.3.7 Assay as analysis of Synthetic Mixture
The synthetic mixture of Letrozole and Clomiphene Citrate was prepared in the ratio of 2.50:50. A synthetic mixture equivalent to 100 mg was prepared by accurately weighing MCC (Micro Crystalline Cellulose) (2.37 mg), Lactose Monohydrate (35.65 mg), Polyvinyl Pyrrolidine (7.9 mg), Talc (0.79 mg) and Magnesium Stearate (0.79 mg) were added in the motor pestle along with the drug Letrozole (2.5 mg) and Clomiphene Citrate (50 mg). were used as excipients. All the components were transferred into a mortar and blended thoroughly using a pestle to obtain a homogeneous synthetic mixture. This mixture was transferred in 100 ml volumetric flask and allowed to sonicate and made up to mark with Methanol. This solution was filtered through Whatmann filter paper. The filtrate was diluted to the mark with Methanol. The mixture contains 25 µg/ml of Letrozole and 500 µg/ml of Clomiphene Citrate.

***3.3.7.1 Preparation of sample solution**:* Accurately 1 ml from the above solution [mixture of Letrozole (25 µg/mL) and Clomiphene Citrate (500 µg/mL)] was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Water. Final concentration of Letrozole was 5.0 µg/mL and Clomiphene Citrate 100 µg/mL then analyzed using the previously described UV-spectrophotometric and chromatographic conditions. The concentrations of LETRO and CLOMI were calculated using a regression equation.

3.3.8 Robustness
The robustness of analytical methods becomes evaluated to decide their ability to face up to minor variations in approach situations. For the HPLC technique, samples have been subjected to evaluation below changed situations, which include adjustments inside the flow rate (± 0.1 mL/min), detection wavelength (± 2 nm), and natural content material (± 2 %) inside the mobile segment. The resulting results on machine suitability parameters have been intently monitored. In the times of Methods I and II, distinct analysts conducted sample analyses to evaluate the robustness of the strategies.

3.3.9 System Suitability Tests
A system suitability test is an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The test includes the Resolution, Column efficiency, Tailing factor and Theoretical plates.

4. RESULTS AND DISCUSSION

4.1 Method I: UV Method

In pharmaceutical analysis, the simultaneous estimation of multiple components using UV spectroscopy is a widely utilized method. Various techniques, including the Simultaneous Equation, Derivative Spectrophotometric approach and the absorbance ratio method, are employed for this purpose. The simultaneous estimation using UV visible spectroscopy offers several advantages, including ease of use, cost-effectiveness, and minimal time and labor

requirements. These attributes make UV visible spectroscopic methods particularly valuable in pharmaceutical research and quality control, allowing for efficient and economical simultaneous determination of multiple components in a given sample.

4.1.1 Selection of wavelength for Letrozole and Clomiphene Citrate

The remarkable absorbance of Letrozole exhibited an absorption maximum at 240 nm (Figure 2), while Clomiphene Citrate showed an absorption maximum at 240 nm and 296 nm (Figure 2). The zero-order UV absorption spectra of Letrozole (5.0 µg/mL) and Clomiphene Citrate (100 µg/mL) in Methanol was showed in Figure 2.

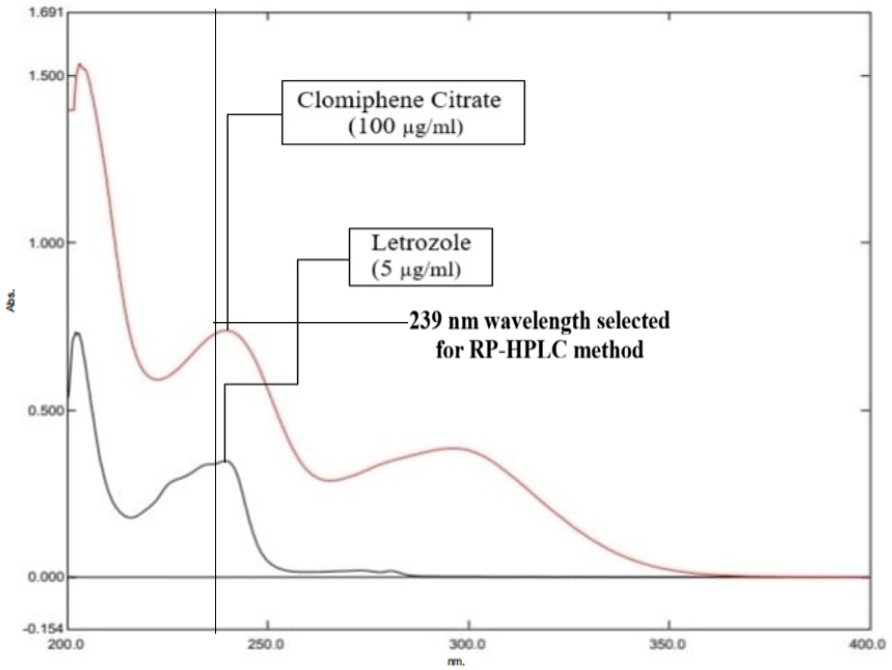

Figure 2: Overlain UV Spectra of Letrozole (5 µg/ml) and Clomiphene Citrate (100 µg/ml) in Methanol (Zero order)

4.1.2 First order derivative UV Method Development

The LETRO and CLOMI overlapping absorption throughout the 200 - 400 nm range is shown by these spectra, which makes it more difficult to quantify the pharmaceuticals using traditional UV spectrophotometry without accounting for the overlap. The sum of the absorbances of the two compounds may be used to calculate the overall absorbance of a solution containing a combination of both at a certain wavelength. In situations where the levels of the two medicinal drugs overlap, the method entails figuring out the quantity of each drug using their zero-order spectra. The resulting absorbance spectra were derived to eliminate the interference of absorbing species. The first derivative corresponding to each absorption spectrum of each drug was recorded, using $\Delta\lambda = 2$ nm and scaling factor 4. The amplitude values were measured at 222 nm ($\lambda 1$) (ZCP of CLOMI) for LETRO and 315 ($\lambda 2$) (ZCP of LETRO) for CLOMI showed in Figure 3.

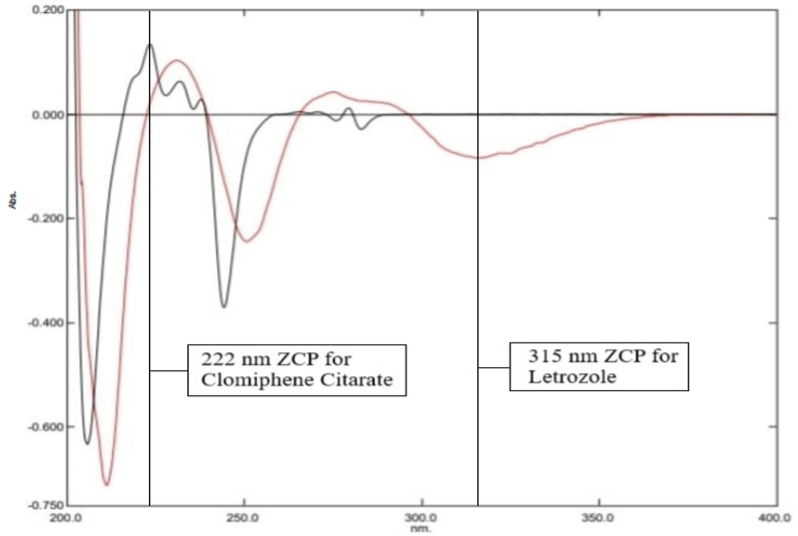

Figure 3: Overlain UV Spectra of Letrozole (5 µg/ml) and Clomiphene Citrate (100 µg/ml) in Methanol (First order)

4.2 Method II: RP-HPLC Method

Pharmaceutical analysis commonly uses simultaneous estimation using RP-HPLC. It enables the use of RP-HPLC to determine the presence of many chemicals in a sample. For the simultaneous estimate of various components, including medications and their contaminants, in pharmaceutical formulations, a number of techniques have been devised and proven effective. Utilizing an appropriate column, mobile phase, and detection equipment, the simultaneous estimation technique by HPLC allows for the separation and quantification of the target substances. In pharmaceutical analysis, Reverse Phase high-performance liquid chromatography (RP-HPLC) is a great instrument for simultaneous estimation that offers confidence and specificity for the identification of chemical entities in Synthetic Mixture.

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also performs better in terms of efficiency, stability and reproducibility. C_{18} (250 mm × 4.6 mm, 5 µm) column was selected because it is a C_{18} stationary phase having the least polarity compared to C_4 and C_8 columns, which provides better retention and separation efficiency for compounds with varying polarity. The C_{18} column allows faster elution of relatively polar compounds compared to non-polar compounds under optimized mobile phase conditions. Additionally, a UV detector was employed, as it enables simple and effective detection of analytes in UV-transparent organic solvents. Therefore, the C_{18} (250 mm × 4.6 mm, 5 µm) column was selected for the efficient separation of Letrozole and Clomiphene Citrate. Isocratic mode was chosen due to its simplicity, reproducibility, and robustness, as well as its suitability for maintaining column stability during longer analytical runs.

4.2.1 Selection of detection wavelength

The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 239 nm both drugs give good peak height and shape. So, 239 nm was selected for simultaneous estimation of Letrozole and Clomiphene Citrate in synthetic mixture.

Overlain UV Spectra of Letrozole (5.0 µg/mL) and Clomiphene Citrate (100 µg/mL) in Methanol has been shown in Figure 2.

4.2.2 RP-HPLC Method Development

Liquid chromatography coupled with UV detection was used to develop a way for simultaneously measuring LETRO and CLOMI. Achieving acceptable peak symmetry and theoretical plates within a realistic time period was the aim. The chromatographic conditions were optimized by experimenting with various stationary and mobile phases. The Kromstar C_{18} (250 × 4.6 mm, 5 µm) column had the best separation with symmetric peaks and the shortest retention period among the reversed phase C_8 and C_{18} columns that were studied. A combination of ACN: Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid) (60:40 % v/v) was the ideal mobile phase. Mixtures of Acetonitrile: Phosphate buffer were also attempted; however, the outcome was asymmetric peaks and a prolonged retention period of LETRO & CLOMI where LETRO eluted first at 4 min and CLOMI at 6.5 min. The RP-HPLC system formed satisfactory system suitability parameters such as [tailing factor was less than 2, Column efficiency was more than 2000, resolution was more than 2].

4.3 VALIDATION OF THE PROPOSED METHODS

4.3.1 Validation Parameters of the UV Method:

4.3.1.1 Linearity and range:

For LETRO and CLOMI, the absorbances ranged from 2.5- 12.50 µg/mL at 222 nm and 50-250 µg/mL at 315 nm showed in Figure 4 and 5, respectively.

Table 1: Linearity and LOD/LOQ data by UV and RP-HPLC Method

Parameters	UV Spectrophotometry		RP-HPLC	
	LETRO at 222 nm	**CLOMI at 315 nm**	**LETRO at 239 nm**	**CLOMI at 239 nm**
Linearity Range	2.50 - 12.50 µg/mL	50 to 250 µg/Ml	2.50 - 12.50 µg/mL	50 to 250 µg/mL
Regression Equation	y = 0.0182x + 0.0109	y = 0.0007x + 0.0137	y = 151.13x + 185.02	y = 12.942x + 67.838
Correlation Coefficient	0.9968	0.9999	0.9986	0.9996
LOD	0.12	2.79	0.14	2.07
LOQ	0.37	8.47	0.44	6.27

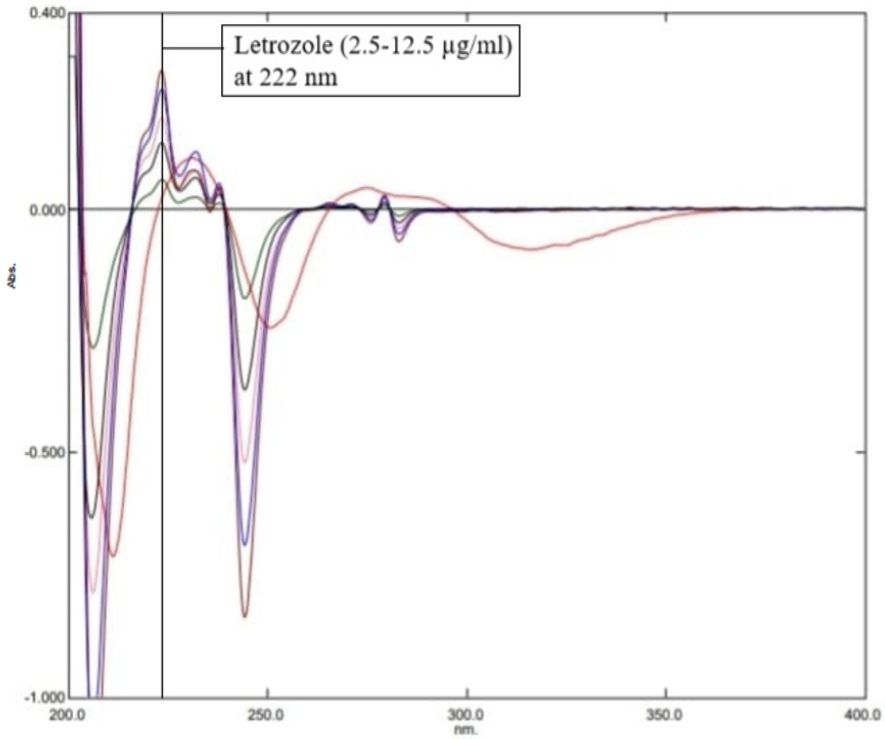

Figure 4: Overlain UV Spectra of Letrozole (2.5-12.5 µg/ml) at 222 nm

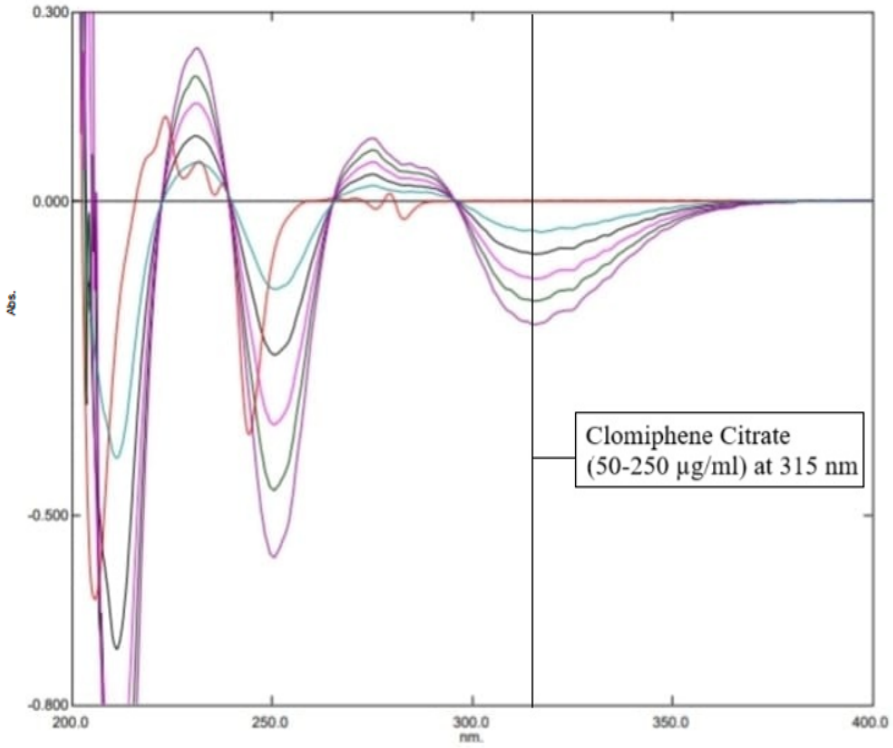

Figure 5: Overlain UV Spectra of Clomiphene Citrate (50-250 µg/ml) at 315 nm

A linear relationship was found and calibration curve was plotted for concentration vs. absorbance. For LETRO, the calibration curve equation y = 0.0182x + 0.0109, while for CLOMI, it was y = 0.0007x + 0.0137. Results showed that the correlation coefficient (R^2) was between 0.9968 and 0.9999 (Table 1).

4.3.1.2 Precision

In terms of precision, both Inter-day, Intraday and Repeatability measurements were conducted at three distinct concentrations 2.5, 5.0 & 7.5 µg/mL for LETRO and 50, 100 & 150 µg/mL for CLOMI in triplicate over three consecutive days and on the same day. The absorbance of the same solutions was measured. For repeatability, 5.0 µg/mL for LETRO and 100 µg/mL for CLOMI were measured. The resulting RSD values for Intraday, Inter-day precision, and Repeatability were showed in Table 2, respectively.

Table 2: Precision study of Letrozole & Clomiphene Citrate for UV Method

Intraday precision					
Conc. (µg/mL)		**Mean Absorbance ±SD (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
2.5	50	|0.055| ± 0.00070	|-0.049| ± 0.00059	1.27	1.20
5.0	100	|0.099| ± 0.00095	|-0.085| ± 0.00078	0.96	0.91
7.5	150	|0.152| ± 0.00125	|-0.120| ± 0.00099	0.82	0.83
Interday precision					
Conc. (µg/mL)		**Mean Absorbance ±SD (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
2.5	50	|0.055| ± 0.00068	|-0.049| ± 0.00058	1.23	1.18
5.0	100	|0.099| ± 0.00097	|-0.085| ± 0.00082	0.98	0.96
7.5	150	|0.152| ± 0.00118	|-0.120| ± 0.00102	0.77	0.85
Repeatability					
Conc. (µg/mL)		**Mean Absorbance ±SD (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
5.0	100	|0.099|±0.00094	|-0.085| ± 0.00078	0.95	0.92

4.3.1.3 LOD and LOQ

The minimum detectable quantity of an analyte within a sample by an analytical method was determined to be 0.12 µg/mL for LETRO at 222 nm and 2.79 µg/mL for

CLOMI at 315 nm, The quantitation limit for a specific analytical method refers to the minimum quantity of the substance in a sample that can be accurately and precisely measured which was found to be 0.37 µg/mL for LETRO at 222 nm and 8.47 µg/mL for CLOMI at 315 (Table 1). The low LOD and LOQ values obtained at the selected wavelengths indicated the adequate sensitivity of the proposed UV spectrophotometric method for the estimation of both drugs.

4.3.1.4 Accuracy

To decide the accuracy of the technique recuperation, change into accomplished by means of standard addition approach. To pre-analysed pattern acknowledged quantity of general LETRO and CLOMI spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of LETRO and CLOMI. Accuracy was carried out by the Recovery Studies (standard addition method). The results were stipulated in triplicate and the accuracy indicated by % recovery. For UV, The % Recovery was obtained in range of 99.73% - 99.82% for Letrozole and 99.96% - 99.99% for Clomiphene Citrate were showed in Table 3.

Table 3: Recovery study data for UV and RP-HPLC Method

UV Method						
Name of Drug	**% Level of recovery**	**Test Amount (µg/mL)**	**Amount of drug taken (µg/mL)**	**Total Std Amt (µg/mL)**	**Total amount Recovered (µg/mL)**	**% Mean Recovery ± SD(n=3)**
Letrozole	50	5	2.5	7.5	7.4	99.82±0.0170
	100	5	5	10	9.9	99.77±0.0249
	150	5	7.5	13.5	13.4	99.73±0.0525
Clomiphene Citrate	50	100	50	150	149	99.99±0.0287
	100	100	100	200	199	99.97±0.7642
	150	100	150	250	249	99.96±0.8784
RP-HPLC Method						
Letrozole	50	5	2.5	7.5	7.489	99.85±0.125
	100	5	5	10	9.99	99.90±0.246
	150	5	7.5	13.5	13.49	99.93±0.357
Clomiphene Citrate	50	100	50	150	149.94	99.96±0.268
	100	100	100	200	199.9	99.95±0.374
	150	100	150	250	249.98	99.99±0.588

4.3.1.5 Assay as Analysis of Synthetic mixture

From assay, Final concentration of Letrozole was 5.0 µg/mL and Clomiphene Citrate 100 µg/mL were run into UV and The Percentage assay of Letrozole and Clomiphene Citrate were found to be 99.80 % and 99.98 %, respectively. Its results are shown in Table 4.

Table 4: Assay as Analysis of synthetic mixture for UV and RP-HPLC Method

UV Method				
Name of Drug	**Amount in synthetic mixture (µg/mL)**	**Mean Amount found (µg/mL)**	**% Assay ± SD (n=3)**	**%RSD**
Letrozole	5	4.99	99.80±0.28	0.28
Clomiphene Citrate	100	99.98	99.98±0.14	0.14
RP-HPLC Method				
Letrozole	5	4.997	99.94±0.17	0.17
Clomiphene Citrate	100	99.99	99.99±0.09	0.09

4.3.2 Validation Parameters of the RP-HPLC Method:

4.3.2.1 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Letrozole and Clomiphene Citrate.

***4.3.2.2 Linearity*:**

The RP-HPLC chromatogram of Letrozole (2.5-12.5 µg/mL) and Clomiphene Citrate (50-250 µg/mL) at 239 nm. The Linearity was performed using Peak Area was found. Calibration graphs were plotted between

concentrations and peak areas. The regression equation of calibration curve was generated y = 151.13x + 185.02 for LETRO and y = 12.942x + 67.838 for CLOMI. The correlation coefficient (R^2) values were observed to be 0.9986 and 0.9996. (Table 1).

4.3.2.3 Precision

A concentration of 2.5, 5.0 & 7.5 µg/mL for LETRO and 50, 100 & 150 µg/mL for CLOMI. At same day three-time interval, the absorbance of the finished solution was measured in a 1.0 cm cell at a chosen wavelength. Likewise, on the first, second, and third days, the peak area of the same solutions was measured. Every solution is made in triplicate and examined. The resulting RSD values for Inter-day and Intraday precision were showed in table 5, respectively.

Table 5: Precision study for Letrozole & Clomiphene Citrate for RP-HPLC

Intraday precision					
Conc. (µg/ml)		**Mean peak area (mAu*sec) ± S.D (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
2.5	50	592.623±6.0827	685.146±7.7676	1.03	1.13
5.0	100	920.449±8.5782	1372.600±12.4398	0.93	0.91
7.5	150	1293.233±10.6926	2014.100±16.4620	0.83	0.82
Interday precision					
Conc. (µg/ml)		**Mean peak area (mAu*sec) ± S.D (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
2.5	50	583.259±6.4110	684.812±8.0445	1.10	1.17
5.0	100	920.279±8.8662	1373.267±13.2035	0.96	0.96
7.5	150	1292.500±10.9672	2014.600±17.3277	0.85	0.86
Repeatability					
Conc. (µg/ml)		**Mean peak area (mAu*sec) ± S.D (n=3)**		**%RSD**	
LETRO	**CLOMI**	**LETRO**	**CLOMI**	**LETRO**	**CLOMI**
5.0	100	921.531±8.7484	1373.183± 12.5794	0.95	0.92

4.3.2.4 Accuracy:

The accuracy of the technique recuperation was decided change into accomplished by means of standard addition approach. To pre-analysed pattern acknowledged quantity of general LETRO and CLOMI spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of fashionable LETRO and CLOMI. The results were studied in triplicate and the accuracy changed into indicated by% recovery (Table 3). Accuracy was carried out by the Recovery Studies. For HPLC, The % Recovery was obtained in range of 99.85%-99.93% for Letrozole and 99.96%-99.99% for Clomiphene Citrate were showed in Table 3. The mean percentage recovery values for both drugs were found to be within the ICH-accepted range of 98-102%, with low standard deviation. These results confirm the accuracy, trueness, and reliability of the RP-HPLC method and indicated that excipients present in the synthetic mixture did not interfere with the estimation of either drug.

4.3.2.5 LOD and LOQ

LOD Values were found to be 0.14 and 2.07 µg/mL for Letrozole and Clomiphene Citrate, respectively. LOQ Values were found to be 0.44 and 6.27 µg/mL, respectively for Letrozole and for Clomiphene Citrate. These results showed in Table 1.

4.3.2.6 Assay:

From assay, Final concentration of Letrozole was 5.0 µg/mL and Clomiphene Citrate 100 µg/mL were injected into HPLC System and The Percentage assay of Letrozole and Clomiphene Citrate were found to be 99.94% and 99.99%, respectively. Results showed in Table 4.

4.3.2.7 Robustness:

Chromatographic analysis was used to analyse the effects of changes in analysts, and the results showed that there was no statistically significant difference in the% RSD of technique II. Additionally, small changes were performed to assess the robustness of the created HPLC procedures. The approaches' robustness was demonstrated by the% RSD, which remained constant despite minor variations in flow rate, run time, and detection. It was determined that the created approaches were essential as a result.

5. CONCLUSION

The UV spectrophotometry and RP-HPLC methods were linear, precise, accurate, and validated in compliance with ICH Q2 (R2) guideline. The RP-HPLC and UV spectrophotometric technique have been developed and validated for routine measurement of LETRO and CLOMI in laboratory-prepared synthetic mixtures. These techniques are easy to use, fast, accurate, and precise. Without interfering, all two methods may determine LETRO and CLOMI concurrently in multi-component. Overall, the validated first-order derivative UV-spectrophotometric and RP-HPLC method were simple, precise, accurate, sensitive, and cost-effective, making it suitable for routine quality control analysis of LETRO and CLOMI in synthetic mixtures. The established protocols should be followed for routine and quality control drug analysis in pharmaceutical compositions with two

components. The low standard deviation and percentage RSD values of the suggested techniques make them appropriate for regular LETRO and CLOMI measurements in laboratory-prepared synthetic mixtures. The statistical results indicated that both methods were equally sensitive, reliable, and can routinely apply for simultaneous estimation of Letrozole and Clomiphene Citrate in Synthetic Mixture.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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