International Journal of Pharma Research and Technology

Development and Validation of UV-Spectrophotometric and RP-UHPLC Method for the Determination of Clomiphene Citrate in Bulk Drug and Tablet Dosage Form

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Abstract

In this study, we explore the potential of clomiphene citrate, a selective estrogen receptor modulator (SERM) and ovulatory stimulant, by developing UV-Spectrophotometric and RP-UHPLC methods. These innovative techniques offer precise and reliable means of determining clomiphene citrate levels in both bulk drug and tablet formulations. UV-Spectrophotometric method was developed employing Analytical Technology Limited and to achieve exceptional chromatographic separation of components, we employed an advanced Agilent tech. gradient system equipped with an auto-injector and DAD detector. A Reverse Phase C18 (100 mm x 4.6mm; 2.5µm) column served as the stationary phase, while a unique mobile phase consisting of methanol and 0.1% OPA water (50:50% v/v) further enhanced the efficiency. In adherence to the rigorous guidelines of the International Conference on Harmonization (ICH), we thoroughly validated both the UV and RP-UHPLC methods. The UV-Spectrophotometric analysis of clomiphene citrate reveals an astonishing maximum absorbance at 234nm, utilizing water: methanol (90:10) solvent blend. The entire process of RP-UHPC with a flow rate of 1.0ml/min meticulously monitored the absorbance at 234nm, successfully eluting clomiphene citrate at an astonishingly rapid rate of 2.9 minutes. The validation parameters, including linearity, accuracy, precision, system suitability, detection limit, quantitation limit, robustness, and ruggedness, all demonstrated exceptional performance, well within the acceptance limits. For the UV method, a remarkable linearity range emerged, spanning concentrations from 5-25µg/ml, boasting an impressive R^2 value of 0.9998. Meanwhile, the RP-UHPLC method exhibited a linearity range from 2.5-12.5µg/ml, showcasing an astounding R^2 value of 0.9991.This developed method revolutionizes the determination and quantification of clomiphene citrate in bulk drug and tablet formulations. The remarkable precision and reliability of our UV-Spectrophotometric and RP-UHPLC methods promise to elevate the understanding and application of clomiphene citrate to unprecedented heights.

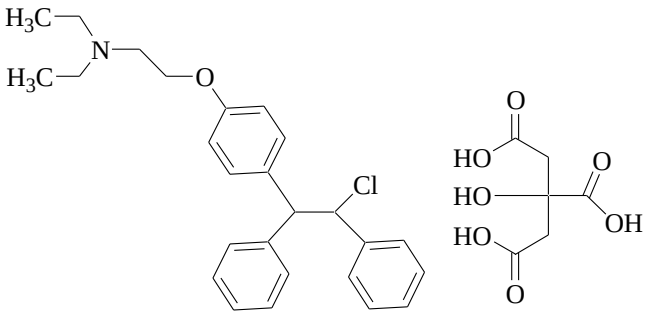
Keywords: UV-Spectrophotometric, RP-UHPLC, Clomiphene citrate, Tablet

Introduction

In the hallowed halls of the Merell Chemistry Department back in 1956, a group of visionary minds led by Frank Palopoli stumbled upon a transformative chemical. Little did they know that their discovery would change the course of medical history forever. Fast forward to 1965, and a momentous event occurred when a New Drug Application (NDA) for this groundbreaking compound was filed. After a rigorous process, in 1967, the green light was given, and Clomiphene citrate was officially approved, becoming a beacon of hope for countless individuals struggling with infertility. With the arrival of Clomiphene citrate on the scene of clinical treatment, the landscape of infertility therapies underwent a profound metamorphosis. A revolution was ignited, particularly in the treatment of polycystic ovarian syndrome (PCOS), offering a newfound ray of optimism to those who had been grappling with the challenges of this condition.[1]

Clomiphene, a selective estrogens receptor modulator (SERM) acts to induce ovulation by selectively interacting with estrogen receptors in the cervix, endometrium, ovary, and brain. This interaction results in both estrogenic and antiestrogenic effects. Moreover, Clomiphene suppresses estrogenic negative feedback and raises gonadotropin levels in the hypothalamus, producing a partial estrogens like effect. Simultaneously, it increases blood testosterone levels and stimulates the release of follicle-stimulating hormone and luteinizing hormone. As an adjuvant, it helps reduce pituitary suppression. During the follicular stage of the ovarian cycle, Clomiphene administration leads to an escalation in LH pulse frequency, indicating its primary function is to promote the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. In summary, clomiphene's multifaceted actions make it an effective agent for stimulating ovulation.[2, 3, 4]

Clomiphene citrate depicted in Fig. 1, known in IUPAC nomenclature as 2-(4-(chloro-1, 2-diphenylethenyl)-phenoxy)-N, N-diethylethanamine, 2-hydroxy-1, 2, 3-propanetricarboxylate, is an artificial powder medication with a white to pale-yellow appearance and no discernible odor. It belongs to the stilbene class and has a chemical formula of $C_{32}H_{36}ClNO_8$, with a molecular weight of 598.09 g/mol. commonly, it is prescribed in the form of 50-mg tablets[5, 6]



Clomiphene Citrate

Figure No.1: Chemical structure of Clomiphene citrate

The literature review underscores the scarcity of UV and HPLC methods available for evaluating Clomiphene citrate. Existing methods either involve

complex analyses in biological matrices or are time-consuming due to combinations with other drugs.[7, 8, 9, 10] As a result, a dedicated endeavour has been undertaken to develop and validate UV and RP-UHPLC methods that offer several advantages: speed, cost-effectiveness, accuracy, precision, and ease of use for assessing Clomiphene citrate independently. The developed method underwent comprehensive evaluation for its linearity, accuracy, precision, system suitability, robustness, and ruggedness, adhering to the ICH guideline Q2 (R1) standards. This demonstrates a high-quality impact by addressing the limitations of previous methods and presenting an efficient and reliable approach for Clomiphene citrate determination.[11]

Material and Method

Chemicals: Clomiphene citrate, in its pure form, was generously provided as a gift sample by Swapnroop Drugs and Pharmaceuticals, located in Aurangabad, India. In the UV-Spectroscopic analysis, HPLC grade water and methanol were utilized as solvents, while for the RP-UHPLC analysis, HPLC grade methanol, 0.1% acetic acid (AA), and 0.1% orthophosphoric acid (OPA) were used. Additionally, a marketed formulation of Clomiphene citrate (FERTOMID-100, Cipla Pharma Ltd.) was procured from a local pharmacist for comparison and reference purposes.

Instruments: The UV-Spectrophotometer used for spectrum recording and absorbance measurements was the Systronic-2201 model, equipped with a Deuterium lamp as the light source. Data processing was facilitated by a computer connected to the spectrophotometer. A Quartz cuvette with a path length of 1 cm was utilized in the experiments. For HPLC analysis, an Agilent Technologies Gradient System with an Auto injector was employed, along with a Reverse phase C18 column (4.6 id x 100mm; 2.5µm particle size), DAD detector, G1310A is pump, and a 20µL injection loop. Additional equipment used included the WENSAR™ High Resolution Balance for weighing purposes and an Ultrasonic electronic instrument i.e. Sonicator for specific applications.

A. UV Method Development and Validation

Selection of solvent

Clomiphene citrate demonstrated good solubility in methanol and moderate solubility in water. To establish and validate a high-quality UV-Spectrophotometric method for clomiphene citrate, a solvent mixture of water and methanol (90:10) was chosen.

Preparation of standard stock solution

10 mg of Clomiphene citrate was carefully weighed and then transferred into 100ml volumetric flask and volume was made up to the mark with water and methanol (90:10) and sonicated 15 min to obtain strength of 100 µg/ml. (flask A). Next, a 2.5 ml aliquot was withdrawn from flask A and added to a 10 ml volumetric flask. The volume was adjusted to the mark again, using the same water and methanol mixture (90:10) (flask B) to obtain a solution of Clomiphene citrate (25µg/ ml).

Preparation of sample solution

Tablet equivalent to 10mg of Clomiphene citrate (13.6mg) was taken in a 100ml volumetric flask and volume made up to the mark with water and methanol (90:10) to obtained 100µg/ml of solution (flask C). An aliquot of 1.5ml from flask C was

transferred into a 10 ml volumetric flask and volume made up to the mark with water and methanol (90:10) to obtained 15µg/ml of solution (flask D).

Determination of Absorption maxima

The clomiphene citrate standard solution, with a concentration of 25µg/ml, underwent a thorough scanning process within the wavelength range of 200 to 400nm. A reference solution of 10ml water was used for comparison during the scan. Notably, the UV spectrum displayed a distinct peak with the highest absorption observed at 234nm (λ_{max}).

Validation

Linearity - A range of concentrations for clomiphene citrate standard solution was prepared by transferring 0.5, 1.0, 1.5, 2.0, and 2.5 ml from flask A into separate 10 ml volumetric flasks. Each flask was then filled up to the mark with a mixture of water and methanol in a ratio of 90:10, resulting in a series of solutions with concentrations ranging from 5-25µg/ml. The absorbance of each solution was measured at 234 nm. A calibration curve was constructed, plotting Concentration against Absorbance, and the correlation coefficient was calculated.

Accuracy - To determine the accuracy, recovery studies were conducted using the spiking method. The sample tablet solution was mixed with standard drug solution at three different levels: 80%, 100%, and 120% concentrations. This was achieved by adding 0.8, 1.0, and 1.2 ml from flask A (the standard drug solution) to 1.0 ml of the sample tablet solution from flask C in a 10ml volumetric flask. The mixture was then made up to the mark with the water and methanol mixture (90:10). The percentage of recovery was subsequently calculated.

Precision - Precision of the method was evaluated through intraday and interday precision assessments, with three different concentrations (10µg/ml, 15µg/ml, 20µg/ml) of clomiphene citrate solutions. These concentrations were prepared by transferring 1.0ml, 1.5ml, and 2.0ml of a standard solution from flask A into separate 10ml volumetric flasks. The volumes were then adjusted to the mark using a water and methanol mixture (90:10). For intraday precision, the prepared solutions were analyzed twice on the same day, while for interday precision; they were analyzed twice on consecutive days. The relative standard deviation (%RSD) was calculated to assess precision.

Repeatability (system suitability) - To determine the repeatability of the method (system suitability), five replicate analyses of clomiphene citrate at a concentration of 20µg/ml were performed. This concentration was prepared by transferring 2.0ml of the standard solution from flask A into a 10ml volumetric flask and adjusting the volume to the mark with a water and methanol mixture (90:10). The obtained results from these replicate analyses were then reported.

Ruggedness–The ruggedness of the method was evaluated by analyzing clomiphene citrate at a concentration of 20 µg/ml. This was achieved by transferring 2.0 ml of the standard solution from flask A into separate 10 ml volumetric flasks. The volumes in each flask were then adjusted to the mark using a water and methanol mixture in a 90:10 ratio. The analysis was conducted by two different analysts, using identical operational and environmental conditions.

Analysis of clomiphene citrate tablet -To determine the Clomiphene citrate content

in a tablet dosage form, the developed and validated method was applied. A solution from flask D was utilized for the analysis of the commercial tablet formulation. The assay was performed, and subsequently, the % label claim was calculated.

B. RP-UHPLC Method Development and Validation

Solubility Studies

Several solvents were experimented with to assess the solubility of clomiphene citrate. The findings from these solubility studies indicated that clomiphene citrate exhibits good solubility in methanol and moderate solubility in water therefore adjusted with 0.1% Orthophosphoric Acid, Buffer pH 2.7 for better separation.

Preparation of Standard Stock Solution

A precise amount of 10 mg of Clomiphene citrate was carefully measured and placed into a 20 ml volumetric flask, labelled as "Flask E." To dissolve the substance and achieve a concentration of 500µg/ml, methanol was added to the flask up to the mark, and the solution was then subjected to sonication. Subsequently, the solution was effectively filtered using a 0.45µ membrane filter to ensure its purity and clarity.

Preparation of sample solution

Tablet equivalent to 10mg of Clomiphene citrate (13.6mg) was carefully taken in a 20 ml volumetric flask and volume was made up to the mark with methanol to obtained 500µg/ml of solution and labelled as flask F. From flask F a precise 0.15ml aliquot was transferred into a 10ml volumetric flask, the volume was then adjusted to the mark using a mobile phase composed of methanol and 0.1% OPA water (50:50) to obtained 7.5µg/ml of solution and labelled as flask G.

Selection of stationary phase:

The stationary phase selected for this method is the Agilent RP C18 column. The column has dimensions of 100 mm x 4.6 mm and a particle size of 2.5 µm. The C18 column is specifically chosen for its exceptional characteristics, including high non-polar retention, symmetric peak shapes, and remarkable reproducibility and stability. These outstanding qualities make it an ideal choice for the RP-UHPLC method, ensuring reliable and consistent results.

Selection of Mobile phase

The standard solution containing clomiphene citrate was tested using different solvents, as well as combinations of solvents, in an attempt to achieve effective separation and a stable peak. Different combinations of methanol and water were experimented with as the mobile phase. Each mobile phase underwent filtration through a 0.45µm membrane filter and was then degassed by sonicating for 20 minutes. The mobile phase was allowed to equilibrate until a steady baseline was achieved. After experimenting with various combinations, the optimal mobile phase was found to be Methanol: 0.1% OPA Water (50:50). This particular combination resulted in well-defined, fully separated peaks with desirable symmetry and significant retention times for the drug.

Selection of Analytical Wavelength

The effectiveness of the HPLC method relies on choosing the appropriate detection wavelength. The most suitable wavelength is the one that yields a strong signal for the specific drug being analysed. In this case, a wavelength of 234 nm was chosen for the analysis, as it resulted in substantial absorbance for the drug.

Optimized Chromatographic Parameters

The chromatographic separation was performed using an Agilent RP C18 Column with dimensions of 100mm x 4.6mm and a particle size of 2.5µm, while the mobile phase consisted of a mixture of methanol and 0.1% OPA water in a 50:50% v/v ratio. The flow rate of the mobile phase was set at 1.0ml/min, and the detection of compounds occurred at a wavelength of 234nm. For each analysis, a 20µl injection volume was used, and the column temperature was kept at ambient conditions. The total run time for each separation was 8 minutes.

Validation

Linearity -Five different volumes 0.05, 0.1, 0.15, 0.2, and 0.25 ml of clomiphene citrate standard solution from flask E were individually transferred into separate 10 ml volumetric flasks. Each flask was then filled up to the mark using a mobile phase, methanol and 0.1% OPA water (50:50). This process resulted in a series of solutions with concentrations ranging from 2.5-12.5 µg/ml. The absorbances of these solutions were measured at a wavelength of 234 nm. A calibration curve was constructed by plotting Concentration versus Area, and the correlation coefficient was subsequently determined.

Accuracy - Recovery studies were conducted using the spiking method, which involved adding standard drug solutions to the sample tablet solution at three different levels: 80%, 100%, and 120%. Specifically, 0.04, 0.05, and 0.06 ml of standard drug solution from flask E were added to 0.05 ml of the sample tablet solution (with a concentration of 2.5 µg/ml) from flask F in a 10ml volumetric flask. The volume was then made up to the mark using a mobile phase, methanol and 0.1% OPA water (50:50). Subsequently, the percentage recovery was calculated based on these measurements.

Precision - Precision of the method was evaluated by assessing both intraday and interday precision. This evaluation was performed at three different concentrations 5µg/ml, 7.5µg/ml and 10µg/ml. To prepare these solutions 0.1 ml, 0.15 ml, and 0.2 ml of a standard solution from flask E were transferred to three separate 10 ml volumetric flasks and then volume was made up to the mark with a mobile phase methanol and 0.1% OPA (50:50).For intraday precision, the prepared solutions were analysed twice on the same day. For interday precision, the solutions were analysed two times on consecutive days. The percent relative standard deviation (%RSD) was then calculated based on the obtained data.

Repeatability (system suitability) - The repeatability of method (system suitability) was evaluated through two repeated analyses of clomiphene citrate at a concentration of 10 µg/ml. This was obtained by transferring 0.2 ml of the standard solution from flask E to a 10 ml volumetric flask, and the volume was then adjusted to the mark using a mobile phase methanol and 0.1% OPA water (50:50). The obtained data was used to calculate various parameters, including the number of theoretical plates, peak symmetry, and tailing factor.

Robustness - The robustness of the method was evaluated by analysing clomiphene citrate at a concentration of 10 µg/ml. This was obtained by taking 0.2 ml of the standard solution from flask E and transferring it to a 10ml volumetric flask and the volume was then filled up to the mark with the mobile phase methanol and

0.1% OPA water (50:50). To deliberately challenge the method, certain modifications were made: the mobile phase composition was altered by ±1%v/v, the flow rate was varied by ±1ml/min, and the wavelength was changed by ±1 nm from the initially optimized chromatographic conditions.

Ruggedness – Ruggedness of the method was evaluated by analysing clomiphene citrate at concentration of 10 µg/ml prepared by transferring 0.2 ml of standard solution from flask E to a 10 ml volumetric flasks and volumes was made up to the mark with mobile phase methanol and 0.1%OPA water (50:50). The analysis was performed under identical operational and environmental conditions, but by two different analysts.

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

The LOD and LOQ were calculated as:

$LOD=3.3\sigma/S$

$LOD=10\sigma/S$

Where,

σ is the standard deviation of response and

S is the slope of the linearity plot.

Analysis of clomiphene citrate tablet - The developed and validated method was applied for the determination of clomiphene citrate in tablet dosage form. For analysis of commercial tablet formulation solution from flask G was taken and assay was performed. Then % label claim was calculated.

Result

A. UV Method Development and Validation –

The Clomiphene citrate Standard solution (5µg/ml) was subjected to UV scanning from 200 to 400nm, and its UV Spectrum was recorded. The analysis of absorption spectra shows the maximum absorption with a prominent peak at 234 nm (Fig. 2).

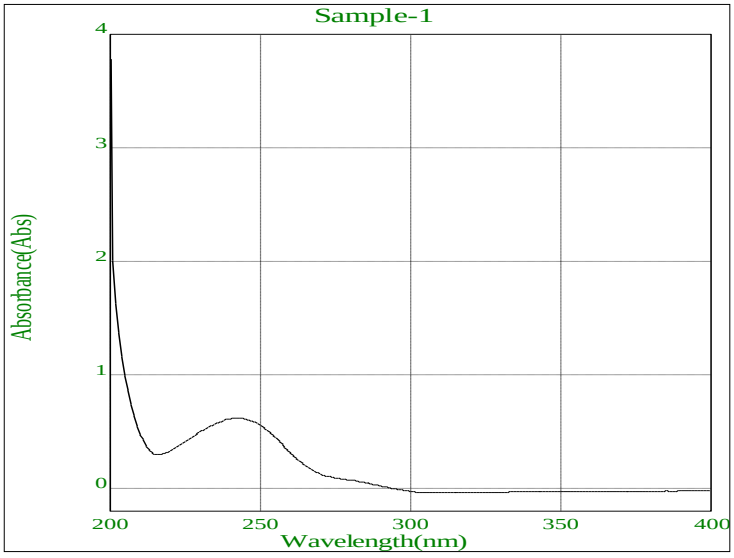


Figure No. 2 - UV Spectrum of clomiphene citrate

UV Method Validation

Linearity -The analytical calibration curve is linear in the 5–25µg/ml range and shows that the correlation coefficient (R^2) is nearly equal to 1 i.e. 0.9998. The linear regression equation is y = 0.0207x + 0.0061. (Fig. 3, Table 1)

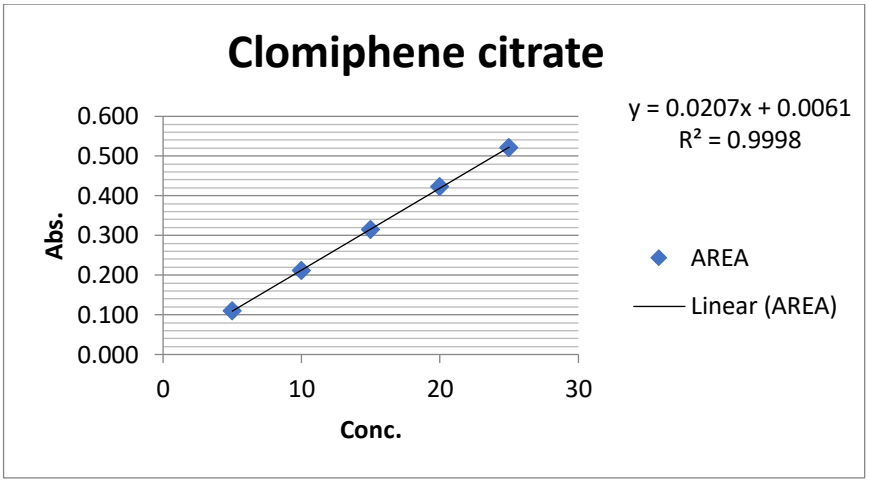


Figure No. 3- Calibration curve of clomiphene citrate by UV-Spectrophotometry

Table No. 1 : Linearity data for clomiphene citrate by UV-Spectrophotometry

Concentration µg/ml	Absorbance		Mean Abs.	S.D.	% RSD
	Abs.-1	Abs.-2			
5	0.1102	0.1106	0.1104	0.0003	0.26
10	0.2109	0.2117	0.2113	0.0006	0.27
15	0.3142	0.3144	0.3143	0.0001	0.04
20	0.4219	0.4229	0.4224	0.0007	0.17
25	0.5208	0.5216	0.5212	0.0006	0.11
Equation	y = 0.0207x + 0.0061				
R^2	0.9998				

Accuracy – The method was found to be accurate as the % recovery was found to be within acceptable limit of 98-102% and also % RSD was not more than 2. Results for recoveries are shown in Table 2.

Table No. 2 : Recovery data for clomiphene citrate by UV-Spectrophotometry

Level (%)	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found Mean*±S.D.	Amount recovered Mean*±S.D.	% Mean* Recovery ± S.D.	% RSD
80%	10	8	18.12±0.007	8.12±0.007	101.45±0.09	0.08
100%	10	10	19.86±0.055	9.86±0.055	98.65±0.55	0.55
120%	10	12	21.91±0.014	11.91±0.014	99.28±0.11	0.11

Precision - Intraday and Interday precision has been expressed as the percent relative standard deviation (%RSD) of peak absorbance response. The method was

found to be precise as the % RSD was not more than 2. Results are shown in Table 3.

Table No.3 - Precision data for clomiphene citrate by UV-Spectrophotometry

Conc. (µg/ml)	Intra-day Precision			Inter-day Precision		
	Absorbance Mean*± SD	%Amount Found	% RSD	Absorbance Mean*± SD	%Amount Found	% RSD
10	0.2127±0.0003	99.81	0.13	0.2142±0.0029	100.51	1.35
15	0.3186±0.0001	100.63	0.02	0.3189±0.0008	100.72	0.24
20	0.4120±0.0003	98.04	0.07	0.4166±0.0059	99.15	1.42

Repeatability (system suitability) –After defining the optimum conditions for the method, repeatability parameters are assessed and % RSD was found within predefined limit i.e. not more than 2. Results are shown in Table 4.

Table No.4 : Repeatability data for clomiphene citrate by UV-Spectrophotometry

Sr. No.	Concentration [µg/ml]	Absorbance	Amount Found	% Amount Found
1	20	0.42110	20.05	100.24
2	20	0.4125	19.63	98.16
3	20	0.4129	19.65	98.26
4	20	0.4225	20.11	100.57
5	20	0.4123	19.62	98.11
Mean		0.41626	19.81	99.07
SD		0.005	0.24	1.22
%RSD		1.22	1.24	1.24

Ruggedness –The % RSD values by Analyst 1 and Analyst 2 were found within the acceptable range which is not more than 2% that shows the parameter was validated. The results are given in Table 5.

Table No. 5 : Ruggedness data for clomiphene citrate by UV-Spectrophotometry

Analyst-I			Analyst-II		
Mean Absorbance	**% Amount Found ± SD**	**% RSD**	**Mean Absorbance**	**% Amount Found ± SD**	**% RSD**
0.4125	98.16±0.0003	0.07	0.4126	98.18±0.0002	0.06

Analysis of clomiphene citrate tablet – The % label claim was found to be 99.16% and % RSD values are not more than 2%, hence the results obtained met the requirements and are shown in Table 6.

Table No.6 : Analysis data for clomiphene citrate tablet by UV-Spectrophotometry

Marketed Formulation	Concentration [µg/ml]	Absorbance	Amount Found	% Label Claim
FERTOMID-100 by Cipla Ltd.	15	0.3148	14.91	99.42
	15	0.3132	14.83	98.90
	Mean	0.3140	14.87	99.16
	SD	0.001	0.055	0.364
	%RSD	0.36	0.367	0.367

B. RP-UHPLC Method Development and Validation–

The developed RP-UHPLC method successfully determined clomiphene citrate with well-defined peak symmetry and a consistently stable baseline under the optimized chromatographic conditions described in Table 7. Figure 4 represents the standard chromatogram attained under optimal conditions, and the corresponding chromatogram data can be found in Table 8.

Table No.7 : Optimized chromatographic parameters

Parameter	Description
Stationary Phase	Agilent C18 Column (100mm x 4.6mm, 2.5µm particle size).
Mobile Phase	Methanol: (0.1%OPA) Water (50:50% V/V)
Flow Rate	1 ml/min
Detection Wavelength	234 nm

Detector	DAD detector
Injection volume	20 µl
Column Temperature	Ambient
Run Time	8 min

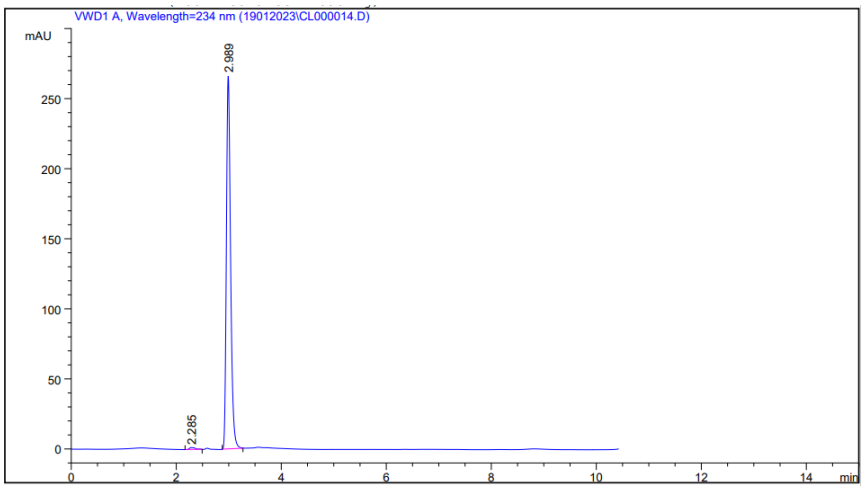


Figure No. 4 : Chromatogram of clomiphene citrate

Table No. 8 : Result for Chromatogram of clomiphene citrate

RT [min]	Area[mV*s]	TP	TF
2.989	1481.72144	7271	0.72

RP-UHPLC Method Validation

Linearity - The analytical calibration curve is linear in the 2.5–12.5µg/ml range and shows thatthe correlation coefficient (R^2) is nearly equal to 1 i.e. 0.9991. The linear regression equation is y = 213.66x + 104.48. (Fig. 5, Table 9)

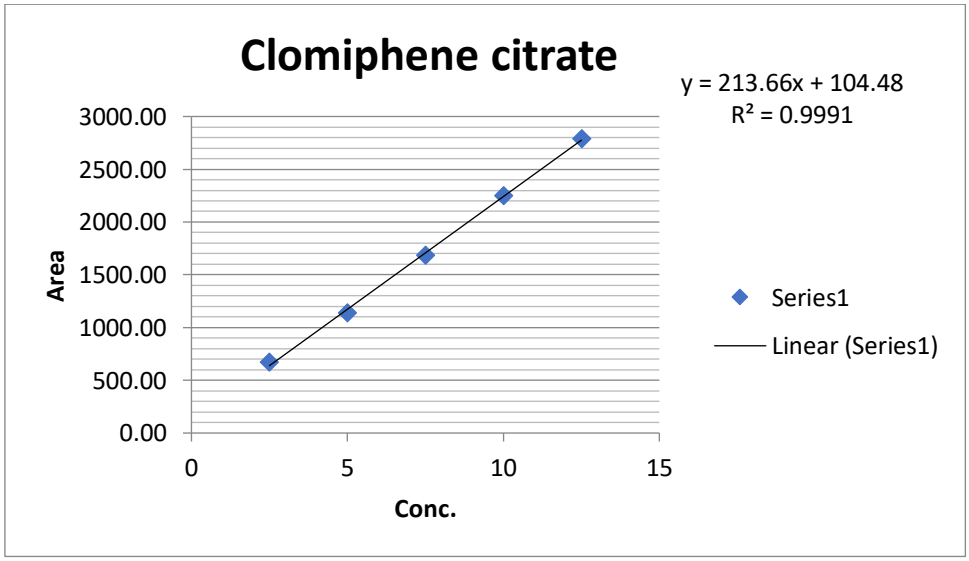


Figure No. 5 - Calibration curve of clomiphene citrate by RP-UHPLC

Table No. 9 :Linearity data for clomiphene citrate by RP-UHPLC

Concentration µg/ml	Peak Area		Mean Area	S.D.	% RSD
	Area-1	Area-2			
2.5	669.792	670.1718	669.98	0.27	0.04
5	1141.422	1140.804	1141.11	0.44	0.04
7.5	1687.687	1686.158	1686.92	1.08	0.06
10	2250.216	2251.216	2250.72	0.71	0.03
12.5	2784.852	2786.997	2785.92	1.52	0.05
Equation	y = 213.66x + 104.48				
R^2	0.9991				

Accuracy – The method was found to be accurate as the % recovery was found to be within acceptable limit i.e. 98-102% and also % RSD was not more than 2. Results for recoveries are shown in Table 10.

Table No. 10 - Recovery data for clomiphene citrate by RP-UHPLC

Level (%)	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found Mean*±S.D.	Amount recovered Mean*±S.D.	% Mean* Recovery ± S.D.	% RSD
80%	2.5	2	4.52± 0.003	2.02± 0.003	101.14±0.17	0.17
100%	2.5	2.5	4.95± 0.008	2.45± 0.008	98±0.31	0.32
120%	2.5	3	5.47± 0.004	2.97± 0.004	99.02±0.13	0.13

Precision - Intraday and Interday precision has been expressed as the percent relative standard deviation (%RSD) of peak area response. The method was found to be precise as the % RSD was not more than 2. Results are shown in Table 11.

Table No. 11 - Precision data for clomiphene citrate by RP-UHPLC

Conc. (µg/ml)	Intra-day Precision			Inter-day Precision		
	Area Mean*± SD	%Amount Found	% RSD	Area Mean*± SD	%Amount Found	% RSD
5	1145.24±0.64	97.45	0.06	1146.24±2.00	97.55	0.17
7.5	1687.74±0.25	98.83	0.01	1690.23±0.45	98.99	0.03

10	2253.50±1.41	100.61	0.06	2257.02±3.71	100.77	0.16

Repeatability (system suitability) –After defining the optimum conditions for the method, repeatability parameters are assessed and % RSD was found within specified limit i.e. not more than 2. Furthermore the tailing factor for peak was less than 2 signifies good peak symmetry, and a number of theoretical plates were consistently greater than 2000 ensuring good column efficacy. Results are shown in Table 12.

Table no. 12 : Repeatability data for clomiphene citrate by RP-UHPLC

Sr. No.	Conc. (µg/ml)	Peak Area	Tailing Factor	Theoretical Plate	Amount found (mg)	% Amount found
1	10	2251.056	0.68	9616	10.06	100.61
2	10	2256.075	0.69	9842		
Mean		2253.57				
SD		3.55				
%RSD		0.16				

Robustness –No significant changes were observed when robustness was assessed at concentration of 7.5 µg/ml. The % RSD was calculated and it is found to be less than 2% thus indicating that the method is robust as shown in Table 13.

Table No.13 : Robustness data for clomiphene citrate by RP-UHPLC

Parameters	Modification	Conc. (µg/ml)	Area mean ±SD	% RSD
Flow rate 1 ml/min	0.9 ml/min	7.5	2629.90±0.93	0.04
	1.1 ml/min	7.5	1325.81±1.05	0.08
Mobile phase composition Methanol:0.1%(OPA)Water (50:50)	(49:51v/v)	7.5	2252.45±1.37	0.06
	(51:49v/v)	7.5	2253.6±3.29	0.15
Wavelength 234 nm	233 nm	7.5	2279.8±0.84	0.04
	235 nm	7.5	2114.03±3.33	0.16

Ruggedness –The % RSD values by Analyst 1 and Analyst 2 were found within the acceptable range which is not more than 2%that shows the parameter was validated. The results are given in Table 14.

Table No. 14 : Ruggedness data for clomiphene citrate by RP-UHPLC

Analyst-I			Analyst-II		
Mean Area	**% Amount Found ± SD**	**% RSD**	**Mean Area**	**% Amount Found ± SD**	**% RSD**
2252.69	100.57±0.52	0.02	2256.80	100.76±1.98	0.09

Limit of detection (LOD) and limit of quantification (LOQ) -The Limit of Detection (LOD) and Limit of Quantification (LOQ) for Clomiphene citrate were determined considering a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. The calculations were based on the equations LOD = 3.3σ/S and LOQ = 10σ/S, where 'σ' represents the standard deviation of the response and 'S' denotes the slope of the linearity plot. The precise values for LOD and LOQ were found to be 0.012 µg/mL and 0.037 µg/mL, respectively.

Analysis of clomiphene citrate tablet – The % label claim was found to be 99.02% and % RSD values are not more than 2%, hence the results obtained met the requirements and are shown in Table 15.

Table No.15 -:Analysis data for clomiphene citrate tablet by RP-UHPLC

Marketed Formulation	Conc. (µg/ml)	Area	Amount found	% Label claim
FERTOMID-100 by Cipla Ltd.	7.5	1688.589	7.416267	98.88
	7.5	1693.059	7.437196	99.16
Mean		1690.82	7.426	99.02
SD		3.161	0.015	0.197
% RSD		0.187	0.199	0.199

Discussion

The study successfully employed developed UV-Spectrophotometric and RP-UHPLC methods to efficiently analyse clomiphene citrate. As a result, the proposed approach offered numerous advantages over the existing one. Unlike the previous method, which relied on expensive acetonitrile or biological fluids as the solvent, the new procedure employed a cost-effective combination of methanol, water, and buffer in the mobile phase. This incorporation of a lower

organic solvent ratio not only enhanced sensitivity but also embraced green chemistry principles, making it an environmentally friendly choice. Additionally, the conventional RP-HPLC method was time-consuming, whereas the proposed RP-UHPLC method proved to be remarkably rapid and more effective, further contributing to its superiority.

Conclusion

In the realm of drug development and the pharmaceutical industry, the significance of developing and validating UV-Spectrophotometric and HPLC methods cannot be overstated. These meticulously developed UV-Spectrophotometric and RP-UHPLC methods have proven to be simple, accurate, and highly precise. Following validation in strict accordance with ICH guidelines, the statistical analysis has shown that the validation parameters meet the required standards, making them highly reliable.

An additional advantage of these methods is that they eliminate the need for pre-procedures, such as extraction, streamlining the analytical process. As a result, these well-established methods are perfectly suited for routine and quality control analysis of Clomiphene citrate, both in bulk and, more specifically, in tablet formulations within the pharmaceutical industry. Their excellence ensures confidence in the assessment and quantification of this essential pharmaceutical compound, further reinforcing the overall drug development process.

Abbreviations

UV: Ultraviolet; HPLC: High Performance Liquid Chromatography; RP-UHPLC: Reverse Phase Ultra High Performance Liquid Chromatography; DAD: Diode Array Detection; IUPAC: International Union of Pure and Applied Chemistry; ICH: International Council for Harmonization; OPA: Orthophosphoric Acid; LOD: Limit of Detection; LOQ: Limit of Quantitation; SD: Standard Deviation RSD: Relative Standard Deviation

Acknowledgements

The authors would like to convey their obligation to Management and Principal, P.S.G.V.P. Mandal's College of Pharmacy, Shahada, Dist. Nandurbar.

References

1. *Dickey R, (1996) Development, pharmacology and clinical experience with clomiphene citrate. Hum Reprod Update2(6):483–506.*
2. *Sir T, Alba F, Devoto L, Rossmanith W, (1989) Clomiphene Citrate and LH Pulsatility in PCO Syndrome. HormMetab Res21 (10):583–583.*
3. *Mbi Feh M K, Wadhwa R, (2022) Clomiphene.StatPearls Publ. Accessed 29 July 2023.*
4. *Girase T,Patil J, Tatiya A, Patil D,Patil M, (2023) Clomiphene Citrate as Nanomedicine Assistance in Ovulatory Disorders and Its Hyphenated Techniques. Mater Proc 14:1-6.*
5. *AnnajiM,(2023) Inclusion Complex of Clomiphene Citrate with Hydroxypropyl-β-Cyclodextrin for Intravenous Injection: Formulation and Stability Studies. AAPS PharmSciTech 24(1):48.*
6. *McLeish M J, (1998) Clomiphene Citrate. Analytical Profiles of Drug Substances and Excipients, 85–120.*
7. *Sayare A S,Undre P B,Ghode P D, Singh S V,Ghode S P, (2021) Development and Validation of RP-HPLC Method for the Estimation of Clomiphene Citrate in Pharmaceutical Dosage Form. Res J Pharm Technol 17(7):3483–3488.*
8. *MailvelanR,RajeshJ,SelvamaniP, Latha S,(2012) HPLC Method Development and Validation for Simultaneous Estimation of*

ubidecarenone and clomiphene citrate in bulk and tablet dosage form.Int J Res Pharm Nano Sci 1(1):61-69.

9. *Venkatakiran V, Puranik S B, Kumar G V S, Sridhar K A,(2013) Development And Validation Of Zero Order Spectrophotometric Method For Estimation Of Clomiphene Citrate In Bulk And Tablet Dosage Form.Int J Pharmtech Res 5(1):27-30.*
10. *Soni A, Chaudhary A, Singla D, Goyal S, (2019) Development And Validation Of The UV Spectrophotometric Method Of Clomiphene Citrate In Simulated Vaginal Fluid And Stress Degradation Studies. Int J Sci Technol Res 8(10):3680-84.*
11. *Validation of Analytical Procedures Q2 (R1). Accessed29 July 2023.*