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Development and Validation of Simultaneous Estimation of Sildenafil Citrate and Clomiphene Citrate Using Uv Spectrophotometric and Rp-Hplc Techniques

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ABSTRACT:
The Combination of Sildenafil Citrate and Clomiphene Citrate was studied under Clinical trial Phase 3; it was proved that therapy improved to clinical outcome on the Endometrial thickness during Ovulation induction in Anovulatory infertility. The present study aimed to develop simple, accurate, and precise UV spectrophotometric and RP-HPLC methods for the simultaneous estimation of these drugs in synthetic mixtures, followed by validation in accordance with ICH Q2 (R2) guideline. In the UV spectrophotometric method, two approaches were applied as Simultaneous Equation Method and First-Order Derivative Method by using Methanol as solvent. For the simultaneous equation method, absorbances were measured at 291 nm (Sildenafil) and 238 nm (Clomiphene). The method showed excellent linearity with correlation coefficients (R^2) of 0.999 and 0.9986 for Sildenafil, and 0.999 and 0.9995 for Clomiphene. The linearity range was 1-5 µg/ml for Sildenafil and 5-25 µg/ml for Clomiphene. Precision (%RSD < 2%) and accuracy (99.33-99.80% for Sildenafil; 99.89-99.96% for Clomiphene) were within acceptable limits. Sensitivity studies indicated low LOD values (0.123-0.169 µg/mL and 0.264-0.259 µg/mL) and LOQ values (0.372-0.513 µg/mL and 0.800-0.783 µg/mL), confirming strong analytical sensitivity. Assay results were 99.50% and 99.80%, respectively. In the first-order derivative method, detection was carried out at 266 nm (Sildenafil) and 290 nm (Clomiphene), with zero-crossing points at 290 nm and 266 nm. The method demonstrated strong linearity (R^2 = 0.9986 and 0.9992), accuracy (99.16-99.92% and 99.86-99.95%), and acceptable LOD values (0.130 µg/mL, 0.101 µg/mL) LOQ values (0.394 µg/mL, 0.307) acceptable. Assay results were 99.55% and 99.90%, respectively. The RP-HPLC method employed an isocratic system with ACN: 0.02 M phosphate buffer (pH 3.4) (60:40 % v/v) at 231 nm. Retention time were 3.5 min (Sildenafil) and 6.5 min (Clomiphene). The method exhibited excellent linearity, precision, and accuracy (99.66-100.04%). LOD values (0.033 µg/mL, 0.133 µg/mL) and LOQ values (0.099 µg/mL, 0.402 µg/mL) were notably low, indicating high sensitivity. Assay values were 99.95% and 99.99%. The developed methods were validated

for specificity, linearity, precision, accuracy, sensitivity (LOD and LOQ), robustness, and assay and found to be reliable for routine quantitative analysis.

1. INTRODUCTION:

Sildenafil Citrate is widely prescribed for the treatment of erectile dysfunction, a condition where a man is unable to achieve or maintain an erection sufficient for sexual intercourse. It works by inhibiting an enzyme called Phosphodiesterase type 5 (PDE5), which leads to increased blood flow to the penis during sexual arousal[1]. Clomiphene Citrate is a medication commonly used in the treatment of infertility, particularly for women who have Anovulation (lack of ovulation) or irregular ovulatory cycles. It works by stimulating ovulation, which is essential for conception[2]. The combination of Sildenafil Citrate and Clomiphene Citrate was studied under clinical trial Phase[3], it was proved that therapy improved to clinical outcome on the Endometrial thickness during Ovulation induction in Anovulatory infertility. Incorporation of Sildenafil Citrate to Clomiphene Citrate regimen of Ovulation induction has a positive impact on endometrial stripe thickness and so pregnancy outcomes. Combining sildenafil citrate with clomiphene citrate in women undergoing ovulation induction can improve endometrial thickness and potentially increase pregnancy rates, especially in cases of unexplained infertility or polycystic ovary syndrome (PCOS). Sildenafil, a vasodilator, may enhance blood flow to the uterus, while clomiphene stimulates ovulation[4-5]. The use of sildenafil as an enhancer of endometrial vascularity helps in improving pregnancy rates in patients with unexplained infertility treated with clomiphene, and counteracts the poor effect of clomiphene on endometrial receptivity. Also, Sildenafil citrate to CC for Ovulation Induction (OI) in PCOS women increases the chemical and clinical pregnancy rates. It also improves the endometrial thickness and ovulation rate through improved endometrial and ovarian Doppler indices. The results concluded that the combination of Sildenafil Citrate and Clomiphene Citrate was safe and effective and showed synergistic result to the improvement of Endometrial Thickness and treatment of unexplained infertility in women[6-8].

A comprehensive literature survey revealed several reported analytical methods for the estimation of Sildenafil Citrate either alone or in combination with other drugs, including RP-HPLC methods[9-12], UV spectrophotometric methods[13-15], stability-indicating RP-HPLC methods[16-18], HPLC in human plasma[19], and Stability indicating UPLC-MS/UV[20]. Similarly, numerous analytical approaches have been described for the quantitative determination of Clomiphene Citrate, such as reversed-phase high-performance liquid chromatography[21], UV Spectrophotometric Method[22], and LC-MS/MS methods[23]. Despite the availability of these sophisticated and well-established analytical techniques, all reported methods focus on the individual estimation of these drugs or their determination in biological matrices. To the best of our knowledge, no validated spectrophotometric and chromatographic method has been reported for the simultaneous quantification of Sildenafil Citrate and Clomiphene Citrate in a synthetic mixture. The absence of a unified, cost-effective, and time-efficient analytical approach for their concurrent estimation highlights a significant analytical gap in the literature.

Therefore, the present study was undertaken to develop and validate novel, accurate, precise, and robust RP-HPLC and simultaneous equation UV spectrophotometric methods for the concurrent estimation of both drugs, in accordance with ICH Q2 (R2)[24] guideline, thereby providing a reliable analytical tool suitable for routine quality control analysis.

2. EXPERIMENTAL MATERIALS AND INSTRUMENTATION

2.1 Chemicals and reagents

Sildenafil Citrate was obtained as a gift sample from Yarrow Chem. Product, Mumbai, India. Clomiphene Citrate was procured from Yarrow Chem. Product, Mumbai, India. HPLC-grade methanol was purchased from Finar Chemicals Pvt. Ltd., Ahmedabad, Gujarat, India. All other chemicals and reagents used in the study were of analytical reagent (AR) grade or HPLC grade and were used without further purification.

2.2 Instrumentation

UV spectroscopic analysis was performed using a Shimadzu UV-1900 UV–Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with UV Probe 2.7 software, a spectral bandwidth of 1 nm, and 1.0 cm matched quartz cuvettes over the wavelength range of 200–400 nm.

Chromatographic analysis was carried out using a Systronics RP-HPLC system (Model SYS-LC-138, Systronics, India) coupled with a UV detector. The pH of the buffer solutions was measured using a Chemi Line pH meter

(Chemi Line Instruments, India). An analytical balance (Scale-Tec, India) was used for accurate weighing of samples. The mobile phase was degassed by sonication using a Digital Pro+ sonicator (Model PS-10A, Broleo, India) prior to use.

2.3 Preparation of Solutions

2.3.1 Preparation of Stock Solution

Precisely weighed quantities of 10 mg each of Sildenafil Citrate and Clomiphene Citrate were quantitatively transferred into a 100 ml volumetric flask and subsequently diluted to volume with methanol to achieve a final concentration of 100 µg/mL. The solutions were sonicated for 5 mins to ensure complete dissolution.

2.3.2 Preparation of calibration curve

The calibration standards in the concentration range of 1-5 µg/mL for Sildenafil Citrate for and 5-25 µg/mL for Clomiphene Citrate, appropriate aliquots of the respective stock solutions were transferred into a series of 10 mL volumetric flasks. For Sildenafil Citrate, aliquots of 0.1, 0.2, 0.3, 0.4, and 0.5 mL were diluted to volume with methanol to yield final concentrations of 1, 2, 3, 4, and 5 µg/mL, respectively. Similarly, for Clomiphene Citrate, aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 mL were diluted to volume with methanol to obtain concentrations of 5, 10, 15, 20, and 25 µg/mL, respectively. The prepared solutions were analyzed under optimized spectrophotometric conditions using a 1 cm matched quartz cuvette. For chromatographic analysis, 20 µL of each working standard solution was injected into the RP-HPLC system under optimized chromatographic conditions.

3. METHODOLOGY

3.1 Method I: UV SEPCTROPHOTOMETRIC METHOD DEVELOPMENT

Pipetted out 0.2 ml solution from stock solution of Sildenafil Citrate (100 µg/ml) and 1.0 ml Clomiphene Citrate (100 µg/ml) into different 10 ml volumetric flask and diluted upto mark with methanol to get the 2 µg/ml of Sildenafil Citrate and 10 µg/ml Clomiphene Citrate. Every solution was scanned between 200 to 400 nm.

3.1.1 Simultaneous equation as Vierordt's method

Solutions of Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) prepared in methanol were subjected to a spectral scan from 200 to 400 nm at a medium speed, utilizing pure methanol as the reagent blank. For the analytical determination, the absorption maxima (λmax) were established at 291 nm for Sildenafil Citrate and 238 nm for Clomiphene Citrate. This procedure applies the Simultaneous Equation technique based on Vierodt's principle, where the precise concentration of each drug within the sample is calculated according to the following mathematical expressions.

Standard Stock solutions of Sildenafil Citrate and Clomiphene Citrate in the concentration range 1-5 µg/mL and 5-25 µg/ml were made in the methanol and absorbance of these solutions was measured at 291 nm and 238 nm. Calibration curves were plotted to confirm the Beer's law and the absorptivity values calculated at the respective wavelengths for both the drugs. Two simultaneous equations as below were formed using these absorptivity values A (1%, 1 cm).

$$\text{At } \lambda 1 \; A1 = ax1bCx + ay1bCy \ldots\ldots\ldots\ldots\ldots\ldots (1)$$
$$\text{At } \lambda 2 A2 = \; ax2bCx + ay2\; bCy \ldots\ldots\ldots\ldots\ldots\ldots \; (2)$$

For measurements in 1 cm cells b=1,
Rearrange eq. (2),

$$Cy = \; A2 - \; ax2Cx/\; ay2$$

Substituting for Cy in eq (1) and rearranging

$$Cx \; = \; A2ay1 - A1\; ay2/ax2\; ay1 - ax1\; ay2 \ldots\ldots\ldots\ldots\ldots\ldots (3)$$
$$Cy \; = \; A1ax2 - A2\; ax1/ax2\; ay1 - ax1\; ay2 \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where C_x and C_y are the concentration of Sildenafil Citrate and Clomiphene Citrate, respectively, A_1 and A_2 are absorbance at 291 nm and 238 nm, respectively, ax_1 and ax_2 are absorptivity of Sildenafil Citrate at 291 nm and 238 nm, respectively; ay_1 and ay_2 are absorptivity of Clomiphene Citrate at 238 nm and 291 nm, respectively. By solving the two simultaneous equations, the concentrations of Sildenafil Citrate and Clomiphene Citrate in sample solutions were obtained.

3.1.2 Simultaneous equation as First Order Derivative Method

Pipetted out 0.2 ml solution from stock solution of Sildenafil Citrate (100 µg/ml) and 1 ml Clomiphene Citrate (100 µg/ml) into different 10 ml volumetric flask and diluted upto mark with Methanol to get the 2 µg/ml of

Sildenafil Citrate and 10 µg/ml of Clomiphene Citrate. Each solution was scanned in the range of 200-400 nm. Overlain UV Spectra of Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) in Methanol (Zero order).

All zero-order spectrum (D_0) were converted to first derivative spectrum (D_1) using delta lambda 2.0 and scaling factor 4. The overlain first derivative spectrums of Sildenafil Citrate and Clomiphene Citrate at different concentration were recorded. The Zero-Crossing Point (ZCP) of Sildenafil Citrate and Clomiphene Citrate were found to be 290 nm and 266 nm, respectively.

3.2 Method III: Reverse Phase High Performance Liquid Chromatography Method

Chromatographic analysis was performed via isocratic elution, wherein various mobile phase configurations including Acetonitrile: Water, Methanol: Water, and Acetonitrile: Phosphate buffer were evaluated in varying ratios. Optimal resolution of both analyte peaks was achieved using a mixture of Acetonitrile: Phosphate Buffer (pH 3.4 adjusted with 10% ortho phosphoric acid) (60:40 %v/v) at a consistent flow rate of 1 mL/min. All solvents underwent filtration through a 0.45 µm membrane and were degassed via sonication for 30 minutes before use. Separation was executed on a Kromstar C_{18} (250 mm × 4.6 mm, 5 µm) stationary phase, with the eluent monitored using a UV Detector and chromatograms specifically extracted at 231 nm. Calibration curves were subsequently established by plotting the measured peak areas against their respective concentrations to derive the corresponding linear regression equations.

3.3 METHOD VALIDATION

The analytical methodologies employed in this research were rigorously validated in accordance with the regulatory standards established by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) under the ICH Q2 (R2)[24] guideline for analytical procedure validation.

3.3.1 Specificity

Specificity denotes the capacity of an analytical procedure to accurately and distinctly quantify the target analyte despite the potential interference of co-existing substances. Within a complex sample, these extraneous components commonly encompass synthesis impurities, degradation products, or various matrix constituents that could otherwise confound the measurement.

3.3.2. Linearity and Range (n=6)

The linearity of the analytical procedure was evaluated through the preparation of five distinct concentrations of standard solutions. Sildenafil Citrate and Clomiphene Citrate demonstrated linear responses within the concentration ranges of 1-5 µg/mL and 5-25 µg/mL, respectively. The proportionality of both analytes was statistically assessed by calculating the slope, y-intercept, and correlation coefficient (R^2) from the resulting calibration curves.

3.3.3. Precision

The precision of both analytical methodologies was evaluated across three distinct parameters: repeatability, intraday (intermediate) precision, and interday (reproducibility) precision. To assess intraday precision, standard solutions of Sildenafil Citrate (1, 2, 3 µg/mL) and Clomiphene Citrate (5, 10, 15 µg/mL) were analyzed in triplicate at three separate time intervals within a single day. Interday precision was similarly established by evaluating the same concentration levels over three consecutive days. Furthermore, repeatability was rigorously determined through six replicate injections of a single concentration level 2 µg/mL for Sildenafil Citrate and 10 µg/mL for Clomiphene Citrate. All precision data were statistically quantified and reported as the percentage relative standard deviation (%RSD) to ensure compliance with ICH Q2 (R2) guideline.

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.

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

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

3.3.6 Accuracy (Recovery study) (n=3)

The accuracy of an analytical procedure denotes the proximity of the experimental result to the accepted reference value or conventional true value. To confirm the accuracy of the proposed method, recovery studies were conducted in accordance with ICH Q2 (R2) guideline at three distinct concentration levels: 50%, 100%, and 150%. These evaluations targeted. Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) using the standard addition technique, with each level analyzed in triplicate. The methodology's accuracy was subsequently established by calculating the percentage recovery of both analytes across these fortified concentrations.

3.3.7 Assay as analysis of Synthetic Mixture

A synthetic mixture containing. Sildenafil Citrate and Clomiphene Citrate in the ratio of 1:5 was prepared. Accurately weighed quantities of Sildenafil Citrate (20 mg) and Aspirin (100 mg) were blended with commonly used excipients, namely microcrystalline cellulose (30 mg), lactose (15 mg), magnesium stearate (2 mg), talc (10 mg), and croscarmellose sodium (7 mg), using a mortar and pestle to obtain a homogeneous mixture. An accurately weighed portion of the prepared blend equivalent to 20 mg of Sildenafil Citrate and 100 mg of Clomiphene Citrate was transferred into a 100 mL volumetric flask. Approximately 70 mL of methanol was added, and the mixture was sonicated to ensure complete dissolution of the drugs. The volume was then made up to the mark with methanol and mixed thoroughly. The resulting solution was filtered through Whatman filter paper to remove insoluble excipients. The obtained stock solution contained 200 µg/mL of Sildenafil Citrate and 1000 µg/mL of Clomiphene Citrate. For sample analysis, 0.1 mL of this stock solution was accurately transferred into a 10 mL volumetric flask and diluted to volume with methanol to yield final concentrations of 2 µg/mL of Sildenafil Citrate and 10 µg/mL of Clomiphene Citrate. The prepared sample solution was analyzed using the optimized RP-HPLC and UV spectrophotometric methods, and the percentage assay of both drugs was calculated.

3.3.8 Robustness

Robustness of the developed RP-HPLC and UV spectrophotometric methods was evaluated by deliberately introducing small and systematic variations in analytical conditions and assessing their effect on the assay results. For the RP-HPLC method, robustness was examined by varying the flow rate (±0.1 mL/min from 1.0 mL/min), mobile phase composition (±2% variation in organic phase), and detection wavelength (±2 nm from 231 nm). The effects of these changes on retention time, peak area, tailing factor, and resolution were studied. For the UV spectrophotometric method, robustness was assessed by varying the detection wavelength (±2 nm from 291 nm for Sildenafil Citrate and 238 nm for Clomiphene Citrate) and evaluating the effect of slight variations in solvent composition.

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 (table 1).

4. RESULTS AND DISCUSSION:

4.1 Selection of wavelength

For the simultaneous equation method, standard solutions of Sildenafil Citrate (2 µg/mL) and Clomiphene Citrate (10 µg/mL) in methanol were subjected to spectral scanning between 200 and 400 nm at medium speed, with methanol employed as the blank solution. For the analytical determination, the absorption maxima (λmax) were established at 291 nm for Sildenafil Citrate and 238 nm for Clomiphene Citrate (Figure 1). This procedure applies the Simultaneous Equation technique based on Vierodt's principle, where the precise concentration of each drug within the sample is calculated. For RP-HPLC method, coupled with UV detection, is fundamentally dependent upon the strategic selection of an optimal detection wavelength. Both analytes exhibited significant molar absorptivity at 231 nm, leading to its selection for the simultaneous quantification of Sildenafil Citrate and Clomiphene Citrate within the synthetic mixture. The spectral rationale for this choice of detection wavelength is showed in Figure 1.

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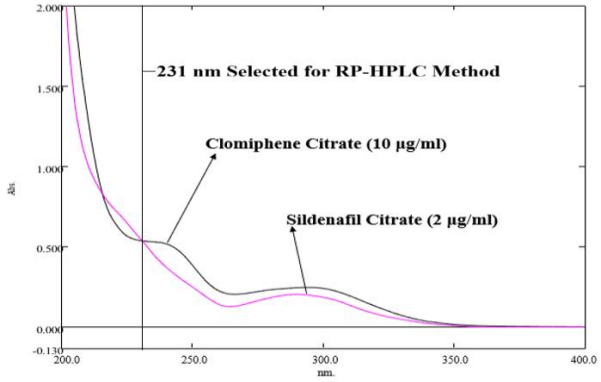

Figure 1: Overlain UV Spectra of Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) in Methanol at 231 nm

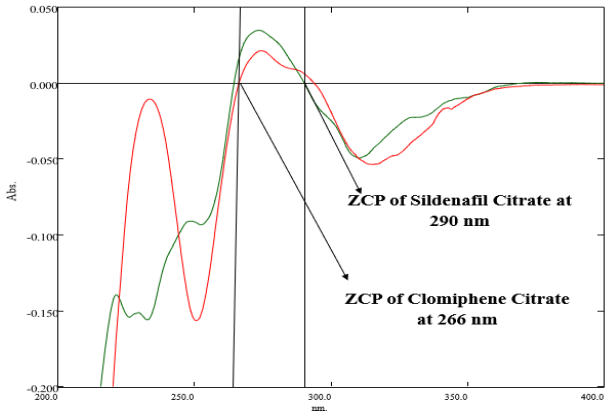

Figure 2: Overlain UV Spectra of Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) in Methanol (First order)

*4.2 Simultaneous equation (*Vierordt's*) method*

For multi-component UV analysis, Vierordt's method is named after the German scientist Karl Vierordt. UV Spectra of Sildenafil Citrate (1-5 µg/mL) and Clomiphene Citrate (5-25 µg/mL) over the linearity and range had been showed in Figure 3 and 4, respectively.

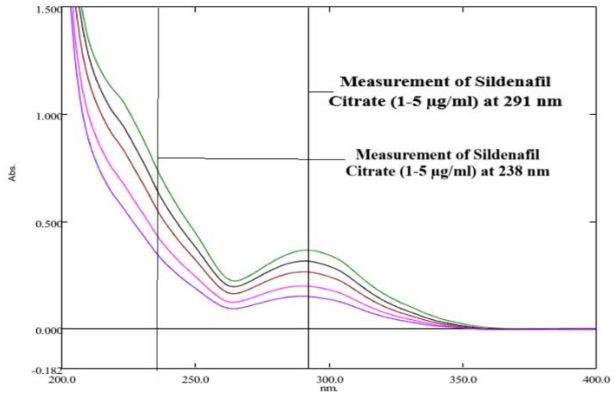

Figure 3: Overlain Spectra of Sildenafil Citrate (1-5 µg/ml) at 291 nm and 238 nm

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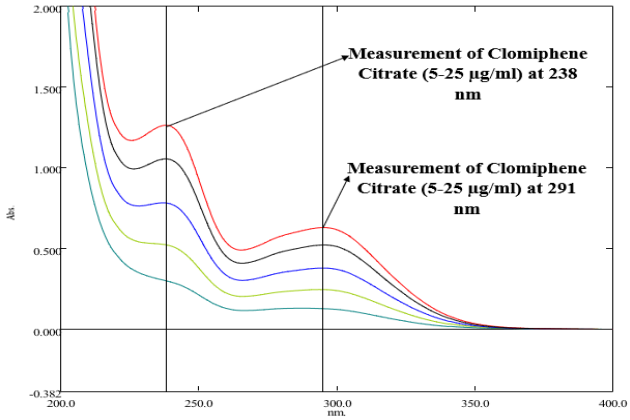

Figure 4: Overlain Spectra of Clomiphene Citrate (5-25 µg/ml) at 238 nm and 291 nm

4.3 Simultaneous equation as First Order Derivative Method

Overlain UV Spectra of Sildenafil Citrate (1-5 µg/ml) and Clomiphene Citrate (5-25 µg/ml) in methanol (First Order) have been shown in Figure no 5 and 6 at the wavelength of 266 and 290.

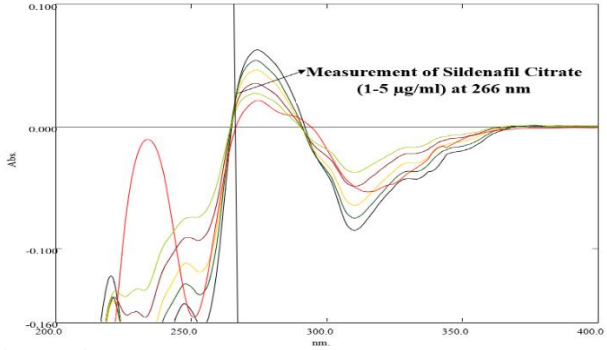

Figure 5: Overlain UV Spectra of Sildenafil Citrate (1-5 µg/ml) at 266 nm

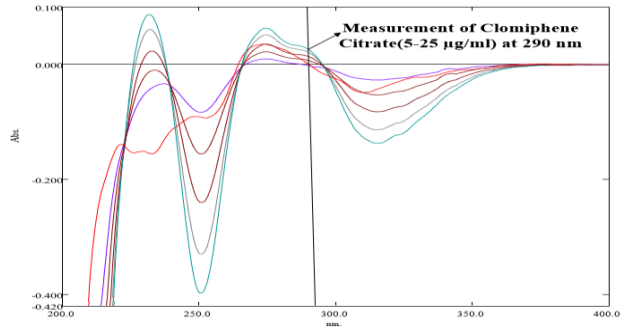

Figure 6: Overlain UV Spectra of Clomiphene Citrate (5-25 µg/ml) at 290 nm

4.3 RP-HPLC Method Development

An RP-HPLC method coupled with UV detection was developed for the concurrent quantification of Sildenafil Citrate and Clomiphene Citrate, with the primary objective of achieving optimal peak symmetry and high theoretical plate counts within an efficient analytical runtime. Chromatographic parameters were refined through the systematic evaluation of various stationary and mobile phase compositions. Among the reversed-phase C_8 and C_{18} columns assessed, the Kromstar C_{18} (250 × 4.6 mm, 5 µm) demonstrated superior performance, yielding highly symmetric peaks and the most favorable retention times. The optimal mobile phase was identified as a mixture of Acetonitrile: Phosphate Buffer (pH 3.4 adjusted with 10% ortho phosphoric acid) (60:40 %v/v) at 231 nm. Although alternative ratios of this buffer and solvent were investigated, they resulted in undesirable peak tailing and excessive retention for both analytes.

4.4 VALIDATION OF THE PROPOSED METHODS

4.4.1 Specificity

Specificity is defined as the ability of an analytical method to unequivocally assess the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. The specificity of the developed RP-HPLC method was evaluated by comparing chromatograms of the mobile phase (blank), placebo (excipients), and the test preparation solution. The chromatogram of the blank showed no peaks at the retention times corresponding to Sildenafil Citrate and Clomiphene Citrate. Similarly, no interfering peaks from excipients were observed at the respective retention times of the analytes in the sample chromatogram. These results demonstrate that the developed method is specific and free from interference due to mobile phase components or formulation excipients, thereby confirming its suitability for the simultaneous estimation of Sildenafil Citrate and Clomiphene Citrate. Retention time was found to be 3.5 min and 6.5 min for Sildenafil Citrate and Clomiphene Citrate, respectively showed in table 1.

Table 1: System suitability parameters for Sildenafil Citrate and Clomiphene Citrate

Sr. No.	System suitability parameters	Sildenafil Citrate	Clomiphene Citrate
1.	Retention time	3.5 min	6.5 min
2.	Theoretical Plates	8143	9162
3.	Tailing Factors	0.9	0.8
4.	Resolution	3.2	

4.4.2 Linearity and range

UV Spectra of Sildenafil Citrate (1-5 µg/ml) and Clomiphene Citrate (5-25 µg/ml) over the linearity and range had been showed in Figure 2 and 3, respectively. For UV, Sildenafil Citrate exhibited a linear response in the concentration range of 1-5 µg/mL at 291 nm and 238 nm. The correlation coefficients (r^2) were found to be 0.999 and 0.9986 at 291 nm and 238 nm, respectively, indicating excellent linearity. The mean absorbance values (n = 6) showed low standard deviation with %RSD values below 1.5%, demonstrating good precision and repeatability. Clomiphene Citrate showed linearity over the concentration range of 5-25 µg/mL at 238 nm and 291 nm, with correlation coefficients (r^2) of 0.999 and 0.9995, respectively.

The RP-HPLC chromatogram of Sildenafil Citrate (1-5 µg/mL) and Clomiphene Citrate (5-25 µg/mL). The Peak Area was found. Calibration graphs were plotted between concentrations and peak areas were observed. The regression equation of calibration curve was generated and Correlation Coefficient for Sildenafil Citrate 0.9996 and for Clomiphene Citrate 0.9998, respectively. The %RSD values were less than 2.0%, confirming acceptable precision and reproducibility of the developed method. The linearity data are summarized in Table 2.

Table 2: Linearity and sensitivity data of Sildenafil Citrate and Clomiphene Citrate

Parameters	**Simultaneous equation as Vierordt's method**				**First Order Derivative Method**		**HPLC**	
	SILDE		**CLOMI**		**SILDE**	**CLOMI**	**SILDE**	**CLOMI**
Wavelength (nm)	291 nm	238 nm	238 nm	291 nm	266 nm	290 nm	231 nm	
Beer's Law Limit (µg/mL)	1-5 µg/ml	1-5 µg/ml	5-25 µg/ml	5-25 µg/ml	1-5 µg/ml	5-25 µg/ml	1-5 µg/ml	5-25 µg/ml
Correlation Coefficient (r^2)	0.999	0.9986	0.999	0.9995	0.9986	0.9992	0.9996	0.9998
LOD (µg/ml)	0.123	0.169	0.264	0.259	0.130	0.101	0.033	0.133
LOQ	0.372	0.513	0.800	0.783	0.394	0.307	0.099	0.402

4.4.2.1 Calculation for Simultaneous Equation Method for Sildenafil Citrate and Clomiphene Citrate in Synthetic Mixture.

Sildenafil Citrate (2 µg/ml) and Clomiphene Citrate (10 µg/ml) in methanol, both the solutions were scanned over

range of 200-400nm against methanol as blank, using medium scan speed. The sampling wavelength for analysis includes 291 nm for Sildenafil Citrate and 238 nm for Clomiphene Citrate. The method employs Simultaneous Equation as per Vierodt's method and the concentrations of drugs in sample solution were determined by using the following formula:

Sildenafil Citrate,

$$C_x = \frac{A2 \times ay1 - A1 \times ay2}{ax2 \times ay1 - ax1 \times ay2}$$

Where ax_1 and ax_2 represented the absorptivity of Sildenafil Citrate at 291 nm and 238 nm, respectively; ay_1 and ay_2 denoted the absorptivity of Clomiphene Citrate at 238 nm and 291 nm, respectively; and A_1 and A_2 corresponded to the absorbance of the sample measured at 291 nm and 238 nm, respectively.
C (Sildenafil Citrate) = 2.08 µg/mL; The concentration of Sildenafil Citrate (Cx), calculated using Vierordt's simultaneous equation method, was found to be 2.08 µg/mL.
For Clomiphene Citrate,

$$C_y = \frac{A1 \times ax2 - A2 \times ax1}{ax2 \times ay1 - ax1 \times ay2}$$

where a_{x1} and a_{x2} are the absorptivity values of Sildenafil Citrate at 291 nm and 238 nm, respectively; $a_{\gamma 1}$ and $a_{\gamma 2}$ represent the absorptivity of Clomiphene Citrate at 238 nm and 291 nm, respectively; and A_1 and A_2 are the absorbance values of the sample measured at 238 nm and 291 nm, respectively.
C (Clomiphene Citrate) = 9.772 µg/mL; The concentration of Clomiphene Citrate (Cy), calculated using Vierordt's simultaneous equation method, was found to be 9.772 µg/mL.

4.4.3 Precision

Methodological precision was evaluated through intraday, inter-day, and repeatability assessments using triplicate analyses of Sildenafil Citrate (1, 2 and 3 µg/ml) and Clomiphene Citrate (5, 10 and 15 µg/ml) across three consecutive days and within a single diurnal period. Absorbance values were recorded for these concentrations to establish intermediate precision, while repeatability was specifically determined using concentrations of 2 µg/ml for Sildenafil Citrate and 10 µg/ml for Clomiphene Citrate. The outcomes, expressed as Relative Standard Deviation (% RSD) for each precision parameters were less than two.

4.4.4 LOD and LOQ

The limits of detection (LOD) and quantification (LOQ) are calculated using the standard deviation responses and slopes obtained from the calibration curves of each drug at their specific wavelengths. The results of LOD and LOQ were displayed in Table 2.

4.4.5 Accuracy

To evaluate the accuracy of the proposed methodology, recovery studies were performed using the standard addition technique, in which pre-analyzed samples were spiked with known concentrations of pure Sildenafil Citrate and Clomiphene Citrate. These assessments were executed at three levels 50%, 100%, and 150% and conducted in triplicate to ensure statistical reliability. The accuracy was expressed as the percentage recovery of the added standards. For the UV spectrophotometric approach, the percentage recovery was found to be within range of 99.33%-99.80% for Sildenafil Citrate and 99.89%-99.96% for Clomiphene Citrate. For RP-HPLC method, the percentage recovery was found to be within the range of 99.66%-99.88% for Sildenafil Citrate and 99.86%-100.04% for Clomiphene Citrate with detailed results provided in Table 3.

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

Vierordt's 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)**
Sildenafil Citrate	50	2	1	3	2.98	99.33±0.27688
	100	2	2	4	3.98	99.50±0.22392
	150	2	3	5	4.99	99.80±0.10309
Clomiphene Citrate	50	10	5	15	14.984	99.89±0.06081
	100	10	10	20	19.984	99.92±0.03064
	150	10	15	25	24.990	99.96±0.01886
First Order Derivative Method						

Sildenafil Citrate	50	2	1	3	2.975	99.16±0.27667
	100	2	2	4	3.978	99.45±0.22239
	150	2	3	5	4.996	99.92±0.10309
Clomiphene Citrate	50	10	5	15	14.98	99.86±0.04722
	100	10	10	20	19.98	99.90±0.03064
	150	10	15	25	24.988	99.95±0.02114
RP-HPLC Method						
Sildenafil Citrate	50	2	1	3	2.99	99.66±0.0164
	100	2	2	4	3.99	99.75±0.0058
	150	2	3	5	4.994	99.88±0.0800
Clomiphene Citrate	50	10	5	15	14.98	99.86±0.0151
	100	10	10	20	19.99	99.95±0.0650
	150	10	15	25	25.01	100.04±0.0474

4.4.6 Assay as Analysis of Synthetic mixture

From assay, the concentration of Sildenafil Citrate 2 µg/mL and Clomiphene Citrate 10 µg/mL were run into UV and RP-HPLC. The Percentage assay of Sildenafil Citrate and Clomiphene Citrate were found to be 99.86% and 99.93% respectively in UV. For RP-HPLC the Percentage assay of Sildenafil Citrate and Clomiphene Citrate were found to be 99.90% and 99.96%, respectively. Its results showed in Table 4.

Table 4: Assay results for UV and RP-HPLC Method

Vierordt's Method				
Name of Drug	**Amount in synthetic mixture (µg/mL)**	**Mean Amount found (µg/mL)**	**% Assay ± SD (n=3)**	**%RSD**
Sildenafil Citrate	2	1.99	99.50±0.00471	0.24
Clomiphene Citrate	10	9.98	99.80±0.01387	0.14
First Order Derivative Method				
Sildenafil Citrate	2	1.991	99.55±0.00189	0.09
Clomiphene Citrate	10	9.99	99.90±0.01203	0.12
RP-HPLC Method				
Sildenafil Citrate	2	1.999	99.95±0.524	0.24
Clomiphene Citrate	10	9.999	99.99±0.158	0.10s

4.4.7 Robustness

Chromatographic analysis was used to analyses 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. The results indicated that minor deliberate variations in method parameters did not produce significant changes in analytical responses. The percentage relative standard deviation (%RSD) values were found to be within acceptable limits (<2%), demonstrating that the developed methods are robust and reliable for routine analysis.

5. CONCLUSION:

The current investigation focused on the development and validation of streamlined, cost-effective, and precise analytical protocols for the concomitant quantification of Sildenafil Citrate and Clomiphene Citrate in a synthetic matrix. While previous literature documents various techniques for these analytes in isolation, a literature gap was identified regarding their simultaneous determination. Consequently, UV-Spectrophotometric and RP-HPLC methodologies were established and validated in strict accordance with ICH Q2 (R2) regulatory standards. For the UV-Spectrophotometric approach, the Vierordt's (simultaneous equation) method was employed, utilizing analytical wavelengths of 291 nm and 238 nm for Sildenafil Citrate and Clomiphene Citrate, respectively. This method exhibited robust linearity over concentration intervals of 1-5 µg/ml and 5-25 µg/ml, respectively, yielding correlation coefficients nearer to 0.999. Comprehensive validation encompassing accuracy, precision, repeatability, and sensitivity (LOD/LOQ) yielded results within established acceptance criteria. Furthermore, recovery experiments and assay data substantiated the method's reliability for estimating both components within the synthetic mixture. Additionally, a highly sensitive RP-HPLC method was optimized using a C_{18} stationary phase and a mobile phase comprised of Acetonitrile: phosphate buffer (60:40 % v/v). The system operated at a flow rate of 1 ml/min with UV detection at 231 nm, resulting in well-resolved peaks and favorable system suitability metrics. The chromatographic technique demonstrated superior linearity, precision, and robustness.

The obtained assay percentages confirmed that this method is highly suitable for standardized quantitative assessments. In conclusion, both newly developed analytical platforms proved to be efficient, accurate, and reproducible. These validated methods are highly recommended for routine quality control and the simultaneous monitoring of Sildenafil Citrate and Clomiphene Citrate in synthetic mixture.

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CONFLICT OF INTEREST:
The authors declare that there is no conflict of interest.

ABBREVIATIONS
ICH: International Council for Harmonization; UV: Ultraviolet, RP-HPLC: Reverse phase High Performance liquid chromatography; API: Active Pharmaceutical Ingredient; LOD: Limit of Detection; LOQ: Limit of Quantification; RSD: Relative Standard deviation SILDE**:** Sildenafil Citrate; CLOMI: Clomiphene Citrate.

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