Ind. J. Pharm. Edu. Res., 2025; 59(3s):s1126-s1133.
https://www.ijper.org

Original Article

An Improved Method Development and Validation for the Quantification of Clomifene in Pharmaceutical Formulation and Biological Matrix by LC-MS/MS

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

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

ABSTRACT

Aim: The major objective of current work was to create sensitive tandem mass spectrometric method using electrospray ionization and liquid chromatography for quantifying Clomifene in biological matrices. **Materials and Methods:** A stationary Phenomenex C_{18} column with dimensions of 50 mm×4.6 mm and 5 µ sizes of particles were used to achieve chromatographic elution. Isocratic eluting of components was done using methanol and 0.10% V/V HCOOH in the fraction of 85:15V/V as a movable phasic system. For drug and internal standard separation, liquid-liquid extraction was carried out using methanol and ethyl acetate (1:4) solvent solution. **Results:** On multiple reaction monitoring, ions of molecular and products were seen at m/z 406.19→125.01 for Clomifene and 450.12→160.03 for Bictegravir internal standard. The drug's linearity graph had an r^2 value of 0.9998 and was rectilinear at concentrations between 400 and 16000 ng/mL. The inter and intra-batch accuracy % relative standard deviation values ranged from 2.54 to 5.21. The percent recovery results of the LQC, MQC and HQC samples were 102.85%, 97.84% and 94.27%, respectively. This approach has made excellent recoveries. **Conclusion:** Clomifene is more stable for a longer period of time and the developed approach was successfully applicable to routine examination of Clomifene in biological samples.

Keywords: Clomifene, LC-MS/MS, USFDA guidelines, Validation, Stability, Linearity, Matrix effect.

Correspondence:
Mrs. Rubina Kauser
Department of Pharmacy, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, INDIA.
Email: arkay0990@gmail.com

Received: 27-11-2024;
Revised: 08-01-2025;
Accepted: 17-04-2025.

INTRODUCTION

Clomifene, also known as clomiphene, is a medication that is used to treat infertility in women who do not ovulate, including those with polycystic ovary syndrome. Use results in a greater chance of twins. It is taken by mouth once a day, with a course of treatment that usually lasts for 5 days.[1,2]

In 1967, the United States Food and Drug Administration gave authorization for the use of clomifene in medical treatment. Under the heading "Ovulation inducers" (on the Complement Listing), it may be found on the list of essential drugs maintained by the World Health Organization[3] (WHO). The beginning of the age of assisted reproductive technology was marked by its introduction.[4] It has also been discovered that clomifene and the pure isomer of enclomiphene, had a potent potential to increase or restore testosterone levels in males who suffered from hypogonadal conditions.[5] Chemically Clomifene designated as (*E, Z*)-2-(4-(2-chloro-1, 2-diphenyl ethenyl) phenoxy)-*N, N*-diethylethanamine with a molecule mass and formula of 405.966 g·mol^{-1} and $C_{26}H_{28}ClNO$ (Figure 1).

Pelvic discomfort and hot flashes are two of the most common adverse effects. Alterations in eyesight, nausea, vomiting, ovarian cancer, difficulty in sleeping and seizures are some of the other potential adverse effects. People who have liver illness, abnormal vaginal bleeding of unknown origin, or who are pregnant are not advised to take it. Clomifene is a nonsteroidal pharmaceutical that belongs to the same family as other drugs known as Selective Estrogen Receptor Modulators (SERMs). The hypothalamus is responsible for the release of gonadotropin, which is followed by the release of gonadotropin from the anterior pituitary. This is how it works.[6-9]

Literature on Clomifene reveals that there are 2 analytical procedures on liquid chromatography for Clomifene citrate[10] and LC-MS/MS[11-16] methods, which were reported for the determination of Clomifene in sample solutions. The present research objective was to develop a sensitive, precise, and accurate LC-MSMS technique for quantitation of Clomifene in biological solutions.

DOI: 10.5530/ijper.20255465

MATERIALS AND METHODS

Reagents and Chemicals

Dr. Reddy's Laboratory in Hyderabad, India provided the Clomifene (purity: 99.87%). From MSN Labs in Telangana, India, Bictegravir of 99.94% was acquired. HCOOH of analytical quality and HPLC grade acetonitrile were purchased from JT Bakers in Telangana, India. The Milli-Q®RO system's built-in water supply was used for the execution of moveable solvent system and washing solutions.

LC-MS/MS Instrument

In this study, a SCIEX API 4000 LC-MS/MS system was employed, featuring a positive Electro-Spray Ionization (ESI) source. The setup included a Shimadzu Prominence HPLC system, equipped with a binary pumping unit, a column oven, and a SILHTC auto sampler. Data collection, integration, and quantification were carried out using Analyst software version 1.6.30.

Liquid Chromatographic System

Chromatographic isolation was carried out using a Phenomenex C_{18} column with dimensions of 50 mm × 4.6 mm and a particle size of 5 µm. The injection volume was set at 5 µL. The flow rate was maintained at 0.8 mL/min, and the column temperature was kept at 25°C. An isocratic separation was performed using a mobile phase composed of methanol and 0.10% V/V formic acid in an 85:15 V/V ratio. Clomifene and Bictegravir, the internal standard, were eluted within a total run time of 3 min. The autosampler and column temperatures were controlled at 5°C and 25°C, respectively.

Instrument Parameters

Clomifene and an internal standard were analyzed using a mass spectrometer in positive mode, employing the Multiple Reaction Monitoring (MRM) technique. The temperature for both the drying and sheath gases was set at 450°C under modified instrument parameters for Clomifene and Bictegravir. The nebulizer pressure was maintained at 25 psi, with flow rates for the drying and sheath gases at 15 and 4 L/min, respectively. A capillary voltage of 3 kV was applied. Each transition had a dwell time of 200 ms. For Clomifene and the internal standard, the collision energies and fragmentor voltages were set at 15 eV/15 eV and 115 V/110 V, respectively. The transitions monitored were m/z 406.19→125.01 for Clomifene and m/z 450.12→160.03 for the internal standard.

Linearity Controls

A fresh stock solution of Clomifene at 1000 µg/mL was prepared by dissolving 100 mg of the drug in 100 mL of mobile phase. Linearity controls were prepared by spiking plasma blank samples with Clomifene standard solutions to achieve final concentrations of 400, 600, 1800, 3600, 6000, 9000, 12500, and 16000 ng/mL.

Quality Controls

The Quality Control (QC) samples were prepared at three different levels: Lower Quality Controls (LQC), Middle Quality Controls (MQC), and Higher Quality Controls (HQC). These QC samples were made in alignment with the calibration standards, resulting in concentrations of 1120, 8000, and 12000 ng/mL for LQC, MQC, and HQC, respectively. The processed controls were stored at -200°C until the time of analysis.

Method of Sample Preparation

To prepare the sample solution, 100 µL of bictegravir (1 µg/mL) was mixed with 200 µL of plasma and vortexed for 2 min. Clomifene and the Internal Standard (IS) were extracted using a solvent system consisting of acetonitrile and 4 mL of ethyl acetate (1:4). The solution was then centrifuged at 5000 rpm for 25 min. After centrifugation, the organic layer was separated and dried using a lyophilizer. The dried residue was reconstituted in 250 µL of mobile phase and transferred into pre-labeled vials. These vials were then infused into the LC-MS/MS system via an auto-sampler.

Method Validation

Method validation involves a thorough evaluation of an analytical or experimental procedure to assess its accuracy, precision, selectivity, and reliability, ensuring that it consistently produces dependable and credible results within established parameters. This process confirms the method's suitability for its intended application and provides confidence in the obtained data. Key characteristics such as selectivity, stability, specificity, linearity, matrix effect, precision, recovery, and accuracy were used to validate the developed technique.[12]

RESULTS

Optimization of Mass Equipment Conditions

To enhance sensitivity and specificity for mass estimation, the MRM approach in positive mode was employed with Clomifene. Standard controls were introduced into the mass spectrometer via syringe pumps to identify precursors and product ions, as ESI

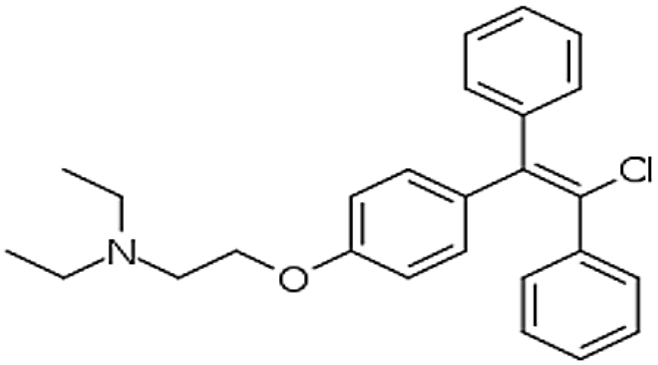

Figure 1: Structure of Clomiphene.

was considered the optimal ionization source for LC-MS/MS. The mass spectra of Clomifene and the Internal Standard (IS) were recorded, with product ions selected for detection at m/z 125.01 and 160.03. Additionally, the mass spectrometer parameters, including ion-spray voltages, temperatures, capillary voltage, heater, collision energy, curtain gases, and nebulizer gases, were optimized to maximize mass response.

Selection of Internal Standard

Bictegravir was selected as the Internal Standard (IS) in this study because method validation results showed no interference at the analyte and bictegravir retention times. Additionally, it exhibited similar chromatographic behavior, ionization efficiency, extraction effectiveness, and retention properties to Clomifene.

Method Validation

Specificity

To evaluate the specificity of the method, plasma blank samples from six different lots were spiked with LLOQQC and bictegravir along with Clomifene. The retention times for the internal standard and Clomifene were found to be 1.18 and 2.5 min, respectively, as shown in Figure 2. The analytical results for Clomifene revealed no significant matrix interference or overlap with the internal standard. Additionally, the responses of all interference peaks were found to be less than 20% of the LLOQQC sample responses.[13]

Linearity and Sensitivity

The method's linearity for Clomifene was established, demonstrating good linearity within the concentration range of 400–16000 ng/mL. The linearity plots were generated by plotting the peak area ratios of Clomifene to the Internal Standard (IS) against concentrations (x), using a $1/C^2$ weighting factor (Table 1). The equation of the calibration graph was $y = 0.000099x + 0.00335$ (Figure 3), with an r^2 value of 0.9998. The lower limit of quantification (LLOQ) for Clomifene was 400 ng/mL (S/N > 10), which was sufficient for accurate quantification of Clomifene in plasma samples.[14]

Accuracy and Precision

Six plasma samples spiked with Clomifene at HQC, MQC, LQC, and LLQC levels were analyzed in one batch and in three subsequent batches to assess the precision and accuracy of intra-batch and inter-batch measurements. The results of the accuracy and precision tests for Clomifene quantification are presented in Table 2. The inter- and intra-batch accuracy and Relative Standard Deviation (RSD) values ranged from 2.54% to 5.21%. [15,16]

Extraction Recovery

Prior to analysis, the biological samples were effectively pretreated. The extraction recoveries were determined by measuring the peak response ratios of Clomifene at HQC, MQC, and LQC levels (n=6) to the extracted spiked sample solutions at the corresponding concentration levels. Similarly, the extraction recovery of the

Table 1: Linearity standard solutions for Clomifene.

LS-ID	Concentration (ng/mL)	Average response	IS response	Area response
LS -1	400	4832	115942	0.041676
LS -2	600	7202	115512	0.062349
LS -3	1800	21462	115643	0.185588
LS -4	3600	42459	115185	0.368616
LS -5	6000	68980	115943	0.594948
LS -6	9000	101631	115825	0.877453
LS -7	12500	144774	115741	1.250845
LS -8	16000	182846	115369	1.58488

LS: Linearity standard, **IS:** Internal standard.

Table 2: Clomifene Precision and Accuracy for Inter-batch and Intra-batch.

Concentration level	Nominal concentration (ng/mL)	Intra-batch			Inter-batch		
		Amount found (ng/mL)	%Accuracy	%RSD	Amount found (ng/mL)	%Accuracy	%RSD
LLOQ	400	385.64	96.41	3.84	415	103.75	5.21
LQC	1120	1061.872	94.81	4.91	1087.072	97.06	2.54
MQC	8000	8298.4	103.73	4.86	8219.2	102.74	3.91
HQC	12000	11445.6	95.38	2.76	11560.8	96.34	4.11

Table 3: Clomifene and IS extraction recoveries.

Concentration level	A	B	% Recovery	% Mean recovery	%RSD
LQC	12689	13050	102.85	98.32	3.58
MQC	90640	88682	97.84		
HQC	135960	128169	94.27		
IS	115324	113202	98.16		

Table 4: Clomifene findings for Matrix Effect.

Clomifene	LQC level			HQC level		
	Analyte MF	IS MF	IS normalized MF	Analyte MF	IS MF	IS normalized MF
B-1	1.13	1.07	1.05	1.02	1.03	0.99
B -2	1.07	1.04	1.03	1.11	1.07	1.04
B -3	1.1	1.12	0.98	1.1	1.01	1.03
B -4	1.12	1.02	1.05	1.07	1.08	0.99
B -5^{x}	1.08	1.06	1.02	1.03	1.02	1.01
B -6^{x}	1.04	1.12	0.96	1.09	1.02	1.07
B -7^{y}	1.06	1.03	1.03	1.08	1.13	0.97
B -8^{y}	1.01	1.05	0.97	1.02	1.09	0.96
Mean	1.016			1		
SD	0.045			0.049		
%RSD	4.46			4.91		

X: Hemolyzed lot; **MF:** matrix factor; **Y:** Lipemic lot; **RSD:** Relative standard deviation.

Table 5: Clomifene stability findings.

Parameter	QC level	P	Q	%RSD	%Stability
Freeze and thaw stability.	LQC	1120	1059.956	3.84	94.64
	HQC	12000	11805.31	2.81	98.38
Stability in refrigerator (1-10°C for 48 hr).	LQC	1120	1145.898	4.91	102.31
	HQC	12000	11921.51	3.64	99.34
Bench-top stability (at <10°C for 20 hr).	LQC	1120	1163.592	2.73	103.89
	HQC	12000	11501.55	4.27	95.85
Long term stability (60 days at -20°C).	LQC	1120	1105.987	4.14	98.75
	HQC	12000	12426.16	3.52	103.55
In-injector stability (at 10°C for 72 hr).	LQC	1120	1040.798	3.94	92.93
	HQC	12000	12248.78	4.06	102.07
Long-term stability (60 days at -70°C).	LQC	1120	1089.579	3.28	97.28
	HQC	12000	11733.04	4.27	97.78

Q: mean concentrations (ng/mL); **P:** nominal concentration (ng/mL) of analytes.

Internal Standard (IS) was determined by comparing the peak response ratios of quality control plasma samples (n=6) to spiked plasma sample solutions at different concentrations. The average extraction recoveries of Clomifene at lower, middle, and higher QC levels were 102.85%, 97.84%, and 94.27%, respectively. At a concentration of 650 ng/mL, the average extraction recovery of the IS was 98.16%. The results are shown in Figure 4 and Table 3.[16]

Matrix Effect

Due to the accuracy of the method, co-eluted matrix components can either reduce or enhance ionization in the mass spectrometer, potentially leading to insignificant responses in the blank matrices. Consequently, the bictegravir normalized matrix factor was determined in eight different sources of human plasma, including two hemolytic and two lipemic batches. The results, shown in Table 4, indicate that the mean IS normalized matrix

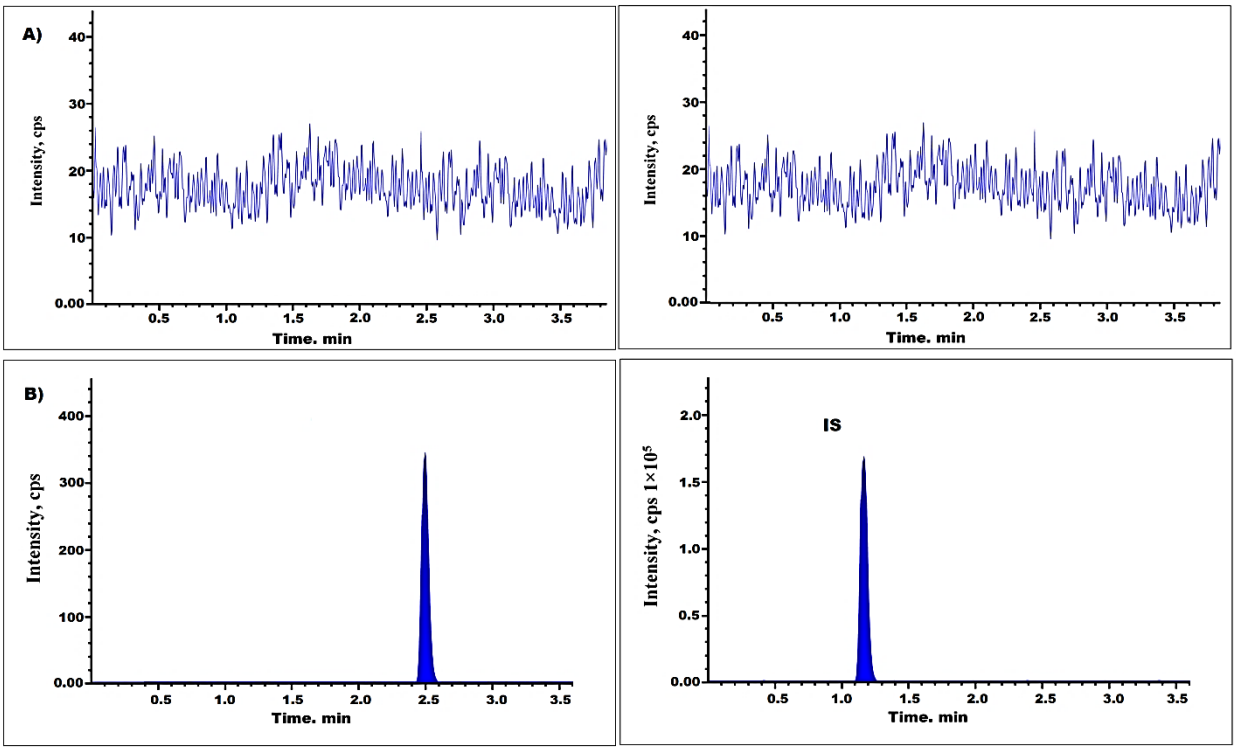

Figure 2: (A) Blank plasma and (B) LLOQ sample chromatograms.

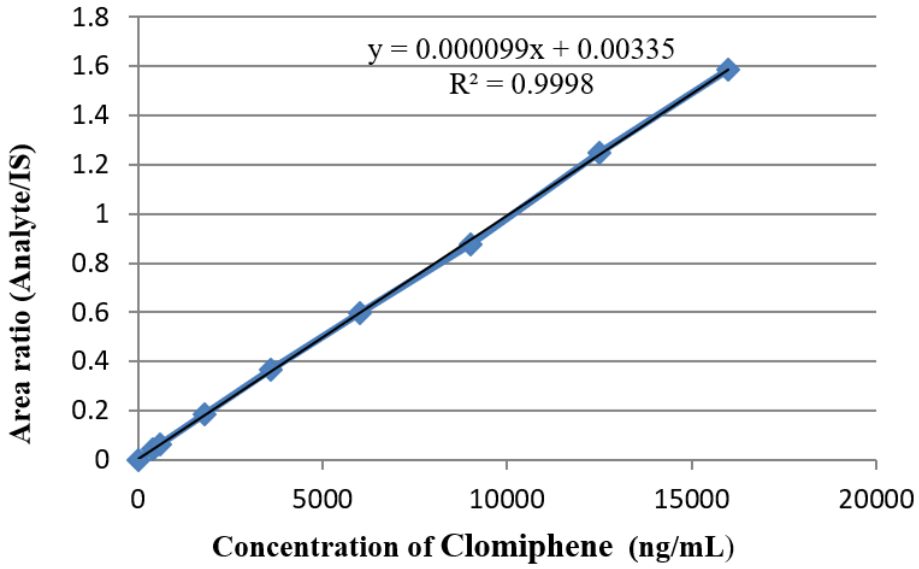

Figure 3: Linearity of Clomiphene.

factors for all drug components ranged from 0.961 to 1.052, with a % RSD of 4.915.[16]

Stability

Clomifene's stability was assessed using both matrix-based and aqueous-based samples. The stock solution in diluents remained stable at 1-10°C for two days, while both Clomifene and the Internal Standard (IS) were stable at 1-10°C for 70 days. Matrix stabilities were evaluated at -70°C and -20°C, over a two-month period using freshly prepared spiked linear standards. The results of the stability study are presented in Table 5. When stored below 10°C and subjected to six freeze-thaw cycles, the drug remained stable for up to 20 hr. Additionally, processed samples did not degrade for up to 72 hr in the auto-sampler at 10°C.

Dilution Integrity

The ULOQ (Upper Limit of Quantification) for Clomifene was determined at double the concentration of the dilution integrity.[12,15] The mean back-computed drug contents for the

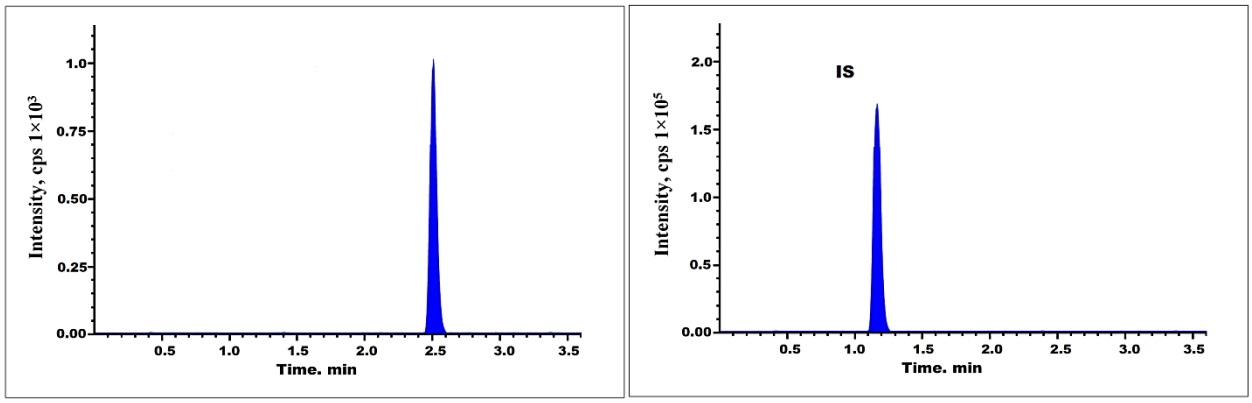

Figure 4 (a): Clomiphene chromatogram at LQC standard.

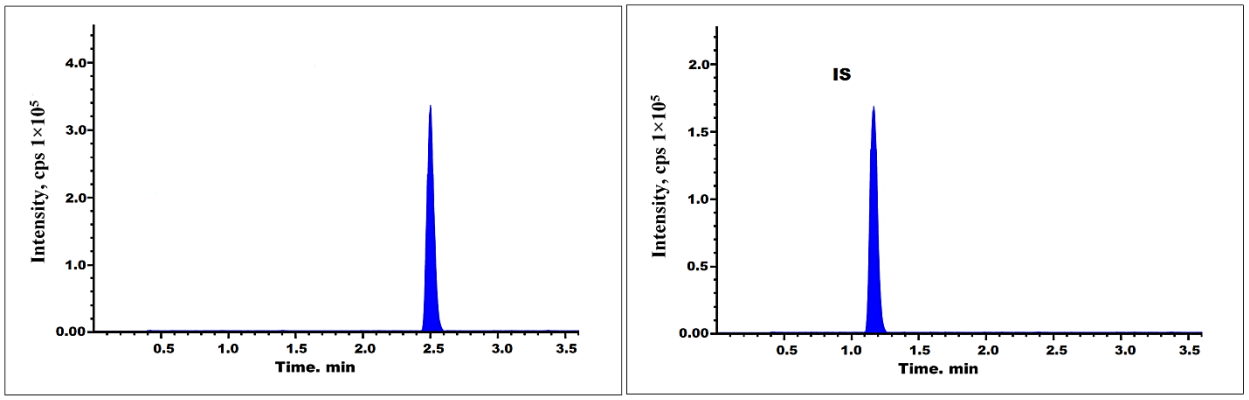

Figure 4 (b): Clomiphene chromatogram at MQC standard.

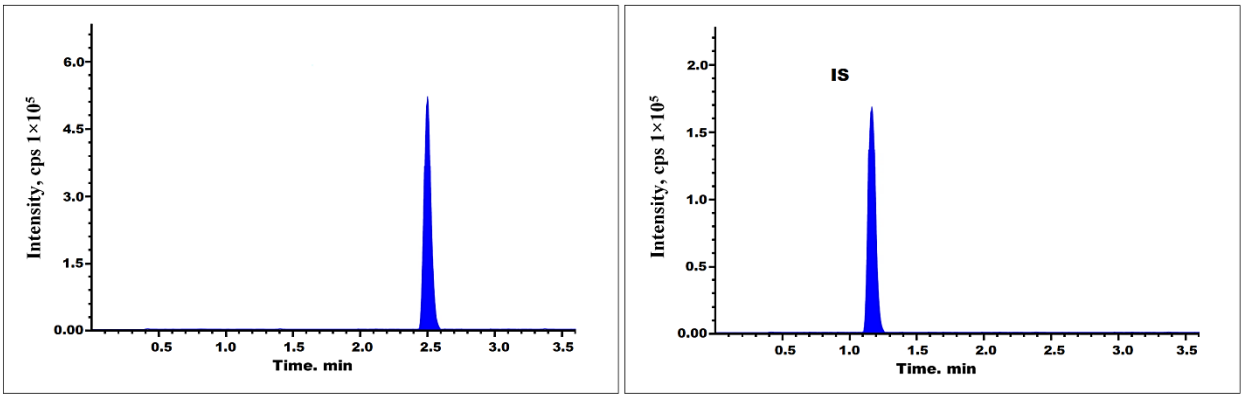

Figure 4 (c): Clomiphene chromatogram at HQC standard.

dilution tested sample solutions ranged from 85 to 115% of the nominal amount after a 1:4 dilution, with a % RSD of 3.86.

DISCUSSION

This study focuses on optimizing mass spectrometry parameters and validating an LC-MS/MS method for accurately quantifying Clomifene using Multiple Reaction Monitoring (MRM). The primary objective was to enhance sensitivity and specificity, ensuring precise quantification in plasma samples. The MRM approach, coupled with Clomifene, was optimized to improve sensitivity and specificity. Standard controls were infused using syringe pumps, and key mass spectrometry parameters—such as ion-spray voltages, temperatures, capillary voltage, collision, curtain gases, and nebulizer gases—were adjusted. Precise precursor and product ions (m/z 142.05 and 160.03) for Clomifene and its Internal Standard (IS) were selected to ensure accurate mass spectra.

Bictegravir was chosen as the IS due to its non-interference at Clomifene's retention time and its similar chromatographic behavior, ionization, extraction efficiency, and retention properties. The method's specificity was confirmed by spiking plasma blank samples with Clomifene and IS, showing no

interference at retention times of 1.18 min for IS and 2.5 min for Clomifene. Interference peaks were less than 20% of the LLOQ sample, indicating excellent specificity.

The method demonstrated excellent linearity for Clomifene across the concentration range of 400 to 16000 ng/mL, with a calibration curve equation (y=0.000099x+0.00335) and an r^2 value of 0.9998. The Lower Limit of Quantification (LLOQ) for Clomifene was determined to be 400 ng/mL, ensuring reliable measurement.

Accuracy and precision were evaluated using intra-batch and inter-batch analyses, with Relative Standard Deviation (RSD) values ranging from 2.54% to 5.21%, confirming the method's consistency. Extraction recoveries for Clomifene were 102.85%, 97.84%, and 94.27% at lower, middle, and higher quality control levels, respectively. IS recovery at 650 ng/mL was 98.16%.

Matrix effect evaluation showed consistent results for Bictegravir normalized matrix factors across different human plasma samples, with a mean RSD of 4.915%. Stability studies confirmed that both Clomifene and IS remained stable under various temperature conditions and freeze-thaw cycles, demonstrating the method's robustness in sample storage and processing.

Dilution consistency was validated with a 1:4 dilution, yielding a mean RSD of 3.86%, indicating the method's reliability in accurate drug concentration measurement. The optimization of mass spectrometry parameters and comprehensive method validation ensures the LC-MS/MS method's specificity, sensitivity, and reliability for precise Clomifene quantification. This method is well-suited for pharmacokinetic and clinical research applications.

CONCLUSION

A sensitive and precise LC-MS/MS method was developed and validated for the effective quantification of Clomifene in human plasma. The method demonstrated high specificity, accuracy, linearity, precision, and stability. The linearity equation (y=0.000099x+0.00335) and correlation coefficient (r^2 = 0.9998) confirmed the method's excellent linearity. For the QC samples (400, 1120, 8000, and 12000 ng/mL), the intra- and inter-day precision (% RSD) ranged from 2.54% to 5.21%, indicating excellent reproducibility. Stability studies, conducted under various conditions, showed stability values ranging from 92.93% to 103.89%. These findings validate the method's reliability, making it suitable for pharmacokinetic and toxicokinetic investigations of Clomifene in various biological matrices for clinical and forensic purposes.

ACKNOWLEDGEMENT

We would like to sincerely thank the faculty of Department of Pharmacy, Annamalai University and St. Pauls College of Pharmacy for their continuous support and invaluable assistance throughout our research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; **HCOOH:** Formic Acid; **SERM's:** Selective Estrogen Receptor Modulators; **HPLC:** High Performance Liquid Chromatography; **MRM:** Multiple Reaction Monitoring; **IS:** Internal Standard; **m/z:** Mass to Charge number of ions; **HQC:** High Quality Control; **MQC:** Middle Quality Control; **LQC:** Lower Quality Control; **LLQC:** Lower Limit Quality Control; **(LLOQ)QC:** Lower Limit of Quantification Quality Control; **RSD:** Relative Standard Deviation; **ULOQ:** Upper Limit of Quantification.

SUMMARY

In the present study, a reliable, sensitive, accurate and robust method was developed and optimized using LC-MS/MS for the quantification of Clomifene in Pharmaceutical formulation and biological matrix. The analytical method was validated according to the ICH guidelines for various parameters. All the values of the validation parameters were found to be within the acceptance limit as per the ICH guidelines. Hence, the developed and validated LC-MS/MS method can be used for routine analysis.

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Cite this article: Kauser R, Chaitanya PSK, Venkatesan P. An Improved Method Development and Validation for the Quantification of Clomifene in Pharmaceutical Formulation and Biological Matrix by LC-MS/MS. Indian J of Pharmaceutical Education and Research. 2025;59(3s):s1126-s1133.