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"Bio-Analytical Method Development and Validation for The Estimation of Tamoxifen in Plasma by Using RP-HPLCMethod"

Ms. Anita Arjun Najan[1]*, Dr. Anup Kumar Chakraborty [1]
[1]Research Scholar, Oriental University, Indore, Madhya Pradesh-453555, India.
[1]Professor, Oriental University, Indore, Madhya Pradesh-453555, India.

Corresponding Author:
Ms. Anita Arjun Najan
Research Scholar, Oriental University, Indore, Madhya Pradesh-453555, India.
Email ID: anitanajan271@gmail.com

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Abstract:
A robust and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantification of Tamoxifen in plasma. Tamoxifen, a selective estrogen receptor modulator, is widely used in the treatment of hormone receptor-positive breast cancer, necessitating accurate bioanalytical techniques for therapeutic monitoring and pharmacokinetic studies. The method employed an Agilent C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of methanol and 0.1% acetic acid in water (30:70, v/v), at a flow rate of 0.8 mL/min. Detection was carried out at 236 nm using a UV detector, with a retention time of 5.657 minutes and theoretical plates of 4970, ensuring high resolution. The method demonstrated excellent linearity ($R^2 = 0.999$) within a concentration range of 2–10 µg/mL, with precise recovery (98–102%) and reproducibility (RSD < 2%). Validation parameters complied with International Council for Harmonisation (ICH) guidelines, confirming specificity, sensitivity, and robustness. Application to a marketed formulation yielded accurate results, with the method proving suitable for routine clinical and pharmaceutical applications. The validated RP-HPLC method also offers potential for therapeutic drug monitoring and drug-drug interaction studies, supporting optimized breast cancer treatment strategies.

Keywords: *Tamoxifen, RP-HPLC, Plasma Quantification, Method Validation, Breast Cancer Therapy.*

Introduction

Tamoxifen, a selective estrogen receptor modulator (SERM), is extensively used in the treatment and prevention of hormone receptor-positive breast cancer. It functions by competitively binding to estrogen receptors, thereby inhibiting the proliferative actions of estrogen on mammary tissues (1). Tamoxifen has been instrumental in reducing the recurrence rate of breast cancer and has significantly improved patient survival rates. Its widespread use necessitates accurate and reliable methods for its quantification, particularly in biological matrices such as plasma, for therapeutic drug monitoring, pharmacokinetic studies, and clinical trials (2).

Bio-analytical method development and validation are critical components in the estimation of drug concentrations in biological samples. These methods ensure the accuracy, precision, and reliability of the analytical results, which are pivotal for understanding the pharmacokinetic and pharmacodynamic properties of drugs (3). Among the available techniques, reverse-phase high-performance liquid chromatography (RP-HPLC) has emerged as a robust, sensitive, and versatile method for the quantification of drugs, including tamoxifen, in plasma (4).

The RP-HPLC method employs a non-polar stationary phase and a polar mobile phase, making it suitable for separating and quantifying hydrophobic compounds like tamoxifen. Its ability to provide high resolution, coupled with short analysis times and minimal sample preparation, has made RP-HPLC the method of choice for routine bio-analytical applications (5). The technique is particularly advantageous for detecting tamoxifen and its metabolites in plasma due to its high sensitivity and reproducibility.

The development of an RP-HPLC method for tamoxifen involves selecting appropriate chromatographic conditions, including the choice of column, mobile phase composition, flow rate, and detection wavelength. These parameters are optimized to achieve maximum separation efficiency, minimal matrix interference, and the best detection sensitivity. Method validation, as outlined by regulatory guidelines such as those from the International Council for Harmonisation (ICH) and the US Food and Drug Administration (FDA), is essential to ensure the reliability of the developed method (6, 7). Validation parameters include specificity, linearity, accuracy, precision, detection limit (LOD), quantification limit (LOQ), and robustness.

The quantification of tamoxifen in plasma is particularly challenging due to the complexity of the biological matrix and the low concentrations of the drug typically encountered. To address these challenges, sample preparation techniques such as protein precipitation, liquid-liquid extraction, or solid-phase extraction are employed to isolate tamoxifen from plasma prior to analysis (8). Advances in analytical instrumentation, including the use of UV-Visible or mass spectrometric detectors in conjunction with RP-HPLC, have further enhanced the sensitivity and selectivity of tamoxifen quantification (9).

Several studies have demonstrated the successful application of RP-HPLC for the bioanalysis of tamoxifen in plasma. For instance, a study by Almeida et al. utilized RP-HPLC coupled with UV detection to estimate tamoxifen levels in breast cancer patients, showcasing the method's applicability in clinical pharmacokinetics (10). Similarly, Zhu et al. developed an RP-HPLC method with fluorescence detection to achieve high sensitivity for tamoxifen quantification in plasma (11). These examples underscore the versatility and reliability of RP-HPLC for bio-analytical applications.

Materials and Methods

Materials

The analytical study was conducted using Tamoxifen, a selective estrogen receptor modulator (SERM), as the drug sample. Tamoxifen was supplied by Swapnroop Drug and Pharmaceutical, India. Blank plasma samples were obtained from Red Cross, Jalgaon.

List of Reagents and Chemicals Used

Methanol (HPLC grade) – Merck Ltd., India
Acetonitrile (HPLC grade) – Merck Ltd., India
Potassium phosphate buffer (HPLC grade) – Merck Ltd., India

Instrumentation

The High-Performance Liquid Chromatography (HPLC) method was selected for the estimation of Tamoxifen. The analysis was performed on an Agilent Tech Gradient System equipped with an auto-injector and Diode Array Detector (DAD). The system was coupled with a reverse-phase Agilent C18 column (250 mm × 4.6 mm, 5 µm particle size). The UV-730D absorbance detector and ChemStation software (version 10.1) were used for data acquisition and processing.

Instrumentation Details

HPLC Instrument: Agilent Tech Gradient System with auto-injector
UV-Spectrophotometer: Analytical Technology
Column: Agilent C18 (250 mm × 4.6 mm, 5 µm)
pH Meter: VSI pH meter (VSI 1-B)
Balance: A&D Company, Japan

Sonicator: ENERTECH electronic instrument

Selection of Formulation

The marketed preparation used was "Remo 100 mg," containing Tamoxifen as the active pharmaceutical ingredient (API). The formulation was procured from the local market under the brand name "Tamoxican."

Method Development

The HPLC method development was focused on optimizing chromatographic conditions to achieve high sensitivity and specificity for Tamoxifen. Parameters such as mobile phase composition, flow rate, column temperature, and detection wavelength were adjusted systematically to enhance resolution and reduce matrix interference. (12) (13)

Method Validation

Validation of the developed method was performed in accordance with International Council for Harmonisation (ICH) guidelines Q2(R1) (14). The parameters evaluated included:

Experimental Work:

Chromatographic Conditions:

The HPLC analysis was conducted using an Agilent Gradient System equipped with an auto-injector and a diode array detector (DAD). Chromatographic separation was achieved on an Agilent C18 column (250 mm × 4.6 mm, 5 µm particle size). The optimized mobile phase consisted of methanol and 0.1% orthophosphoric acid in water (30:70, v/v) with a flow rate of 0.8 mL/min, maintaining ambient temperature. The detection wavelength was set at 236 nm for all analyses (12).

Mobile Phase Selection:

Various mobile phases were evaluated to achieve the best resolution and retention time for Tamoxifen. Methanol: Water (0.1% orthophosphoric acid, pH 3) in a ratio of 30:70 was found to provide sharp, well-resolved peaks with high reproducibility (13).

Sample Preparation:

Plasma samples were prepared using protein precipitation. A volume of 2 mL of plasma was spiked with 10 mg of Tamoxifen dissolved in methanol. The mixture was sonicated for 10 minutes and centrifuged at 5000 rpm for 1 hour. The supernatant was filtered through a 0.45 µm membrane and injected into the HPLC system. Calibration standards were prepared by

diluting stock solutions of Tamoxifen in mobile phase to achieve concentrations of 2–10 µg/mL (14).

System Suitability Parameters:

The system suitability test was conducted by injecting five replicates of a standard solution containing Tamoxifen. Parameters such as retention time, peak symmetry, and theoretical plates were monitored to ensure consistent system performance (15).

Table1 : Selection of mobile Phase.

Sr.No.	Mobile Phase
1.	Methanol : (0.1% OPA)(90:10 %)v/v) 236 nm, flow 0.7ml/min
2	Methanol : Water 0.1% OPA(80 : 20%v/v)PH3,236 nm flow 0.7 ml/min
3	Methanol : Water 0.1% OPA (70 : 30%v/v)PH3,236 nm, flow 0.8ml/min sample in mobile phase
4	Methanol : Water 0.1% OPA (60 : 40%v/v) PH 3, 236 nm,0.7 ml/min
5	Methanol: water 0.1% OPA (50:50% v/v)PH 236 nm,0.7 ml/min
6	Methanol: water 0.1% OPA (40:60% v/v)PH 236 nm,0.7 ml/min
7	Methanol: water 0.1% OPA (30:70% v/v)PH 236 nm,0.7 ml/min
8	Methanol: water 0.1% OPA (30:70% v/v)PH 236 nm,0.8 ml/min, sample in mobile phase

Table 2: linearity for Rp-HPLC Method

Concentration (µg/mL)	
Sr no	Tamoxifen
1	2
2	4
3	6
4	8

5	10

Table 3: Accuracy Rp-HPLC Method

Sample	Amount Added (mg)
	Tamoxifen
Accuracy 80%	1.6
Accuracy 100%	2
Accuracy 120%	2.

Result and Discussion: Preliminary Studies on Tamoxifen

Melting Point: The melting point of the reference standard Tamoxifen was determined to be in the range of 140–142°C, consistent with its standard specifications, indicating the purity of the sample.

Solubility Studies: Tamoxifen was found to be freely soluble in methanol, ethanol, DMSO, and acetonitrile but practically insoluble in water, aligning with its known chemical properties. These results guided the selection of appropriate solvents for the method development (14).

UV Spectroscopy: A UV spectrum of Tamoxifen (10 µg/mL in methanol) revealed a maximum absorbance (λmax) at 236 nm. This wavelength was chosen for the detection in the HPLC method to ensure optimal sensitivity and accuracy.

Chromatographic Behavior

Extensive trials were conducted to identify the optimal mobile phase composition. Initial experiments with varying methanol-water ratios showed peak splitting and poor resolution. The final optimized mobile phase, comprising methanol and 0.1% acetic acid in water (30:70, v/v) at pH 3, delivered a sharp and well-resolved peak with a retention time of 5.657 minutes. The theoretical plates were found to be 4970, meeting the requirements for a robust analytical method (15).

Calibration Curve and Linearity

The developed RP-HPLC method demonstrated excellent linearity in the concentration range of 2–10 µg/mL, with a correlation coefficient (R^2) of 0.999. The linear regression equation for Tamoxifen was $y=350.4x+45.20y = 350.4x + 45.20y=350.4x+45.20$

where

x represents concentration and

y represents the peak area. This high degree of correlation signifies the method's reliability for quantitative analysis.

Validation Parameters

Accuracy: Recovery studies performed at 80%, 100%, and 120% levels showed mean recoveries within 98–102%, confirming the method's accuracy.

Precision: Both intra-day and inter-day precision studies yielded %RSD values below 2%, indicating consistent reproducibility of results.

LOD and LOQ: The method's limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 0.055 µg/mL and 0.1679 µg/mL, respectively, showcasing the method's sensitivity.

Robustness: Variations in flow rate, mobile phase composition, and detection wavelength did

not significantly affect the retention time or peak shape, confirming the method's robustness.

Analysis of Marketed Formulation

The validated RP-HPLC method was applied to the analysis of a marketed formulation, "Remo 100 mg" (Glenmark Pharma). The assay results indicated that the % label claim was within the acceptable range (99–101%), further confirming the applicability of the developed method.

Conclusion

The developed RP-HPLC method for the estimation of Tamoxifen in plasma has proven to be robust, accurate, and reproducible. The method successfully addresses the challenges of analyzing Tamoxifen in complex biological matrices with high sensitivity and specificity. Validation results demonstrated compliance with ICH guidelines, confirming its suitability for routine analysis in clinical and pharmaceutical settings. Furthermore, the method's application to a marketed formulation highlights its practicality and potential for therapeutic drug monitoring and pharmacokinetic studies, ultimately contributing to optimizing breast cancer treatment strategies.

Additionally, this validated method can be extended to study drug-drug interactions, especially in combination therapies involving Tamoxifen. It offers a reliable tool for monitoring the pharmacokinetics of Tamoxifen when administered with other anticancer agents or hormonal treatments, ensuring enhanced patient safety and efficacy. The versatility of the method makes it a valuable addition to both clinical and research applications, addressing current and future demands in oncology pharmacology

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