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Development and Validation of an HPLC Method for Quantifying Endoxifen in pharmaceutical formulation: Analytical and Forced Degradation Studies

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Abstract:

This study presents the development and validation of a high-performance liquid chromatography (HPLC) method for the quantification of Endoxifen, a potent active metabolite of Tamoxifen, in pharmaceutical formulation. Endoxifen, known for its enhanced therapeutic potential in treating estrogen receptor-positive (ER+) breast cancer, requires precise analytical methods for accurate measurement in plasma, serum, and tissue samples. The method was optimized using a Zodiac C18 column and a mobile phase consisting of 0.1% trifluoroacetic acid buffer and acetonitrile in a 78:22 ratio, with UV detection at 278 nm. The chromatographic conditions included a flow rate of 1.0 mL/min and a column temperature of 30°C. Validation of the method was conducted following ICH Q2 (R1) guidelines, demonstrating excellent specificity, linearity, precision, and accuracy. The method exhibited a linear response over the concentration range of 2.5 to 15 µg/mL, with a correlation coefficient of 1. Precision studies, including repeatability and intra-day and inter-day variations, showed low % RSD values, confirming the method's consistency. Accuracy was affirmed through recovery studies, with mean recoveries close to 100% at all tested levels (50%, 100%, and 150% of the label claim). The robustness of the method was evaluated by varying the detection wavelength, showing negligible impact on performance. Forced degradation studies revealed that Endoxifen is susceptible to degradation under acidic, basic, oxidative, thermal, and photolytic conditions. Oxidative degradation had the most significant effect, with a 17.24% reduction in Endoxifen's peak area. These findings provide insights into Endoxifen's stability and highlight the need for stability-indicating methods. The method's limit of detection (LOD) was 0.017 µg/mL and limit of quantitation (LOQ) was 0.051 µg/mL, demonstrating high sensitivity. In conclusion, the validated HPLC method offers a reliable approach for Endoxifen quantification in biological matrices, crucial for pharmacokinetic studies and patient safety. The forced degradation analysis supports the development of stability-indicating methods and informs strategies to maintain Endoxifen's efficacy throughout its lifecycle.

Introduction:

Endoxifen, a potent active metabolite of Tamoxifen, has garnered significant attention in recent years due to its enhanced therapeutic potential in the treatment of estrogen receptor-positive (ER+) breast cancer. (1) Unlike Tamoxifen, which requires metabolic conversion to its active forms, Endoxifen exhibits superior pharmacological activity, making it a promising candidate for targeted cancer therapy. Endoxifen exerts its therapeutic effects primarily by inhibiting the proliferation of ER+ breast cancer cells, which has made it a focal point of numerous clinical and pharmacological studies aimed at improving cancer treatment outcomes. (2)

The accurate quantification of Endoxifen in biological matrices such as plasma, serum, and tissue samples is crucial for understanding its pharmacokinetics, optimizing dosage regimens, and ensuring patient safety. Analytical method development and validation are fundamental steps in this process, ensuring that the methods used are reliable, reproducible, and compliant with regulatory requirements. High-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection or mass spectrometry (MS) is widely employed for this purpose, offering high sensitivity and specificity essential for detecting low concentrations of Endoxifen in complex biological samples. (3-5)

Method validation is a rigorous process that involves evaluating various parameters, including accuracy, precision, specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), and robustness. These parameters are assessed according to guidelines established by regulatory bodies such as the International Council for Harmonisation (ICH) and the United States Food and Drug Administration (FDA). The validated method must demonstrate reliability in different settings and across multiple laboratories, ensuring consistent results that can be trusted in both clinical and research environments. (6-7)

In addition to method development and validation, it is equally important to study the stability of Endoxifen under various stress conditions through forced degradation studies. These studies play a pivotal role in understanding the degradation pathways of Endoxifen and identifying potential degradation products that could impact its efficacy and safety. Forced degradation involves subjecting Endoxifen to extreme conditions, including acidic, basic, oxidative, thermal, and photolytic environments. These conditions simulate the challenges that the drug may encounter during manufacturing, storage, transportation, and administration, providing valuable insights into its shelf life and optimal storage conditions. (8)

The results of forced degradation studies are essential for the development of stability-indicating methods, which are capable of distinguishing Endoxifen from its degradation products. Such methods are crucial for ensuring the integrity and potency of Endoxifen throughout its lifecycle, from production to patient administration. Additionally, understanding the degradation profile of Endoxifen aids in the formulation of strategies to minimize degradation and extend the drug's shelf life, thereby enhancing its clinical utility.

This study aims to develop and validate a robust analytical method for the quantification of Endoxifen in pharmaceutical formulation, while also conducting comprehensive forced degradation studies to evaluate its stability. The findings of this research are expected to contribute to the growing body of knowledge surrounding Endoxifen, supporting its development as a therapeutic agent and ensuring its safe and effective use in clinical practice.

Materials and Methods

Materials:

Endoxifen was obtained as gift samples from Intas Pharmaceuticals, India. Different HPLC grade solvents like Acetonitrile, potassium dihydrogen phosphate buffer, trifluro acetic acid, HPLC water was obtained from Ricca Chemical Company. HPLC grade water was used to prepare all the solutions. The formulation of Endoxifen was also obtained from Intas Pharmaceuticals, Ahmedabad as gift sample.

Preparation of Standard stock solutions

Accurately weighed quntitty of 100 mg of Endoxifen was weighed and transferred into suitable volumetric flask and dissolved in to sufficient volume of mobile phase and made upto volume to obtain 1 mg/ml of

Endoxifen. Standard solutions were diluted using mobile phase to obtain working standard solutions (2.5-15 mcg/ml) and all solutions were protected from light.

Preparation of Chromatographic procedure

The chromatographic separation was carried out using a Zodiac C18 column (150 mm × 4.6 mm, 5 µm) from Zodiac Life Sciences. The mobile phase, consisting of a 0.1% trifluoroacetic acid (TFA) buffer and acetonitrile (ACN) in a 78:22 ratio, was pumped at a flow rate of 1.0 mL/min, with the pH adjusted to 3.5. The buffer was prepared by adding 1 mL of TFA to 1000 mL of water, followed by filtration through a 0.45 µm nylon filter and degassing before use. The detection wavelength was set at 278 nm, and a 10 µL sample volume was injected for analysis. The column temperature was maintained at 30°C, with ACN used as the diluent.

Preparation of Sample Solution

Powder equivalent to 10 mg of Endoxifen was weighed and transferred in a 100 ml volumetric flaskand sufficient mobile phase was added to dissolve the drug. It was sonicated for 20 minutes and final volume was made to the mark with mobile phase. The solution was filtered through 0.45 µ membrane filter. 10 ml of aliquot from above stock solution was further diluted to 100 ml with mobile phase to get the final concentration of 10 mcg/ml.

Method Validation

Method validation is a critical process in analytical chemistry that ensures the reliability and accuracy of the method used for the analysis of specific compounds. This process involves a series of tests and procedures designed to demonstrate that the analytical method is suitable for its intended purpose. Validation parameters typically include assessments of specificity, linearity, accuracy, precision, detection limit, quantitation limit, robustness, and system suitability. The validation process confirms that the method can produce consistent, reproducible, and accurate results under specified conditions. This is essential for ensuring the credibility of the analytical data, particularly in regulatory environments where the method's performance must be rigorously documented. In this study, the method was validated following ICH Q2 (R1) guidelines to ensure it meets the necessary standards for reliable quantification of the target analytes in pharmaceutical formulation. (9)

Specificity

The specificity of the analytical method was evaluated by analyzing a blank solution (diluent only), a placebo (sample matrix without the analyte), and a standard solution of the analyte. (Figure 1) The blank and placebo chromatograms were examined to ensure no peaks appeared at the retention time of the analyte, confirming that there was no interference from the diluent or matrix components. Additionally, the analyte was subjected to forced degradation under various stress conditions, including acid and base hydrolysis, oxidation, thermal degradation, and photolysis. The degraded samples were analyzed to determine if any degradation products co-eluted with the analyte peak. The method's specificity was confirmed by demonstrating that the analyte peak was well-resolved from any other peaks, with no significant interference observed in the chromatograms. No interference peak was observed during the placebo and blank analysis. The blank samples of degradation solutions was also analysed and no additional peak was observed on the retention time of Endoxifen along with degradation peaks.

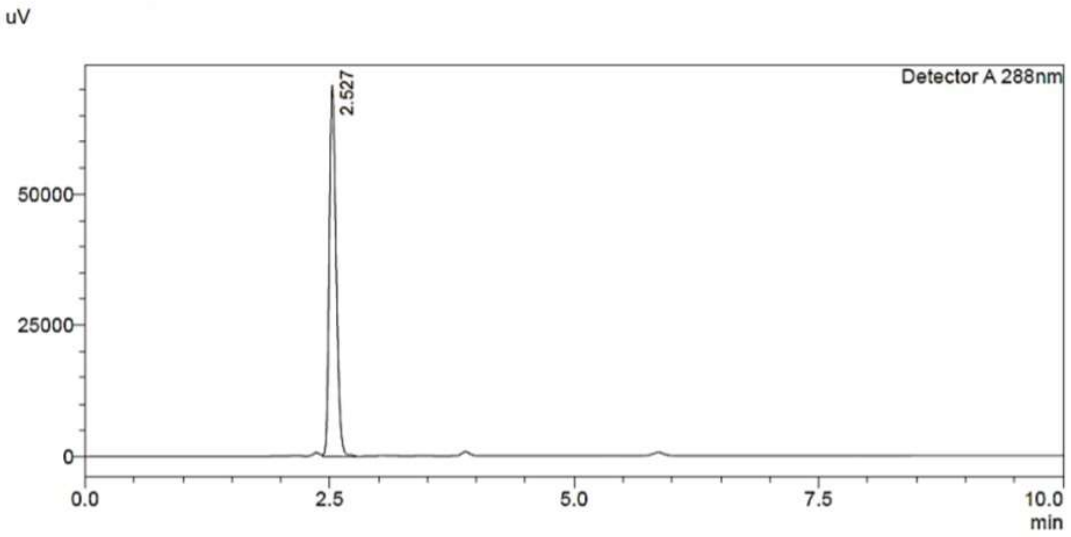


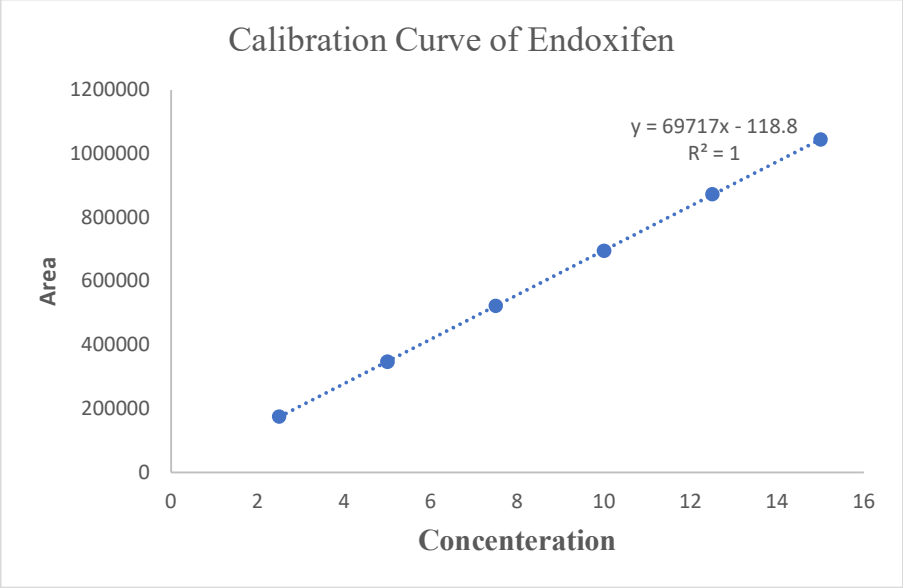
Figure 1: Chromatogram of Endoxifen

Linearity

To prepare the calibration curve, aliquots of 0.25, 0.5, 0.75, 1, 1.25, and 1.5 mL were taken from the standard stock solution and transferred to separate 10 mL volumetric flasks. Each flask was then filled to the mark with the mobile phase to achieve final concentrations ranging from 2.5 to 15 µg/mL. The area under the curve for each concentration was measured using the chromatographic method. A calibration curve of area versus concentration was plotted, and the linearity response was assessed by analyzing this calibration curve for Endoxifen. The correlation coefficient (R^2) and the regression line equation were then determined to evaluate the linear relationship between the area and concentration of Endoxifen.

Table 1: Linearity Data for Endoxifen (n=6)

Concentration (µg/ml)	**Avg. area (µV*sec.) ± SD**	**% RSD**
2.5	175340±2039.66	1.16
5	347122±1762.12	0.51
7.5	523109±2135.77	0.41
10	695463±4671.86	0.67
12.5	873031±2797.11	0.32
15	1045373±1790.36	0.17



The calibration curve for Endoxifen was established with concentrations ranging from 2.5 to 15 µg/mL. The results showed that at 2.5 µg/mL, the average area was 175,340 ± 2,039.66 µVsec., with a % RSD of 1.16. At 5 µg/mL, the average area was 347,122 ± 1,762.12 µVsec., corresponding to a % RSD of 0.51. For 7.5 µg/mL, the average area was 523,109 ± 2,135.77 µVsec., with a % RSD of 0.41. At 10 µg/mL, the average area was 695,463 ± 4,671.86 µVsec., resulting in a % RSD of 0.67. At 12.5 µg/mL, the average area was 873,031 ± 2,797.11 µVsec., with a % RSD of 0.32. Finally, at 15 µg/mL, the average area was 1,045,373 ± 1,790.36 µVsec., and the % RSD was 0.17. These data indicate that the method provided precise and reproducible results across the entire concentration range. The regression coefficient was 1.

Precision

Repeatability

Six replicate solutions of 10 µg/ml for Endoxifen were prepared and analysed using the optimized chromatographic conditions. The areas of the solutions were determined and % RSD was calculated.

Table 2: Repeatability Study data of Endoxifen

Sr. no.	Conc (µg/ml)	Area (µV*sec.)	Average (µV*sec.) ±SD	% RSD
1	10	696389	695223±3055.00	0.44
2	10	693893		
3	10	690938		
4	10	699375		
5	10	697358		
6	10	693385		

Intra-day and Inter-day Precision

To evaluate intra-day and inter-day precision, three concentrations of Endoxifen (5, 10, and 15 µg/mL) were prepared by diluting the standard stock solution with the mobile phase. For intra-day precision, each

concentration was analyzed in triplicate within a single day under the same experimental conditions, and the average areas were calculated along with their standard deviations (SD) and percentage relative standard deviations (% RSD). For inter-day precision, the same concentrations were analyzed in triplicate on three separate days, with the method being run under consistent conditions each day. The average areas, SD, and % RSD were determined for each concentration across the three days. This procedure ensured the assessment of the method's reproducibility and reliability both within a single day and over multiple days. Table 3 indicates the % RSD value for all the concentration was less than 2 which indicates method follows precision criteria.

Table 3: Intra-day and Inter-day Precision study data of Endoxifen

Intra-day precision (n=3)			
Sr. no.	**Conc.(µg/ml)**	Mean area (µV*sec.) ± S.D	% RSD
1	5	344126 ± 1363.34	0.40
2	10	693522 ± 1315.92	0.19
3	15	1046107 ± 2511.83	0.24
Inter-day precision (n=3)			
4	5	344718.3 ± 2738.01	0.79
5	10	694071 ± 1411.16	0.20
6	15	1047204 ± 1631.64	0.15

Accuracy

The accuracy of the method was evaluated through a recovery study using a marketed formulation at three different levels of standard addition, ranging from 50% to 150% of the label claim. Initially, twenty tablets were accurately weighed, and an amount of powder equivalent to 10 mg of Endoxifen was transferred to a 100 mL volumetric flask. The powder was dissolved in the mobile phase, and the solution was sonicated for 20 minutes to ensure complete dissolution. The final volume was then adjusted to the mark with mobile phase. To determine recovery, fixed aliquots of this sample solution were transferred to 10 mL volumetric flasks. Endoxifen standard solution was added to these flasks at levels corresponding to 50%, 100%, and 150% of the label claim, and the volumes were made up to the mark with mobile phase. Each concentration level was prepared in triplicate (n=3). The prepared samples were then analyzed using the optimized chromatographic conditions. The percentage recovery of Endoxifen was calculated by comparing the amount of Endoxifen recovered from the spiked samples to the amount added, thereby confirming the accuracy of the method.

Table 4: Accuracy study data of Endoxifen

Level (%)	**Test amount (µg/ml)**	**Spiked std. amount (µg/ml)**	**Total amount (µg/ml)**	**Amount recovered (µg/ml)**	**% Recovery**	**% Mean recovery ± SD. (n=3)**
50	5	2.5	7.5	7.42	98.93	99.91 ± 1.08
	5	2.5	7.5	7.58	101.07	
	5	2.5	7.5	7.48	99.73	
100	5	5	10	9.94	99.40	99.20 ± 0.26
	5	5	10	9.89	98.90	

	5	5	10	9.93	99.30	
150	5	7.5	12.5	12.44	99.52	99.60 ± 0.21
	5	7.5	12.5	12.48	99.84	
	5	7.5	12.5	12.43	99.44	

At 50% level, with a test amount of 5 µg/mL and 2.5 µg/mL spiked, the recoveries were 7.42 µg/mL, 7.58 µg/mL, and 7.48 µg/mL, resulting in an average recovery of 99.91% ± 1.08%. For the 100% level, with 5 µg/mL test amount and 5 µg/mL spiked, the recoveries were 9.94 µg/mL, 9.89 µg/mL, and 9.93 µg/mL, averaging 99.20% ± 0.26%. At 150% level, with 5 µg/mL test amount and 7.5 µg/mL spiked, recoveries were 12.44 µg/mL, 12.48 µg/mL, and 12.43 µg/mL, resulting in a mean recovery of 99.60% ± 0.21%. These results confirm the method's accuracy across all tested levelsas shown in table no: 4.

Robustness

The robustness of the analytical method was evaluated by varying the detection wavelength. Standard solutions of Endoxifen at a concentration of 10 µg/mL were analyzed using the chromatographic conditions originally optimized for a baseline wavelength of 278 nm. The analysis was performed at three different wavelengths: 276 nm, 278 nm, and 280 nm. For each wavelength, the standard solutions were injected, and the peak areas and retention times were recorded.

Table 5: Robustness Data for Endoxifen

Change in wavelength	**Amount taken (µg/ml)**	**Amount found (µg/ml)**	**% Assay ± SD (n=3)**
276	10	9.84	98.44 ± 0.47
278	10	9.92	99.22 ± 0.66
280	10	9.86	98.65 ± 0.17

LOD and LOQ

Calibration curve was repeated for three times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows.

LOD=3.3 * SD/Mean slope of calibration curve
LOQ=10 * SD/Mean slope of calibration curve

SD = Standard deviation of y intercepts of calibration curves

LOD and LOQ were shown in table no: 6

Table 6: LOD and LOQ value for Endoxifen

Sr. No.	Parameter	Mean slope	SD	Result (µg/ml)
1	LOD	69717	364.86	0.017
2	LOQ			0.051

Assay

The assay was performed on the three batches of Endoxifen. Powder equivalent to 10 mg of Endoxifen was weighed and transferred in a 100 ml volumetric flaskand sufficient mobile phase was added to dissolve the drug. It was sonicated for 20 minutes and final volume was made to the mark with mobile phase. The solution was filtered through 0.45 µ membrane filter. 10 ml of aliquot from above stock solution was further diluted to 100 ml with mobile phase. The sample solution of 10 mcg/ml was assayed as per proposed method and % assay was calculated.

Table 7: Assay result for Endoxifen

Sr. No	Amount taken (µg/ml)	Amt. found (µg/ml)	% Assay	Mean %Assay ±SD
1	10	9.92	99.21	99.96±1.03
2	10	10.11	101.13	
3	10	9.95	99.53	

Forced degradation Study

Forced degradation study was performed on the sample solution. The sample was exposed to hydrolytic, oxidative, thermal and photolytic condition. All the procedures was carried out in triplicate.

Acid Degradation: To assess acid degradation, 5 mL of the stock solution of Endoxifen was mixed with 2 mL of 2 N hydrochloric acid. The mixture was refluxed for 8 hours at 60°C in a dark condition to prevent photolytic degradation. After refluxing, the solution was cooled to room temperature and neutralized with an equivalent volume of 2 N sodium hydroxide. Suitable aliquots of the resultant neutralized degradation sample were then pipetted out and the volume was made up to the mark with the mobile phase.

Base Degradation: For base degradation, 5 mL of the Endoxifen stock solution was treated with 2 mL of 2 N sodium hydroxide and refluxed for 8 hours at 60°C, also in the dark to exclude photolytic effects. Following refluxing, the solution was cooled to room temperature and neutralized with an equivalent volume of 2 N hydrochloric acid. The resultant degradation sample was then diluted with the mobile phase to the desired volume.

Oxidative Degradation: To evaluate oxidative degradation, 5 mL of the Endoxifen stock solution was mixed with 2 mL of 5% hydrogen peroxide. The mixture was refluxed for 30 minutes at 60°C, under dark conditions to avoid photolytic degradation. After refluxing, the solution was cooled to room temperature, and suitable aliquots were diluted with the mobile phase.

Thermal Degradation: For thermal degradation, the Endoxifen sample was placed in an oven at 80°C for 2 hours in the dark. After the treatment, the sample was allowed to cool to room temperature and then diluted with the mobile phase to achieve a concentration of 10 µg/mL before analysis.

Photolytic Degradation: For photolytic degradation, the Endoxifen sample was exposed to UV light in a UV chamber for 8 hours. Post-exposure, the sample was diluted with the mobile phase to a concentration of 10 µg/mL and analyzed.

Table 8: Forced degradation study results for Endoxifen

Parameter	Standard Area	Endoxifen Peak Area (mV) After Degradation± standard deviation	% Degradation
Acid Degradation	697438	620162± 1284.45	11.08
Base Degradation		582849±1453.57	16.43
Peroxide Degradation (Oxidative)		577200±1938.29	17.24

Thermal Degradation		644502±947.84	7.59
Photolytic Degradation		633832±1005.62	9.12

Conclusion:

The developed and validated analytical method for Endoxifen demonstrates robust performance across various parameters, ensuring accurate quantification and reliable results. The method's specificity, linearity, precision, and accuracy have been thoroughly evaluated and found to meet the stringent criteria outlined by ICH guidelines. The linear calibration curve, with a correlation coefficient of 1, confirms the method's reliability across the tested concentration range. Precision studies, including repeatability and intra-day and inter-day analyses, yielded low % RSD values, indicating the method's consistency and reproducibility. The accuracy of the method was validated through recovery studies, with mean recoveries close to 100% across all tested levels, affirming its capability to produce precise results.

Forced degradation studies have provided valuable insights into the stability of Endoxifen under various stress conditions. The degradation profiles under acidic, basic, oxidative, thermal, and photolytic conditions demonstrate that Endoxifen is susceptible to degradation, with oxidative degradation showing the highest impact. These findings are crucial for understanding the drug's stability and for the development of stability-indicating methods. The method's robustness, tested by varying the detection wavelength, further supports its reliability in different analytical conditions.

Overall, the validated method offers a reliable approach for the quantification of Endoxifen in biological matrices, essential for pharmacokinetic studies and ensuring patient safety. The insights gained from the forced degradation studies enhance our understanding of Endoxifen's stability and inform strategies to maintain its efficacy throughout its lifecycle. This comprehensive validation and degradation analysis contribute significantly to the development of Endoxifen as a therapeutic agent and support its safe and effective use in clinical settings.

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