Available online on 15.07.2024 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Research Article

# Method Development and Validation for Estimation of Etifoxine in Capsule Dosage Form by Using RP-HPLC/UV

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Article Info:

Article History:

Received 22 April 2024
Reviewed 03 June 2024
Accepted 28 June 2024
Published 15 July 2024

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Abstract

A RP-HPLC/UV method was developed and validated to quantify the etifoxine in capsule dosage form. The chromatographic separation was carried out using a Hypersil ODS $C_{18}$ column (250×4.6 mm, 5 µm) with a mobile phase consisting of an ammonium acetate buffer: acetonitrile in a volumetric ratio of 40:60%. The UV wavelength chosen for detection was at 255 nm. The flow rate was set at 1 ml/min. The retention time for etifoxine was determined to be 2.074 min. Linearity was detected within the concentration range of 7.5-45 µg/mL for etifoxine. The approach has been confirmed to be linear, accurate, precise, robust, and has established limits of detection and quantitation. The established procedure was uncomplicated, cost-effective, and suitable for the routine analysis of etifoxine in capsule dosage form.

**Keywords:** Etifoxine, RP-HPLC and Validation.

Cite this article as:

Choudary ML, Srinivas M, Kumari BU, Method Development and Validation for Estimation of Etifoxine in Capsule Dosage Form by Using RP-HPLC/UV, Journal of Drug Delivery and Therapeutics. 2024; 14(7):64-70 *DOI: http://dx.doi.org/10.22270/jddt.v14i7.6703*

## INTRODUCTION

Anxiolytics are a class of drugs used to treat various conditions, including generalized anxiety and panic disorders. Sedatives (hypnotics) are another class of medications used for treating conditions ranging from patients on mechanical ventilators to sleeplessness. Anxiety is characterized by a state of arousal and the perception of danger, serving an adaptive function by preparing the body to respond to potential threats. On the other hand, processing unpleasant information by the brain, especially through the amygdala circuits, is linked to a depressive subjective state. The DSM-5 classifies specific phobias, panic disorder, social anxiety disorder, generalized anxiety disorder, separation anxiety disorder, and adjustment disorder with anxiety as pathological anxious conditions that can be diagnosed when symptoms become severe[1]. Anxiety and adjustment disorders are prevalent globally and often coexist with other medical conditions. Despite limited understanding of the neurological pathways underlying anxiety, treatment has advanced significantly in recent years. Barbiturates were commonly used for their calming effects in the early twentieth century, but their drawbacks, such as addiction and overdose risks, led to their replacement with benzodiazepines. A recent study identified new neural pathways involved in anxiety, expanding therapeutic options and offering new opportunities for treatment. Optimizing treatment is crucial due to the adverse effects associated with current anxiolytics. Initially, therapy focused on enhancing inhibitory control, primarily by targeting the "γ-aminobutyric acid receptor type A ($GABA_AR$)" in the central nervous system. Etifoxine (see figure 1), chemically known as "6-chloro-N-ethyl-4-methyl-4-phenyl-4H-benzo[d][1,3]oxazin-2-amine", is used as a medication to reduce anxiety and prevent seizures[2]. Originally created in the 1960s to address anxiety problems, this treatment is currently undergoing research to explore its potential in promoting repair of peripheral nerves and alleviating pain caused by chemotherapy. It produces effects similar to benzodiazepine medications but with distinct side effects. The exact mechanism of action of etifoxine has not been completely understood[3]. Etifoxine (ETX) works by enhancing the activation of $GABA_A$ receptors directly, but it does this through a different binding site that is separate from the usual benzodiazepine binding site. Etifoxine binds to the peripheral benzodiazepine receptor (PBR), also known as the "18 kDa translocator protein (TSPO)" on the outer mitochondrial membrane. This binding triggers the production of neurosteroids, which then alters $GABA_A$ receptors[4].

The biological and pharmacological effects of ETX have been the subject of numerous investigations undertaken and published in recent years. However, this aspect of quantitative analysis of ETX has not been extensively studied in research articles, as far as we are aware. One non-validated approach that combines an Agilent TC-C18 column with a mobile phase consisting of methanol and water (70:30, v/v) has been reported (in Chinese)[5]. Only one LC-MS/TOF method has been reported for the analysis of ETX under stress conditions[6]. Establishing a quick and easy HPLC analytical technique for ETX is essential. A good peak separation between the drug and its degradation products has been recommended in recent years for any HPLC method development of active pharmaceutical ingredients[7-9]. However, when measuring ETX at low concentrations, these

methods prove to be imprecise and insensitive. Additionally, they are expensive, time-consuming, and not suitable for routine analysis. As a result, we chose to utilize a low-cost UV-coupled HPLC technology that is widely accessible in laboratories with limited financial resources. This method requires regular monitoring of ETX in the capsule formulation. With these factors in mind, the objective of this research is to establish an RP-HPLC method for measuring ETX in tablet formulations following ICH recommendations under Q specification[10].

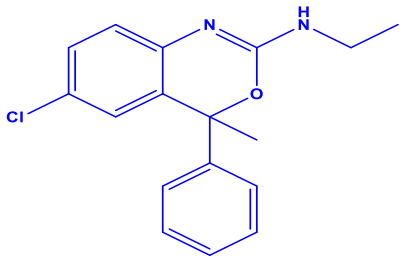


**Figure 1:** Structure of etifoxine

## MATERIALS AND METHODS

### Chemicals and reagents

The source of ETX was Ascentyo Biosciences in Hyderabad, India. Throughout the analysis, all HPLC-grade solvents were employed, including ammonium acetate, potassium di-hydrogen ortho phosphate, acetonitrile, methanol, and water Merck Ltd. (Mumbai, India).

### Instruments

The Shimadzu HPLC system (Model No. LC-20AD) in conjunction with a UV detector (Model No. SPD-M20A) was used for the study. Version 2 of the Empower program was used to acquire data. A Hypersil ODS C18 column (diameters: 250 mm × 4.6 mm, 5 µm) made up the experimental setup. A 20 µL sample loop-equipped Rheodyne injection valve was used to introduce the samples. An analytical balance made by Mettler Toledo was used for the weighing process.

### Chromatographic conditions

A mobile phase consisting of ammonium acetate buffer (ACN) at a volumetric ratio of 40:60 v/v was utilized in an isocratic mode. At room temperature (25°C), the analysis was carried out using a mobile phase flow rate of 1.0 mL/min. A 20 µL sample injection into the HPLC apparatus was necessary for each trial. As seen in figure 2, the UV detector was set up to identify ETX in the column's effluents at a wavelength of 255 nm.

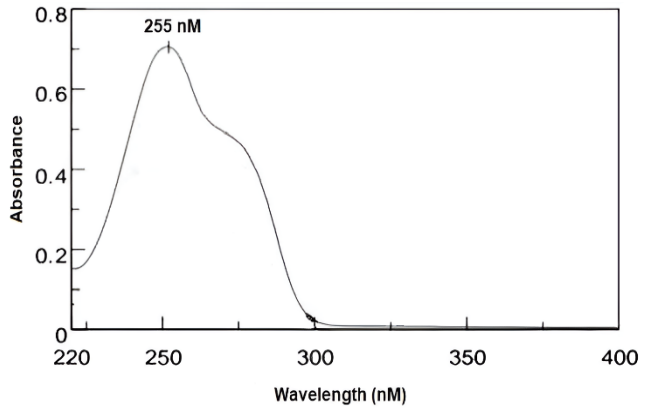


**Figure 2:** UV spectra of etifoxine

### Ammonium acetate buffer (10mM; pH 4.0) preparation:

After accurately weighing and dissolving 0.77 g of ammonium acetate in 1 L of HPLC-grade water, glacial acetic acid was used to adjust the pH of the resulting solution to 3.0. Any suspended particles were then removed from the solution by filtering it using a nylon filter (0.45 µ).

### Mobile Phase preparation

The mobile phase was prepared by sonicating acetonitrile as an organic modifier for 10 minutes to degas, after combining the ammonium acetate buffer and acetonitrile in a 40:60 v/v ratio.

### Diluents preparation

ACN and water were combined in a ratio of 60:40 (% v/v).

### Preparation of standard stock solution

A 15 mg of ETX powder with purity more than 99% was placed in a 100 mL volumetric flask (VF). Next, the flask was filled with the diluent and subjected to sonication for 20 minutes. The volume was set to 100 mL by adding the diluent. Then, 2 mL of the solution was moved to a 10 mL volumetric flask and topped up with 10 mL of diluent to reach a concentration of 30 µg/mL of ETX.

### Preparation of sample solution from dosage form

Ten 50 mg Stresam® capsules were taken, and the contents were emptied and placed in a dry watch glass. The capsule powder equivalent to 15 mg of ETX was weighed and then added to a 100 ml volumetric flask along with 50 ml of diluents to create a standard stock solution of 150 µg/mL. For an hour, the mixture was periodically sonicated to ensure the ETX was fully soluble. Subsequently, a 0.45 µm membrane filter was used to filter it, and 100 milliliters of diluent were added to make a stock solution. After that, 2 mL of the solution was transferred to a 10 mL volumetric flask and topped off with 10 mL of diluent to achieve a concentration of 30 µg/mL of ETX.

### Selection of the detection wavelength

For the HPLC analysis, an ETX solution of 30 µg/mL was employed. Using the mobile phase as a reference, the solution was examined using a UV spectrophotometer operating in the wavelength range of 190 to 400 nm. The scanning procedure aimed to identify the wavelength at which the ETX most efficiently absorbs UV light in order to facilitate its identification for ensuing HPLC investigations. Figure 2 illustrates the drug's highest absorbance at 255 nm.

### Analytical method development

Several parameters must be optimized while keeping a set of constants in mind while designing a procedure for ETX HPLC analysis. For the best chromatographic separation, the composition of the mobile phase, the choice of column, and the flow rates were carefully adjusted. However, a number of parameters were held constant, including the type of detector, injection volume (20 µL), oven temperature (25 ± 2 ºC), and elution mode, in order to ensure consistency and enable validation. Every set of chromatographic conditions has a corresponding spectrum captured at the designated detection wavelength. Peak height, column pressure, precision, resolution, analysis duration, and solvent efficiency per run were other parameters taken into account during the method's development.

**Validation**

After acceptable chromatographic conditions were established, the method was validated following the ICH Q2 requirements[10]. Additionally, the stability of reagents and solvents was investigated as well.

***Evaluation of system suitability***

System suitability studies were carried out to confirm the HPLC system's reliability. To quantify column efficiency, plate count, and tailing factor, injections of 30 µg/mL were made six times. The outcomes showed consistency by verifying that the system met the predetermined standards and stayed within the given parameters.

***Specificity and selectivity***

The successful and interference-free detection of ETX in the sample served as validation of the method's specificity and selectivity. While the blank, which was merely the diluent, showed no reaction or interference, the chromatogram of the ETX reference standard produced a positive result. Figure 3 displays the chromatogram corresponding to the standard.

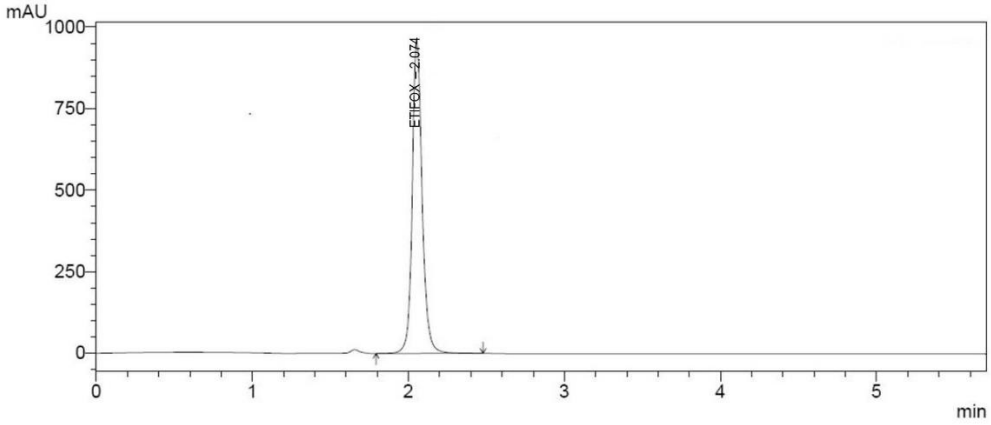


**Figure 3:** Optimized chromatogram of etifoxine

***Linearity***

By creating dilutions from the standard stock solution ranging from 7.5 to 45 µg/mL, the linearity of the ETX was evaluated. During the HPLC process, peak area responses were measured for every concentration. In Figure 4, the linearity graph is displayed.

***Precision***

Six consecutive injections of the reference solution were used to assess accuracy; the results showed excellent precision and consistency, with a low percentage RSD of 0.63. The accuracy criteria of 2.0% was reached by six injections of the test solution, demonstrating a % RSD of 0.47 and validating the method's dependability in sample analysis.

***Intermediate precision (IP)***

Two analysts analyzed the standard solution on different days and in different laboratories using different HPLC machines to determine the intermediate precision. With a minor variation of 0.81% and an RSD that was within the allowable limit of 2.0% on both days, they both obtained nearly identical test results. Even though the outcomes were linked to time-based modifications, this remained the case.

***Accuracy or recovery studies***

A triplicate recovery investigation was carried out in order to confirm the HPLC procedure. Pre-analyzed samples were injected with ETX at 15, 30, and 45 µg/mL. To verify accuracy, the average recovery percentage was computed from these tests.

***Robustness***

The robustness of the HPLC process was assessed by purposefully varying the wavelength and flow rate. As seen by negligible changes in the chromatogram, tailing factor, and plate count, the method's resilience was preserved despite fluctuations in flow rate and wavelength, retaining accuracy and precision.

**Application of the developed method**

An easy way to estimate the amount of ETX in commercially accessible capsules was to use the established HPLC method. The standard procedure described in the materials and methods section was followed in the production of the sample solutions. Three injections of each sample were made into the HPLC apparatus to ensure the accuracy and consistency of the results.

## RESULT AND DISCUSSION

**Method development and optimization**

***Mobile phase***

Several columns were used during technique development to achieve excellent ETX separation, including Phenomenox Luna $C_{18}$ and Hypersil ODS $C_{18}$, with dimensions of 250 × 4.6 mm and a particle size of 5 µm. The peak should occur when the medication either fully ionizes or unionizes because the pH of the mobile phase greatly influences the peak. The pKa of ETX (about 5.18) was taken into consideration when evaluating two different mobile phase solutions: 0.1% OPA in water (pH 2.5) and phosphate buffer (0.025 M) pH 3.0 (pKa–pH > 2). Methanol and acetonitrile were both tested as organic solvents for the mobile phase. To decrease the drug's retention time ($t_R$) and enhance peak form, the composition of the mobile phase was changed. Chromatographic data software was used to calculate system suitability characteristics, such as tailing factor at 10% peak width ($Tf_{10\%}$), theoretical plate count, and percent relative standard deviation (%RSD) of areas of six replicates of the standard solution in order to determine the best approach. Short run times, minimal organic solvent usage, an asymmetry factor near 1.0, and a maximum theoretical plate count were all taken into account. Based on the results, ammonium acetate buffer and ACN (40:60% v/v) was finalized as the optimized mobile phase for the estimation of ETX in capsules.

***Effect of column***

Several columns, including C4 and C18, were tested for the ETX elution. A peak form that was adequate could not be achieved when the C4 column was tested. The high affinity of ETX for the stationary phase may account for the larger peak of ETX with tailing in the C4 column even at greater organic ratio in the mobile phase. Less retention time and an excellent peak shape were displayed by the C18 column. When contrasted with the Luna (Phenomenex) chemistry, the C18 column using Hypersil ODS column chemistry displayed superior peak form and peak separation.

***Effect of flow rate***

When the flow rate was adjusted from 1 mL/min to 1.2 mL/min, there was no discernible change in the form of the ETX peak. Thus, a 1 mL/min flow rate was maintained.

***Column oven temperature***

The temperature of the column oven had no discernible effect on peak shape. Peak form altered only slightly when the temperature was raised from 25° to 40°. Because a greater temperature could shorten the column's lifespan, the column oven temperature was maintained at 25°.

***System suitability parameters***

By computing the percentage RSD (relative standard deviation) based on six consecutive injections of the reference solution, the accuracy of the HPLC system was assessed. To be considered acceptable, the Relative Standard Deviation (RSD) may not be greater than 2%. The RSD of the standard solution was within the allowed range, indicating precision within the specified criterion, demonstrating the procedure's dependability for precisely quantifying ETX in samples. Table 1 presents the findings. With a retention time (RT) of 2.074 minutes, ETX demonstrated quick identification and successful separation. The HPLC technique for ETX measurement was adjusted to select the right wavelength, fine-tune parameters, and mobile phase composition with care in order to provide quick analysis and good resolution. Strict analytical requirements are satisfied by the accurate and effective measurement of ETX provided by this comprehensive optimization.

**Table 1.** System suitability parameters for Etifoxine

| S.No. | Parameter | Etifoxine | Acceptance criteria |
|---|---|---|---|
| 1. | Retention time (RT) | 2.074 | -- |
| 2. | Theoretical plates (N) | 8674 | NLT 2000 |
| 3. | Tailing factor (T) | 1.68 | NMT 2.0 |
| 4. | Linearity range (µg/mL) | 7.5-45 | -- |
| 5. | Detection Limit (µg/mL) | 0.05 | -- |
| 6. | Quantification limit (µg/mL) | 0.15 | -- |
| 7. | Regression data: Slope | 49626 | -- |
| 8. | Regression data: Intercept | 22313 | -- |
| 9. | Regression data: Correlation coefficient | 0.9993 | -- |

***Linearity***

For ETX, a linearity graph was made, with the area under the curve (AUC) on the y-axis and the concentration in µg/mL on the x-axis. Within the range of 7.5–45 µg/mL, a linear relationship between drug concentrations and peak area responses was discovered. The results, which are summarized in Table 2 and illustrated in Figure 4, highlight how important linearity is for analytical methods. By examining peak areas, this function ensures precise evaluation of drug levels across a wide concentration range. Precise ETX analysis is made possible by the HPLC approach's strong linearity in the authorized concentration range and strong correlation between concentration and peak area response.

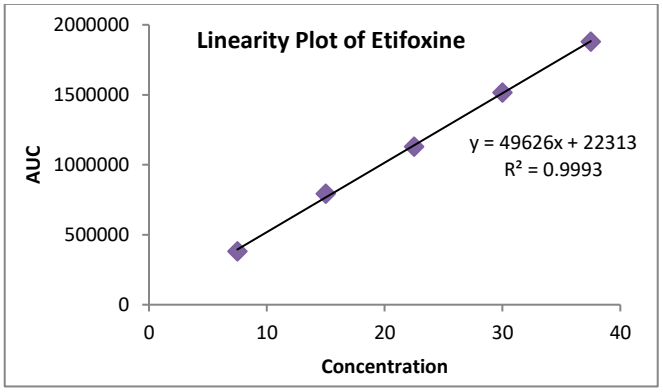


**Figure 4:** Linearity graph of etifoxine

**Table 2.** Linearity of etifoxine

| S.No. | Drug | Values of X and Y variables | | | | | | | Correlation coefficient |
|---|---|---|---|---|---|---|---|---|---|
| 1. | ETX | Variable | 1 | 2 | 3 | 4 | 5 | 6 | 0.999 |
| | | X | 7.5 | 15 | 22.5 | 30 | 37.5 | 45 | |
| | | Y | 380052.4 | 792405.4 | 1127305 | 1515055 | 1879719 | 2217436 | |

***Precision***

The precision of the procedure was judged to be good, taking into account the repeatability of the sample and standard preparations. Table 3 provides a summary of the validation parameters. The outcomes show how dependable and consistent the HPLC method is when it comes to ETX analysis. The method may be reliably applied for accurate quantification in a variety of sample types thanks to this validation.

**Table 3.** Precision study

| S.No. | System Precision | | Method Precision | |
|---|---|---|---|---|
| | Rt | AUC | Rt | AUC |
| 1 | 2.068 | 1507260 | 2.048 | 1512164 |
| 2 | 2.059 | 1517611 | 2.074 | 1519781 |
| 3 | 2.076 | 1520382 | 2.061 | 1521042 |
| 4 | 2.081 | 1495594 | 2.082 | 1513408 |
| 5 | 2.078 | 1506159 | 2.071 | 1523355 |
| 6 | 2.074 | 1518652 | 2.073 | 1532080 |
| **Mean** | 2 | 1510943 | 2 | 1520305 |
| **SD** | 0.01 | 9643.906 | 0.01 | 7247.303 |
| **% RSD** | 0.386 | 0.638271 | 0.578 | 0.476701 |

***Intermediate precision***

The ability of the HPLC method is demonstrated by its consistent performance across numerous labs, equipment, and analyzers—even on different days. Its moderate precision attests to its suitability for frequent usage, ensuring consistently accurate and reliable results under various experimental conditions. Table 4 demonstrates that the method and instrument's precision are demonstrated by the intermediate precision percentage RSD values, which are less than 2.0%.

**Table 4.** Intermediate precision or Ruggedness study

| Analyst Name | Analyst I | | | Analyst II | | |
|---|---|---|---|---|---|---|
| **Area of Std.** | 1520405 | | | 1545356 | | |
| **S.No.** | **Concentration (µg/mL)** | **AUC** | **Assay (%)** | **Concentration** | **AUC** | **Assay (%)** |
| 1 | 30 | 1487259 | 97.82 | 30 | 1531822 | 100.75 |
| 2 | 30 | 1540147 | 101.30 | 30 | 1539538 | 101.26 |
| 3 | 30 | 1515037 | 99.65 | 30 | 1540816 | 101.34 |
| 4 | 30 | 1525739 | 100.35 | 30 | 1533083 | 100.83 |
| 5 | 30 | 1538395 | 101.18 | 30 | 1543159 | 101.50 |
| 6 | 30 | 1530585 | 100.67 | 30 | 1551997 | 102.08 |
| | **Mean** | 1522860 | 100 | **Mean** | 1540069 | 101 |
| | **SD** | 19681.32 | 1.29 | **SD** | 7341.42 | 0.48 |
| | **% RSD** | 1.292 | 1.292 | **% RSD** | 0.477 | 0.479 |
| Difference between mean assay of two different analysts = 0.81 % | | | | | | |

***Accuracy***

A mean recovery percentage of 100.54% was obtained from the experiment, demonstrating the accuracy of the HPLC method in determining the ETX content within the expected range. The table 5 data, which display mean recovery percentages and spiking concentrations, validate the method's suitability for quantitative analysis by showcasing the effective recovery of ETX from various spiked samples. The accuracy of the recovery falling within the allowable range verifies the method's satisfactory performance in measuring the ETX content.

**Table 5.** Accuracy study

| S.No. | Level | Amount added (µg/mL) | Mean Amount recovered (µg/mL) | Mean % Recovery |
|---|---|---|---|---|
| 1. | 50% | 15 | 15.13 | 100.88 |
| 2. | 100% | 30 | 30.25 | 100.49 |
| 3. | 150% | 45 | 45.14 | 100.26 |

***Robustness***

The results of extensive trials are presented in Table 6, which also lists the various variables that were looked at and how they impacted the method's effectiveness. The HPLC method's established reliability attests to its suitability for routine usage and guarantees consistent and reliable results even in the event of considerable modifications to the operating parameters. As seen by the small variations in peak areas and retention times, the method can yield reliable results in a variety of conditions. This HPLC method differs from previous analytical techniques in that it has shorter retention times, more theoretical plates (which may indicate better resolution), and a mobile phase that makes it easier to separate ETX from other components. Therefore, it is more appropriate for the routine measurement of ETX in a variety of sample types due to its increased efficiency and accuracy.

**Table 6.** Robustness Study

| Parameters | Variation | Mean Peak area | %RSD | Tailing factor | No of Theoretical Plates |
|---|---|---|---|---|---|
| Wavelength minus | 250 nm | 1563647 | 0.86 | 1.57 | 6415.01 |
| Wavelength plus | 260 nm | 1566501 | 0.73 | 1.61 | 6625.68 |
| Flow rate minus | 0.8 min/mL | 1540970 | 0.61 | 1.16 | 6303.74 |
| Flow rate plus | 1.2 min/mL | 1551851 | 0.75 | 1.16 | 7123.63 |
| Organic phase ratio change (less) | Ammonium acetate buffer: Acetonitrile (50:50) | 1564720 | 0.39 | 1.58 | 6314.69 |
| Organic phase ratio change (more) | Ammonium acetate buffer: Acetonitrile (30: 70) | 1556779 | 0.46 | 1.58 | 7307.86 |
| Column change | Merck $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | 1559318 | 0.26 | 1.57 | 7255.87 |
| Temperature minus | 20 °C | 1569564 | 0.28 | 1.07 | 6375.79 |
| Temperature plus | 30 °C | 1578550 | 0.62 | 1.22 | 6856.42 |

**Analysis of marketed formulation**

In the commercial formulation assay, ETX yielded a mean of 98.68% after six determinations. The drug's%RSD was found to be within allowable bounds by the results. Table 7 illustrates this conclusion, which states that the excipients do not interfere.

**Table 7.** Analysis of marketed formulation

| Commercial Formulation | Ingredients | Labeled Amount (mg) | Amount Found (mg) | Found % |
|---|---|---|---|---|
| Stresam® | Etifoxine | 50 mg | 49.34 mg | 98.68 |

## CONCLUSION

The ICH guidelines were followed in the development and validation of the HPLC technology. A Shimadzu HPLC system and a UV detector (Model No. SPD-M20A) were used in the process, and a wavelength of 255 nm was used for detection. Using isocratic elution, a Hypersil ODS C18 column (250 x 4.6 mm, 5 µm) was employed with an injection volume of 20 µl. The ultimate process is straightforward, accurate, affordable, quick, dependable, and uncomplicated. Among its many features are its short duration (less than 7 minutes) and superb quality. The approach is suitable for normal laboratory analysis of etifoxine and quality control, as evidenced by the % RSD values for all validation parameters meeting the necessary standards.

## Acknowledgement

The authors are grateful to Teja Educational Society, Hyderabad for providing facilities to carry out this work.

## Conflicts of Interest

The authors declare no conflict of interest.

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