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Research Article

Development and Validation of a Reverse Phase High-Performance Liquid Chromatographic Method for Quantitative Estimation of Etifoxine Hydrochloride in Capsule Dosage Form

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Abstract
Etifoxine Hydrochloride (ETH) is a non-benzodiazepine anxiolytic agent widely used for the management of anxiety disorders. The drug exhibits a unique dual mechanism of action involving positive allosteric modulation of GABAA receptors and stimulation of neurosteroid synthesis, resulting in effective anxiolytic activity with fewer sedative adverse effects compared to conventional benzodiazepines. Despite its increasing therapeutic use, limited analytical methods have been reported for the quantitative estimation of Etifoxine Hydrochloride in pharmaceutical dosage forms. The present study aimed to develop and validate a simple, rapid, accurate, precise, and robust reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of Etifoxine Hydrochloride in capsule dosage forms. Methods: Chromatographic separation was achieved using a Thermo Hypersil Gold C18 column (4.6 × 250 mm) with a mobile phase consisting of water adjusted to pH 3.2 with 0.1% orthophosphoric acid and acetonitrile (60:40, v/v) under isocratic conditions. The flow rate was maintained at 1.0 mL/min, and detection was performed at 260 nm using a PDA detector. The developed method was validated according to ICH Q2(R1) guidelines for system suitability, linearity, accuracy, precision, ruggedness, robustness, and specificity. Etifoxine Hydrochloride exhibited a well-resolved chromatographic peak with a retention time of approximately 4.9 min. The method demonstrated excellent linearity over the selected concentration range with a correlation coefficient (R^2) of 0.999. Accuracy studies showed recoveries ranging from 99.70% to 99.87%, while precision studies yielded %RSD values below 2%. The assay of the marketed capsule formulation showed a drug content of 99.60% of the labeled claim. Robustness and ruggedness studies confirmed the reliability and reproducibility of the method. The developed RP-HPLC method was found to be simple, precise, accurate, specific, and economical. The method is suitable for routine quality control analysis and estimation of Etifoxine Hydrochloride in pharmaceutical capsule formulations.
Keywords: Etifoxine Hydrochloride, RP-HPLC, Method Validation, Capsule Formulation, ICH Guidelines, Pharmaceutical Analysis.

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1. Introduction

Anxiety disorders are among the most prevalent psychiatric illnesses worldwide and significantly affect quality of life, social functioning, and occupational performance. Although benzodiazepines remain effective anxiolytic agents, their long-term use is frequently associated with sedation, dependence, cognitive impairment, and withdrawal symptoms. Consequently, considerable efforts have been directed toward the

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development of safer anxiolytic drugs with improved tolerability profiles [1].

Etifoxine Hydrochloride is a non-benzodiazepine anxiolytic agent chemically known as 6-chloro-N-ethyl-4-methyl-4-phenyl-1H-3,1-benzoxazin-2-imine hydrochloride. The drug possesses a molecular formula of C17H18Cl2N2O and a molecular weight of 337.24 g/mol. Unlike conventional benzodiazepines, Etifoxine exerts its anxiolytic effect through a dual mechanism involving direct positive allosteric modulation of GABAA receptors and indirect stimulation of neurosteroid biosynthesis via activation of the translocator protein (TSPO) located in mitochondrial membranes. This unique mechanism contributes to effective anxiolytic activity while minimizing sedation and cognitive impairment [2].

Recent clinical investigations have demonstrated the efficacy of Etifoxine Hydrochloride in the management of adjustment disorders, generalized anxiety disorders, and stress-related conditions. Due to its favorable safety profile and reduced risk of dependence, the drug has attracted considerable attention in modern psychopharmacotherapy. Consequently, pharmaceutical industries have introduced capsule formulations containing Etifoxine Hydrochloride for therapeutic use [3].

Reliable analytical methods are essential for quality control, formulation development, stability assessment, and regulatory compliance of pharmaceutical products. High-performance liquid chromatography (HPLC) remains one of the most widely employed analytical techniques due to its high sensitivity, specificity, reproducibility, and suitability for quantitative estimation of drugs in complex pharmaceutical matrices.

Pang et al. developed a simple and accurate HPLC method for determination of Etifoxine Hydrochloride and its related impurities in capsule dosage forms using an Agilent TC-C18 column and methanol-water mobile phase. The method demonstrated excellent linearity (r = 0.9999), recovery of approximately 99.8%, and low detection limits, indicating its suitability for routine pharmaceutical analysis [4].

Djabrouhou and Guermouche reported a stability-indicating LC-MS/TOF method for characterization of degradation products of Etifoxine Hydrochloride under oxidative, acidic, alkaline, thermal, and photolytic stress conditions. The method successfully identified major degradation products and established the stability-indicating nature of the analytical procedure [5].

Choudary et al. recently developed and validated an RP-HPLC method for estimation of Etifoxine Hydrochloride in capsule dosage forms using a Hypersil ODS C18 column with ammonium acetate buffer and acetonitrile mobile phase. The method demonstrated satisfactory linearity, precision, accuracy, robustness, and sensitivity, indicating its applicability for routine quality control analysis [6].

Although a few chromatographic methods have been reported for Etifoxine Hydrochloride, there remains a need for a simple, economical, and reproducible RP-HPLC method employing readily available reagents and simplified chromatographic conditions. Furthermore, limited analytical reports are available for routine estimation of Etifoxine Hydrochloride in marketed capsule formulations.

Therefore, the present study was undertaken to develop and validate a simple reverse-phase high-performance liquid chromatographic method for quantitative estimation of

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Etifoxine Hydrochloride in capsule dosage forms. The developed method was validated according to International Council for Harmonisation (ICH) Q2(R1) guidelines with respect to specificity, linearity, accuracy, precision, ruggedness, robustness, and system suitability parameters. The validated method was subsequently applied for routine analysis of a marketed capsule formulation containing Etifoxine Hydrochloride.

2. Materials And Methods

2.1. Materials

Etifoxine Hydrochloride reference standard (purity 99.3% w/w) was procured from Arrow Chem, Mumbai, India. The marketed capsule formulation containing Etifoxine Hydrochloride 50 mg (Anxyfree® Capsules, Intas Pharmaceuticals Ltd., India) was purchased from a local pharmacy and used for assay studies.

All chemicals and solvents employed during the study were of analytical reagent (AR) or HPLC grade. Acetonitrile (HPLC grade), methanol (HPLC grade), orthophosphoric acid (OPA), triethylamine, disodium hydrogen phosphate, and purified water were obtained from reputed commercial suppliers and used without further purification.

2.2. Instrumentation

Chromatographic analysis was performed using a Waters 600 HPLC system equipped with a 996 photodiode array (PDA) detector and Empower chromatography software. Separation was achieved using a Thermo Hypersil Gold C18 column (4.6 mm × 250 mm, 5 µm particle size).

2.3. Determination of Maximum Absorption Wavelength (λmax)

A stock solution containing Etifoxine Hydrochloride (500 µg/mL) was prepared using HPLC-grade methanol.An aliquot was appropriately diluted to obtain a concentration of 5 µg/mL and scanned in the UV region between 200 and 400 nm against methanol as blank.The UV spectrum exhibited maximum absorbance at 260 nm**(Figure 1)**, which was selected as the analytical wavelength for chromatographic detection.

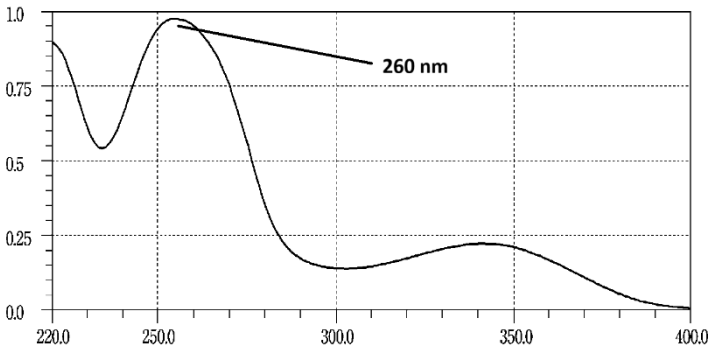

Figure 1. UV absorption spectrum showing λmax of Etifoxine Hydrochloride at 260 nm.

2.4. HPLC Method Development

2.4.1.Selection of Diluent

Various solvents including methanol, acetonitrile, and purified water were evaluated for solubility studies. Etifoxine Hydrochloride exhibited excellent solubility in acetonitrile;

therefore, acetonitrile was selected as the diluent for preparation of standard and sample solutions.

2.4.2. Optimization of Chromatographic Conditions

Different chromatographic conditions were investigated to obtain satisfactory peak symmetry, retention characteristics, and system suitability parameters**(Table 1)**.Several mobile phase compositions comprising methanol, acetonitrile, and acidified water were evaluated. Initial trials employing methanol alone resulted in poor peak resolution. Mobile phases containing acetonitrile and water improved chromatographic performance but exhibited unsatisfactory peak symmetry. Ultimately, a mobile phase consisting of water adjusted to pH 3.2 with 0.1% orthophosphoric acid and acetonitrile (60:40, v/v) produced a sharp**(Figure 2)**, symmetrical peak with acceptable retention time and theoretical plate count **(Table 2)**.

Table 1. Optimization trials performed during HPLC method development.

Trial No.	Mobile Phase	Observation
1	Methanol (100%)	Poor separation
2	ACN:Water pH 3.0 (80:20)	Peak asymmetry
3	ACN:Water pH 3.0 (70:30)	Poor resolution
4	ACN:Water pH 3.2 (40:60)	Optimized chromatogram

Table 2: Optimized Chromatographic Conditions

Parameter	Condition
Column	Thermo Hypersil Gold C18 (4.6 × 250 mm)
Mobile Phase	Water pH 3.2 (0.1% OPA): ACN (60:40, v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	260 nm
Injection Volume	20 µL
Column Temperature	Ambient
Run Time	10 min
Detector	PDA Detector

2.5. Preparation of Standard Solution

Accurately weighed 5 mg of Etifoxine Hydrochloride reference standard was transferred into a 100 mL volumetric flask and dissolved in acetonitrile. The volume was adjusted with the same solvent to obtain a stock solution equivalent to 50 µg/mL.Appropriate dilutions were prepared from the stock solution for validation studies.

2.6. Preparation of Sample Solution

The entire contents of Anxyfree® capsule equivalent to 50 mg of Etifoxine Hydrochloride were transferred into a 100 mL volumetric flask and dissolved in acetonitrile.The solution was sonicated, centrifuged at 5000 rpm for 10 min, and filtered through a 0.45 µm membrane filter. Suitable dilutions were prepared to obtain a final concentration equivalent to 50 µg/mL.

2.7. Assay of Marketed Capsule Formulation

Twenty microliters each of standard and sample solutions were injected separately into the HPLC system after equilibration of the chromatographic column.The chromatograms were

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recorded and the peak areas were measured. The drug content in the marketed formulation was calculated by comparing sample peak areas with those of the standard solution.

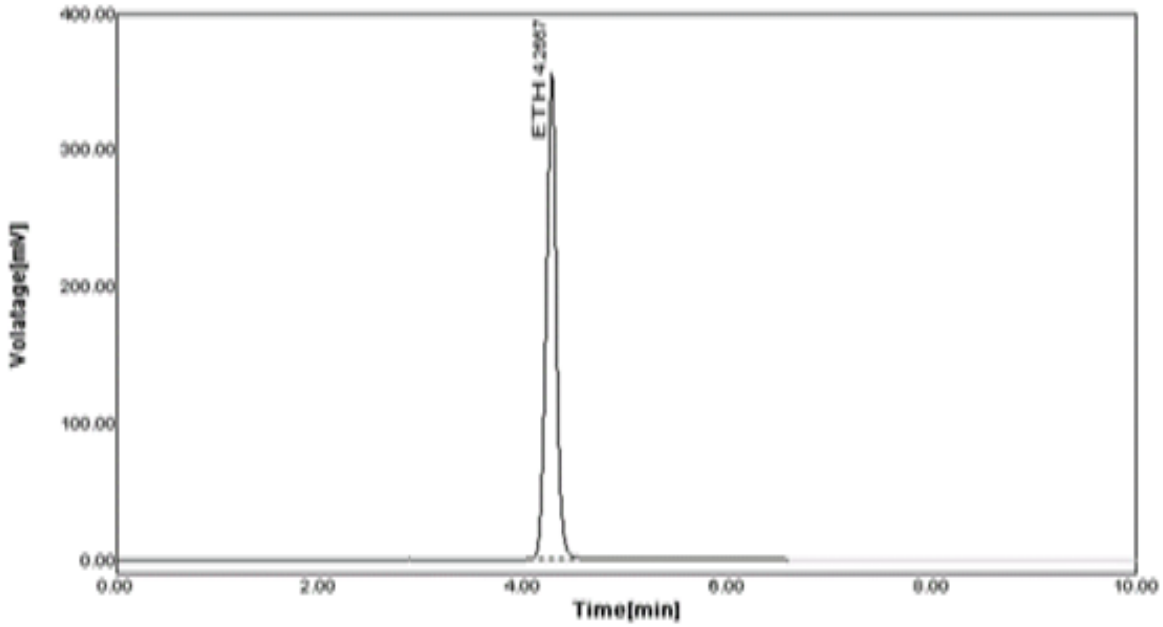

Figure 2. Representative chromatogram of standard Etifoxine Hydrochloride showing retention time at approximately 4.9 min.

2.8. Validation of Developed RP-HPLC Method

Method validation was performed according to ICH Q2(R1) guidelines. System suitability was evaluated by five replicate injections of standard solution. Parameters including retention time, peak area, theoretical plate count, and tailing factor were recorded. Linearity was assessed by analyzing solutions over the concentration range corresponding to 80–150% of the target concentration. Calibration curves were generated by plotting concentration versus peak area. Accuracy was determined by standard addition at 80%, 100%, and 120% levels. Percentage recovery and %RSD values were calculated. Precision studies included system precision and method precision (Repeatability) results were expressed as %RSD. Intermediate precision was evaluated using different analysts, instruments, and days of analysis. Robustness was studied by deliberate variation of flow rate (±10%), mobile phase composition (±10%) and detection wavelength (±2 nm). Specificity was evaluated through placebo interference studies to confirm the absence of interfering peaks at the retention time of Etifoxine Hydrochloride.

2.9. Statistical Analysis

Results obtained during validation studies were expressed as mean, standard deviation (SD), and percentage relative standard deviation (%RSD). Acceptance criteria were evaluated according to ICH Q2(R1) recommendations.

3. Results And Discussion

3.1. Method Development and Optimization

The primary objective of the present investigation was to develop a simple, rapid, accurate, and reproducible RP-HPLC method for quantitative estimation of Etifoxine Hydrochloride (ETH) in capsule dosage forms. Various chromatographic conditions were investigated to

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obtain a symmetrical peak with acceptable retention time, theoretical plate count, and tailing factor.

Initially, methanol was evaluated as the sole mobile phase component; however, poor chromatographic performance and inadequate peak resolution were observed. Subsequently, different combinations of acetonitrile and water at varying ratios were investigated. Improved peak characteristics were obtained with acetonitrile-water systems, although peak asymmetry and poor resolution were observed in certain trials. Optimization of aqueous phase pH using orthophosphoric acid significantly improved peak shape and chromatographic efficiency**(Table 1)**.

The optimized chromatographic conditions consisted of a Thermo Hypersil Gold C18 column (4.6 × 250 mm), mobile phase containing water adjusted to pH 3.2 with 0.1% orthophosphoric acid and acetonitrile (60:40, v/v), flow rate of 1.0 mL/min, and UV detection at 260 nm. Under these conditions, Etifoxine Hydrochloride produced a sharp, symmetrical peak with a retention time of approximately 4.9 min**(Table 2) (Figure 2)**.

3.2. System Suitability Studies

System suitability testing was performed to verify the adequacy of the chromatographic system before analysis. Five replicate injections of the standard solution were analyzed under optimized chromatographic conditions.

The mean retention time was found to be 4.994 min. The average peak symmetry factor was 0.960, while the theoretical plate count exceeded 7700, indicating efficient chromatographic separation. The %RSD for peak area was only 0.26%, demonstrating excellent repeatability and reproducibility of the chromatographic system. The obtained values complied with pharmacopeial acceptance criteria, confirming the suitability of the developed chromatographic method for routine analysis**(Table 3)**.

Table 3. System Suitability Parameters

Parameter	Mean Value
Peak Area	408384
Retention Time (min)	4.994
Symmetry Factor	0.960
Theoretical Plates	7797.53
%RSD of Peak Area	0.26

3.3. Assay of Marketed Capsule Formulation

The validated RP-HPLC method was applied successfully for quantitative estimation of Etifoxine Hydrochloride in marketed capsule formulation (Anxyfree® Capsule 50 mg).The average assay value was found to be 99.60% of the labeled claim**(Table 4)**, demonstrating satisfactory content uniformity and formulation quality. The assay values were within acceptable limits and demonstrated applicability of the developed method for routine quality control testing.

Table 4. Assay Results of Marketed Capsule Formulation

Replicate	Assay (mg)	% Purity
1	50	100.00
2	49	99.33

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3	49	99.33
4	50	100.00
5	49	99.33
Average	49.40	99.60
SD	0.005	0.36
%RSD	0.36	0.36

3.4. Accuracy Studies

Accuracy of the proposed method was evaluated using the standard addition technique at three concentration levels corresponding to 80%, 100%, and 120% of the target concentration.The percentage recovery ranged between 99.70% and 99.87%, indicating excellent accuracy and absence of interference from formulation excipients. The recovery values satisfied ICH acceptance criteria (98–102%), confirming the accuracy of the developed method.

3.5. Precision Studies (System Precision and Method Precision)

System precision was evaluated by repeated injections of the standard solution. The mean peak area was 409270, standard deviation was 367.7 and %RSD was found to be 0.09. The low %RSD value indicates excellent repeatability of the chromatographic system.Method precision was determined by analyzing three independently prepared sample solutions. The mean assay values were 99.53, standard deviation of 0.57 and %RSD of 0.38. The low variability confirms the repeatability and reliability of the proposed analytical procedure.

3.6. Ruggedness (Intermediate Precision)

Ruggedness was evaluated by different analysts using different chromatographic systems on different days. The mean % assay was found to be 99.40% and %RSD was found to. The, low %RSD values indicate that normal operational variations do not significantly affect analytical performance.

3.7. Linearity and Range

Linearity was evaluated over the concentration range corresponding to 80–150% of the target analytical concentration.A direct proportional relationship was observed between concentration and peak area response. The calibration curve exhibited excellent linearity with a correlation coefficient (R^2) of 0.999. The results demonstrate excellent linearity within the selected analytical range.

Table 5. Linearity Data for Etifoxine Hydrochloride

Concentration Level (%)	Response
80	Linear
100	Linear
120	Linear
140	Linear
150	Linear

3.8. Robustness Studies

Robustness was evaluated by deliberate variation of chromatographic conditions including flow rate, mobile phase composition, and detection wavelength.Variation of flow rate between 0.9 and 1.1 mL/min did not significantly affect retention time, peak symmetry, or

theoretical plate count. The mean retention time at 0.9 and 1.1 ml/min flow rate was 5.46 and 4.13 min respectively. For robustness study for mobile phase composition, at -10% ACN and +10% ACN the retention time were found to be 5.08 and 4.80 min respectively. For robustness of wavelength variation, at 258 nm and 262 nm the retention time were 4.80 and 5.00 min respectively. The method remained unaffected by deliberate changes in analytical conditions, confirming its robustness.

3.9. Specificity

Specificity was assessed by placebo interference studies.No interfering peaks were observed at the retention time corresponding to Etifoxine Hydrochloride, confirming the specificity of the developed analytical method**(Figure 3)**.

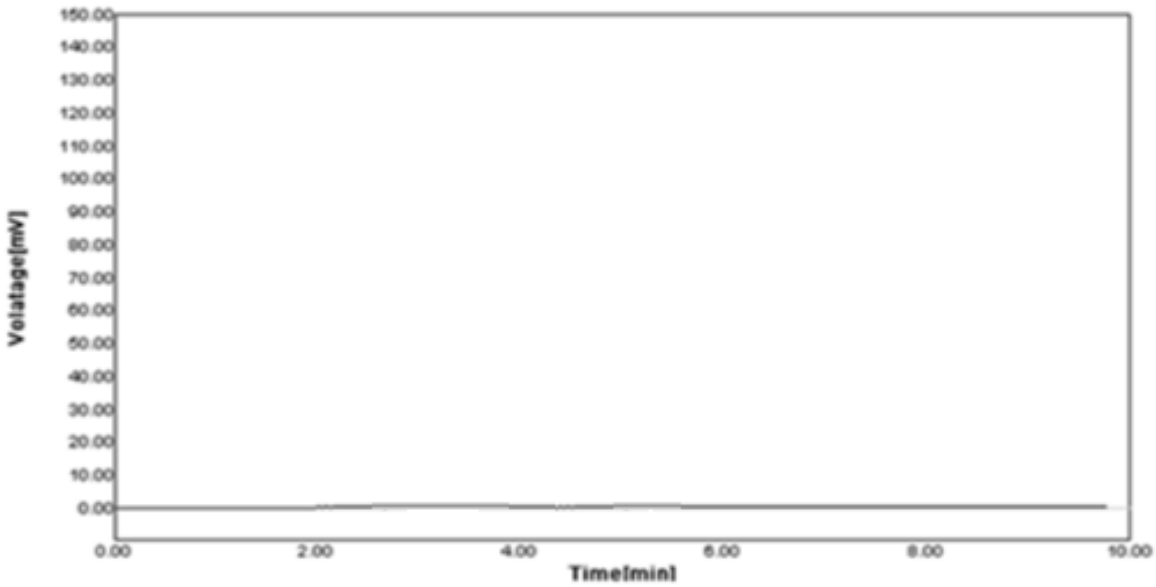

Figure 3. Placebo chromatogram showing absence of interfering peaks at the retention time of Etifoxine Hydrochloride.

3.10. Comparative Discussion with Reported Methods

The analytical performance of the developed RP-HPLC method was compared with previously reported methods for estimation of Etifoxine Hydrochloride.

Pang et al. reported an HPLC method employing methanol-water systems for estimation of Etifoxine Hydrochloride in capsule formulations. Although satisfactory accuracy and precision were obtained, the method required comparatively longer analysis time. Djabrouhou and Guermouche developed a stability-indicating LC-MS method suitable for degradation studies but requiring sophisticated instrumentation [4].

Recently, Choudary et al. developed an RP-HPLC method for Etifoxine Hydrochloride using a Hypersil ODS C18 column and buffered mobile phase. The present method provides comparable analytical performance while utilizing a simple mobile phase composition consisting of acidified water and acetonitrile [5].

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The developed method demonstrated excellent validation characteristics including assay (99.60%), recovery (99.70–99.87%), precision (%RSD < 2%), ruggedness (%RSD 0.34%), and linearity (R^2 = 0.999). Furthermore, the short retention time of approximately 4.9 min contributes to reduced solvent consumption and increased sample throughput, making the method highly suitable for routine pharmaceutical quality control laboratories.

4. Discussion

The present investigation was undertaken to develop and validate a simple, rapid, precise, accurate, and economical RP-HPLC method for quantitative estimation of Etifoxine Hydrochloride (ETH) in capsule dosage forms. The developed method successfully achieved all analytical objectives and demonstrated satisfactory validation characteristics in accordance with ICH Q2(R1) guidelines [7].

Etifoxine Hydrochloride has recently emerged as an important non-benzodiazepine anxiolytic agent due to its unique dual mechanism of action involving positive modulation of GABAA receptors and stimulation of neurosteroid biosynthesis. Despite its increasing clinical use, only a limited number of analytical methods have been reported for its quantitative estimation in pharmaceutical dosage forms [1,2]. Consequently, there exists a continuing need for reliable and validated analytical procedures suitable for routine quality control applications.

The developed RP-HPLC method employed a Thermo Hypersil Gold C18 column with a mobile phase consisting of water adjusted to pH 3.2 with 0.1% orthophosphoric acid and acetonitrile (60:40, v/v). Under optimized chromatographic conditions, Etifoxine Hydrochloride produced a sharp, symmetrical peak with a retention time of approximately 4.9 min. The relatively short retention time offers significant practical advantages, including reduced analysis time, lower solvent consumption, and increased sample throughput.

System suitability studies demonstrated excellent chromatographic performance. The average retention time was 4.994 min, peak symmetry factor was 0.960, and theoretical plate count exceeded 7700. The %RSD for peak area was only 0.26%, confirming the reproducibility of the chromatographic system. These results comply with USP and ICH recommendations for chromatographic analysis [7].

The assay of the marketed capsule formulation (Anxyfree® Capsule 50 mg) yielded an average drug content of 99.60% of the labeled claim. The obtained assay values indicate satisfactory content uniformity and compliance with pharmacopeial quality requirements. Similar assay results have been reported by Pang et al., who developed an HPLC method for determination of Etifoxine Hydrochloride in capsule dosage forms and reported excellent agreement with label claims [4].

Accuracy studies conducted using the standard addition technique demonstrated recovery values ranging from 99.70% to 99.87%. These findings indicate the absence of interference from excipients and confirm the accuracy of the proposed analytical method. The recovery results are comparable to those reported by Choudary et al., who observed recovery values close to 100% during validation of an RP-HPLC method for Etifoxine Hydrochloride capsules [6].

Precision studies revealed excellent repeatability and reproducibility. System precision studies produced a %RSD of 0.09%, while method precision yielded a %RSD of 0.38%.

Intermediate precision (ruggedness) studies performed by different analysts on different days generated a %RSD of 0.34%. All values were significantly below the ICH acceptance criterion of 2%, indicating outstanding analytical precision [4]. Similar observations have been reported in pharmaceutical validation studies involving RP-HPLC methods for anxiolytic drugs and related pharmaceutical compounds [8,9].

Linearity studies demonstrated a direct proportional relationship between concentration and chromatographic response over the selected analytical range. The correlation coefficient ($R^2 = 0.999$) confirmed excellent linearity and reliability of the analytical method. These findings are consistent with previous chromatographic investigations where correlation coefficients greater than 0.998 were considered indicative of acceptable analytical performance [10].

Robustness studies were conducted by deliberate variation of flow rate, mobile phase composition, and detection wavelength. No significant changes in chromatographic behavior were observed following these modifications. Theoretical plate count remained above 2000 and tailing factor remained below 2.0 under all experimental conditions. These findings demonstrate the robustness of the method and confirm its suitability for routine laboratory applications [11,12].

Specificity was established through placebo interference studies. No interfering peaks were observed at the retention time corresponding to Etifoxine Hydrochloride, confirming the selectivity of the developed analytical procedure. Such specificity is particularly important in pharmaceutical quality control where formulation excipients may potentially interfere with chromatographic analysis [13].

A comparison of the present method with previously reported analytical procedures reveals several advantages. Stability-indicating LC-MS methods reported by Djabrouhou and Guermouche provide excellent characterization of degradation products but require sophisticated instrumentation and higher operational costs [5]. In contrast, the present RP-HPLC method utilizes readily available chromatographic equipment and a simple mobile phase system, making it more practical for routine quality control laboratories. Similarly, recently reported RP-HPLC methods for Etifoxine Hydrochloride often employ buffered systems or more complex chromatographic conditions [14]. The current method achieves comparable analytical performance while maintaining simplicity and economy.

Overall, the developed RP-HPLC method demonstrated excellent specificity, accuracy, precision, linearity, robustness, and ruggedness. The method fulfills all validation requirements specified by ICH Q2(R1) guidelines and can be successfully applied for routine quality control analysis of Etifoxine Hydrochloride in pharmaceutical capsule formulations.

5. Conclusion

A simple, rapid, precise, accurate, robust, and economical RP-HPLC method was successfully developed and validated for quantitative estimation of Etifoxine Hydrochloride in capsule dosage forms.

Chromatographic separation was achieved using a Thermo Hypersil Gold C18 column with a mobile phase consisting of water adjusted to pH 3.2 with 0.1% orthophosphoric acid and acetonitrile (60:40, v/v). The developed method produced a well-resolved

chromatographic peak with a retention time of approximately 4.9 min and demonstrated excellent analytical performance.

Validation studies confirmed that the method satisfies all ICH Q2(R1) requirements with respect to specificity, accuracy, precision, ruggedness, robustness, linearity, and system suitability. The assay of the marketed capsule formulation demonstrated drug content of 99.60% of the labeled claim, indicating satisfactory quality and content uniformity.

The method offers several advantages including simple mobile phase preparation, short analysis time, low solvent consumption, high reproducibility, and ease of operation. These characteristics make the method highly suitable for routine quality control analysis, formulation development studies, stability investigations, and regulatory applications.

To the best of our knowledge, only a limited number of validated RP-HPLC methods have been reported for estimation of Etifoxine Hydrochloride in capsule formulations. Therefore, the proposed method provides a valuable analytical tool for pharmaceutical industries and quality control laboratories engaged in the analysis of Etifoxine-containing products.
Future studies may focus on the development of stability-indicating methods, bioanalytical applications, and simultaneous estimation of Etifoxine Hydrochloride with related pharmaceutical compounds in combined dosage forms.

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