RESEARCH PAPER

SIMULTANEOUS RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ETIFOXINE HYDROCHLORIDE AND ALPRAZOLAM IN API MIXTURE

Harshwardhan Balaso Burange[1]*, Vikram Veer[2], Ashok Bhosale[3]

[1,2,3]Department of Pharmaceutical Quality Assurance, PDEA's Shankarrao Ursal College of Pharmaceutical Sciences and Research Centre, Kharadi, Pune, Maharashtra, India

Corresponding Author Email: harshwardhanburange@gmail.com

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ABSTRACT:

Background: Running separate HPLC assays for each drug in a combination product wastes reagent, instrument time, and increases the risk of between-run variability. Etifoxine hydrochloride and alprazolam are used together in managing generalized anxiety disorder with somatic symptoms, till now there is no developed and validated RP-HPLC method for these two drugs simultaneously

Objectives: This research aims for the creation and verification of an isocratic RP-HPLC method for the concurrent measurement of etifoxine hydrochloride and alprazolam within a joined API blend. The procedure is established because the ICH Q2(R1) requirements must be met for scientific accuracy.

Materials and Methods: Within the laboratory, a Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) was utilized alongside a mobile phase composed of phosphate buffer (pH 4.0) and acetonitrile (25:75 v/v). This liquid mixture flowed at a rate of 1.0 mL/min while the temperature remained at 40°C. Scientific investigators claim that monitoring at 247 nm is necessary because this wavelength represents the Q-absorption point where both pharmacological substances show considerable light intake. The validation process included system suitability, specificity with PDA peak purity, linearity, accuracy, repeatability, intermediate precision, and robustness to ensure the validation remains reliable.

Results: Etifoxine hydrochloride eluted at 6.87 min and alprazolam at 10.42 min, with a resolution factor of 3.2. Both analytes were linear from 50–150% of working concentration (r = 0.99999913 and 0.99999996). Mean recoveries were 99.2–99.5% for etifoxine HCl and 99.1–99.9% for alprazolam. Precision %RSD stayed below 0.7% throughout, and all robustness variations remained within ±2.0% of target assay values.

Conclusion: The method separates both analytes cleanly in a single 16-minute run on standard QC instrumentation one run where two used to be needed.

Keywords: Alprazolam, Etifoxine hydrochloride, ICH Q2(R1), Method validation, Reversed-phase HPLC, Simultaneous estimation.

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INTRODUCTION

Simultaneous quantification of two active pharmaceutical ingredients in a single chromatographic run is analytically more efficient than running separate assays it cuts reagent use, instrument time, and between-run variability. For anxiolytic drugs, this efficiency matters beyond the bench: these compounds are prescribed at low doses, and any quantification error has a direct bearing on patient safety. [1]

Etifoxine hydrochloride and alprazolam are two structurally distinct anxiolytics used together in the clinical management of generalized anxiety disorder with somatic symptoms.[2] Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine; $C_{17}H_{13}ClN_4$; MW 308.77 g/mol; pKa 5.01) is a high-potency triazolobenzodiazepine that binds to the benzodiazepine site on the GABA-A receptor, increasing chloride ion influx and reducing neuronal excitability.[3] Etifoxine hydrochloride (6-chloro-N-ethyl-4-methyl-4-phenyl-4H-3,1-benzoxazin-2-amine hydrochloride; $C_{17}H_{17}ClN_2O{\cdot}HCl$; MW 337.25 g/mol; pKa 5.18) acts through two

independent mechanisms: positive allosteric modulation at the $\alpha^{+}\beta^{-}$ interface of the GABA-A receptor distinct from the benzodiazepine binding site and stimulation of neurosteroid biosynthesis via the translocator protein (TSPO).[4] Because etifoxine does not occupy the benzodiazepine site, it can be co-administered with alprazolam without displacement, which is the pharmacological basis for using this combination clinically.

Both drugs absorb UV radiation but at different primary wavelengths etifoxine HCl at 214 nm and alprazolam at 234 nm. When scanned together between 200–400 nm, a common absorption point was identified at 247 nm where both compounds show proportional UV response, making it the appropriate detection wavelength for simultaneous quantification.

The regulatory framework for this work is ICH Q2(R1), which defines the validation parameters a method must satisfy: specificity, linearity, accuracy, precision, and robustness. [5] ICH Q1A(R2) further requires that methods used in stability testing be stability-indicating. [6] For any simultaneous assay, the chromatographic signal for each API must be free from interference by the other drug and by degradation products a criterion addressed directly in the specificity studies.

The published literature contains individual RP-HPLC methods for each drug but none for their combination. For etifoxine HCl, Pang Jing et al. reported a method on an Agilent TC-C18 column using methanol: water (70:30) at 254 nm, with linearity from 9–90 mg/L and mean recovery of 99.82%.[7] Djabrouhou et al. developed an LC-MS/TOF stability-indicating method on a Kromasil C18 column identifying four degradation products.[8] Choudary et al. used a Hypersil ODS C18 column with ammonium acetate buffer: acetonitrile (40:60) at 255 nm, with linearity from 7.5–45 µg/mL.[9] For alprazolam, Shingate et al. reported a simultaneous stability-indicating method with propranolol at 221 nm,[10] Rele et al. developed a tablet assay using phosphate buffer:acetonitrile (60:40) at 225 nm,[11] and Rupsi et al. estimated alprazolam and melatonin together on a C18 column at 277 nm.[12] Abirami et al. estimated alprazolam and melatonin together using methanol: water (70:30) at 222 nm.[13] No published method covers the simultaneous RP-HPLC estimation of etifoxine HCl and alprazolam in a combined API mixture with full ICH Q2(R1) validation. The present work fills that gap, describing the development and validation of an isocratic RP-HPLC method using a Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) with phosphate buffer pH 4.0: acetonitrile (25:75 v/v) at 1.0 mL/min, UV detection at 247 nm, and a total run time of 16 minutes.

MATERIALS AND METHODS

Chemicals and Reagents

Etifoxine Hydrochloride working standard was procured from MSN Organic Pvt. Ltd. and Alprazolam working standard from Lee Pharma Ltd. HPLC-grade acetonitrile and methanol were obtained from Merck Life Science, along with AR-grade orthophosphoric acid. HPLC-grade water was supplied by Rankem. Nylon membrane disc filters (0.45 µm) and PVDF syringe filters (0.45 µm) were sourced from MDI.

Instrumentation

Chromatographic analysis was performed on a Waters e2695 HPLC system with a Waters 2998 Photodiode Array (PDA) detector, controlled via Empower PRO software. UV characterisation used a Shimadzu UV1900i double-beam spectrophotometer with 10 mm matched quartz cells. Weighing was done on a Mettler Toledo XSE205DU analytical balance and pH was measured using a Thermo Scientific Orion Star A211 digital pH meter.

Chromatographic Conditions

Separation was achieved on a Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) at 40°C. The mobile phase was phosphate buffer (pH 4.0) and acetonitrile (25:75 v/v), delivered isocratically at 1.0 mL/min. Detection was at 247 nm using the PDA detector. Injection volume was 10 µL, run time 16 minutes, and sample compartment temperature 25°C.

Preparation of Mobile Phase

Sodium dihydrogen phosphate (1.19 g) was dissolved in 1000 mL of HPLC-grade water, pH adjusted to 4.0 with orthophosphoric acid, and filtered through a 0.45 µm nylon membrane. The buffer was combined with acetonitrile (25:75 v/v), filtered again, and sonicated for 15 minutes to degas before use.

Preparation of Diluent

HPLC-grade water and acetonitrile were mixed in a ratio of 40:60 (v/v). This mixture was used as the diluent for all standard and sample preparations.

Preparation of Standard and Sample Solutions

For the standard, 50 mg of Etifoxine HCl and 60 mg of Alprazolam working standards were co-dissolved

in approximately 80 mL diluent in a 100 mL volumetric flask, sonicated for 30 minutes, and made up to volume. A 5.0 mL aliquot was diluted to 50 mL with diluent to yield a working standard of 50 ppm Etifoxine HCl and 60 ppm Alprazolam. The solution was filtered through a 0.45 µm nylon membrane, discarding the first 2.0 mL of filtrate.

For the sample, 125 mg Etifoxine HCl API and 150 mg Alprazolam API were dissolved in approximately 150 mL diluent in a 250 mL volumetric flask, sonicated for 60 minutes with intermittent shaking at room temperature, and made up to volume. A 10.0 mL aliquot was diluted to 100 mL with diluent to give 50 ppm Etifoxine HCl and 60 ppm Alprazolam. Filtered through a 0.45 µm nylon membrane, discarding the first 2.0 mL.

Method Validation

The method was validated per ICH Q2(R1) guidelines for an assay procedure. LOD and LOQ were not included as they are not required for assay validation under ICH Q2(R1). System suitability required %RSD of peak areas ≤ 2.0%, tailing factor ≤ 2.0, theoretical plates ≥ 2000, and resolution ≥ 2.0 across five replicate injections. Specificity was assessed through retention time identification, blank interference check, and PDA-based peak purity (purity angle < purity threshold). Linearity was evaluated at five concentration levels from 50–150% of working concentration (Etifoxine HCl: 25–75 µg/mL; Alprazolam: 30–90 µg/mL), with acceptance criterion r ≥ 0.999 and %Y-intercept within ±2.0%. Accuracy was assessed by recovery studies at 50%, 100%, and 150% of working concentration in triplicate, with acceptance criterion 98.0–102.0% mean recovery. Precision used six independent preparations on the same day for repeatability; intermediate precision was assessed by a second analyst on a different system on a separate day, with %RSD ≤ 2.0% and absolute mean difference ≤ 2.0% required for both. Robustness was evaluated by deliberate variation of flow rate (±0.1 mL/min), detection wavelength (±2 nm), and column temperature (±2°C), with assay difference ≤ ±2.0% from control as the acceptance criterion.

RESULTS

Drug Characterisation

Physical Characteristics

Etifoxine HCl presented as a white, odourless powder and Alprazolam as an off-white, odourless powder, consistent with their respective pharmacopoeial descriptions.

Solubility Studies

Both drugs failed to dissolve completely in water and methanol after extended sonication. Acetonitrile alone dissolved both analytes cleanly. A binary mixture of water: acetonitrile (40:60 v/v) also gave complete dissolution and was selected as the diluent for all subsequent preparations.

Infrared Spectroscopy

FTIR confirmed the identity of Etifoxine HCl through characteristic absorptions at 2938.69 cm^{-1} (C–H stretch), 1682.33 cm^{-1} (C=N stretch), 1328.24 cm^{-1} (C–N stretch), and 1209.63 cm^{-1} (C–O stretch). For Alprazolam, identity was confirmed by bands at 2924.19 cm^{-1} (C–H stretch), 1644.95 cm^{-1} (C=N stretch), 1587.73 cm^{-1} (C=C stretch), 1376.98 cm^{-1} (C–H bend), and 771.01 cm^{-1} (C–Cl stretch). (Figure 1, 2)

UV Spectroscopy and Wavelength Selection

UV spectra were recorded from 200–400 nm. Etifoxine HCl showed λmax at 285 nm (0.4795 AU) and Alprazolam at 234 nm (0.8409 AU). A common isoabsorptive point was identified at 247 nm (0.3494 AU) where both analytes absorb proportionally. This wavelength was selected for all HPLC detection work. (Figure 3,4,5)

Method Development and Optimisation

Six chromatographic trials were conducted systematically. The first five trials on Zorbax SB-CN and Waters Symmetry columns with varying mobile phase compositions produced inadequate resolution, poor peak shapes, or incomplete elution. The final optimised conditions used a Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) with phosphate buffer pH 4.0: acetonitrile (25:75 v/v) as the mobile phase at 1.0 mL/min flow rate, UV detection at 247 nm, column temperature 40°C, and a 16-minute run time. Under these conditions, Etifoxine HCl eluted at 6.87 min (tailing factor 1.1; theoretical plates 6354) and Alprazolam at 10.42 min (tailing factor 1.0; theoretical plates 10,423), with adequate resolution between the two peaks. These conditions were adopted for full validation. (Table 1 and Figure 6).

Method Validation

System Suitability

Five replicate injections gave %RSD of peak areas of 0.4% and 0.6% for Etifoxine HCl and Alprazolam respectively well within the 2.0% limit. Tailing factors were 1.2 and 1.0, theoretical plates were 6204 and 10,574 (both exceeding the 2000-plate minimum), and resolution between peaks was 3.2. (Table 2).

Specificity

Retention times agreed within ±2.0% between standard and sample: Etifoxine HCl at 6.85 min (standard) vs. 6.86 min (sample); Alprazolam at 10.44 min (standard) vs. 10.48 min (sample). No interfering peaks were detected in the blank chromatogram at either retention time. PDA peak purity confirmed spectral homogeneity purity angles were below purity thresholds for both analytes in both standard and sample injections (Etifoxine HCl: 2.65/4.02 standard, 2.58/3.91 sample; Alprazolam: 2.13/3.96 standard, 2.08/3.71 sample). (Table 3 and Figure 7,8, 9,10,11)

Linearity

Both analytes showed excellent linearity across 50–150% of working concentration. For Etifoxine HCl (25–75 µg/mL): r = 0.99999913, slope 17,083, intercept 670, %Y-intercept 0.08%. For Alprazolam (30–90 µg/mL): r = 0.99999996, slope 50,995, intercept 207.2, %Y-intercept 0.007%. Both met the acceptance criterion of r ≥ 0.999 and %Y-intercept within ±2.0%. (Table 4 and 5 and Figure 12 and 13)

Accuracy

Mean recoveries across 50%, 100%, and 150% levels were 99.5%, 99.5%, and 99.2% for Etifoxine HCl and 99.1%, 99.8%, and 99.9% for Alprazolam. All 95% confidence intervals fell within the 98.0–102.0% acceptance range. (Table 6)

Precision

Repeatability gave mean %assay of 99.4% (%RSD 0.5%) for Etifoxine HCl and 100.0% (%RSD 0.7%) for Alprazolam across six preparations. Intermediate precision by a second analyst on a separate day gave 99.5% (%RSD 0.5%) and 99.4% (%RSD 0.6%) respectively, with absolute mean differences of 0.1% and 0.6% from method precision both within the 2.0% limit. (Table 7)

Robustness

Deliberate variations in flow rate (±0.1 mL/min), detection wavelength (±2 nm), and column temperature (±2°C) all produced assay differences within ±2.0% from control for both analytes. The largest deviation was 2.0% for Etifoxine HCl at a flow rate of 1.1 mL/min. System suitability criteria were met under every condition tested.

Table 1: Final optimized RP-HPLC chromatographic conditions column, mobile phase, flow rate, detection wavelength, injection volume, column temperature, and run time.

Chromatographic Conditions:

Column	Hypersil ODS C18 (150 mm X 4.6 mm), 5µm
Mobile Phase	Buffer pH 4.0 : Acetonitrile (25:75 v/v)
Flow Rate	1.0 mL/min
Injection Volume	10 µL
Wavelength	247 nm
Column Temp	40ºC
Sample Temp	25ºC
Run Time	16.0 minutes
Seal Wash	Water: Acetonitrile (90:10) v/v
Needle Wash	Water: Acetonitrile (10:90) v/v

Table 2: System suitability parameters for Etifoxine Hydrochloride and Alprazolam %RSD of peak area, tailing factor, theoretical plates, and resolution (n = 5 replicate injections)

Tailing Factor	**1.2**	**1.0**
Theoretical plates	**6204**	**10574**
Resolution (Rs)	**3.2**	
Active substance	Etifoxine HCL	Alprazolam
Injection No.	**Area**	**Area**
1	852541	3085745
2	862585	3112541
3	859658	3105362
4	860285	3076580
5	858858	3065054

Mean	**858785**	**3089056**
%RSD	**0.4**	**0.6**

Table 3: Specificity data retention times for identification, blank interference results, and PDA peak purity angles and thresholds for Etifoxine Hydrochloride and Alprazolam in standard and sample solutions.

Component	**Retention time (min)**	**Tailing Factor**	**Purity angle**	**Purity threshold**
Blank	-	-	-	-
Standard solution				
Etifoxine HCL	6.85	1.1	2.65	4.02
Alprazolam	10.44	1.0	2.13	3.96
Sample solution				
Etifoxine HCL	6.86	1.2	2.58	3.91
Alprazolam	10.48	1.1	2.08	3.71
Individual peak identification				
Etifoxine HCL	6.89	1.1	2.72	4.16
Alprazolam	10.46	1.0	2.15	4.01

Table 4: Linearity data for Etifoxine Hydrochloride concentration levels (25–75 µg/mL), peak areas, calibration curve parameters (slope, intercept, correlation coefficient r, % Y-intercept, RSS).

Level	**Conc (µg/mL)**	**Area**	**Mean**
50%	25	427602	427634
		427665	
75%	37.5	641837	641709
		641580	
100%	50	854563	854633
		854702	
125%	62.5	1068321	1067713
		1067104	
150%	75	1282016	1282289
		1282562	
Corr. Coeff			0.99999913
Intercept			670
Slope			17083
% Y-intercept			0.08
RSS			796,459.60

Table 5: Linearity data for Alprazolam concentration levels (30–90 µg/mL), peak areas, calibration curve parameters (slope, intercept, correlation coefficient r, % Y-intercept, RSS).

Level	**Conc (µg/mL)**	**Area**	**Mean**
50%	30	1530021	1529988
		1529954	
75%	45	2295102	2295375
		2295648	
100%	60	3059236	3059621
		3060005	
125%	75	3824123	3824482
		3824841	
150%	90	4590164	4590056

		4589947	
Corr. Coeff			0.99999996
Intercept			207.2
Slope			50995
% Y-intercept			0.007
RSS			452,868.30

Table 6: Accuracy (% Recovery) data for Etifoxine Hydrochloride at 50%, 100%, and 150% of working concentration individual and mean % recovery with 95% confidence interval (n = 3 per level).

Level (%)	**mg added**	**Mg recovered**	**Area-1 Area-2**		**Average Peak Area**	**% Recovery**	**Mean recovery %**	**SD**	**95% Cl**
			Inj-1	**Inj-2**					
50	25.06	25.00	427630	427598	427614	99.78	99.5	0.260	98.86 – 100.15
	25.04	24.85	425023	425001	425012	99.26			
	24.98	24.86	425436	425088	425262	99.55			
100	50.02	49.58	846214	849720	847967	99.13	99.5	0.541	98.15 – 100.85
	50.10	49.73	849610	851236	850423	99.34			
	50.02	50.08	856623	856414	856518	100.13			
150	75.04	74.91	1281691	1280541	1281116	99.83	99.2	0.655	97.56 – 100.81
	75.00	73.89	1263254	1264025	1263640	98.53			
	74.96	74.37	1271240	1272541	1271891	99.22			

Table 7: Accuracy (% Recovery) data for Alprazolam at 50%, 100%, and 150% of working concentration individual and mean % recovery with 95% confidence interval (n = 3 per level).

Level (%)	**mg added**	**Mg recovered**	**Area-1 Area-2**		**Average Peak Area**	**% Recovery**	**Mean recovery %**	**SD**	**95% Cl**
			Inj-1	**Inj-2**					
50	30.12	29.88	1531857	1531265	1531561	99.22	99.1	0.459	97.97 – 100.25
	30.04	29.62	1518245	1517865	1518055	98.61			
	29.94	29.79	1527415	1526202	1526809	99.51			
100	60.06	60.10	3080451	3079584	3080018	100.07	99.8	0.384	98.82 – 100.73
	59.92	59.86	3068542	3067415	3067979	99.91			
	60.14	59.74	3061254	3061874	3061564	99.34			
150	90.06	89.50	4586256	4587458	4586857	99.39	99.9	0.504	98.60 – 101.11
	90.10	90.45	4635652	4635021	4635337	100.39			
	90.02	89.82	4602315	4603658	4602987	99.78			

Table 8: Method Precision (Repeatability) data for Etifoxine Hydrochloride and Alprazolam % assay of six independent preparations with mean, SD, and %RSD.

Sample	**Etifoxine HCl Area**	**Etifoxine HCl % Assay**	**Alprazolam Area**	**Alprazolam % Assay**
Sample 1	857405	100.1	3085365	100.1
Sample 2	850365	99.1	3100054	100.8
Sample 3	848925	99.3	3076814	99.7
Sample 4	850245	99.2	3113540	100.7
Sample 5	847202	98.9	3076842	99.9
Sample 6	858585	98.8	3051547	99.0
Mean % Assay		**99.4**		**100.0**
SD		**0.45**		**0.66**
% RSD		**0.5**		**0.7**

Table 9: Intermediate Precision (Ruggedness) data for Etifoxine Hydrochloride and Alprazolam %assay of six preparations by Analyst I and Analyst II on different HPLC systems and columns, with mean, SD, 95% CI, and absolute mean difference.

Parameter	**Etifoxine HCl Analyst I (HPLC-015 / AD/HC-036)**	**Etifoxine HCl Analyst II (HPLC-021 / AD/HC-042)**	**Alprazolam Analyst I (HPLC-015 / AD/HC-036)**	**Alprazolam Analyst II (HPLC-021 / AD/HC-042)**
Sample 1	100.1	99.6	100.1	98.2
Sample 2	99.1	99.9	100.8	99.5
Sample 3	99.3	98.9	99.7	99.5
Sample 4	99.2	99.6	100.7	99.4
Sample 5	98.9	99.0	99.9	99.6
Sample 6	99.8	100.2	99.0	99.9
Mean % Assay	99.4	99.5	100.0	99.4
SD	**0.46**	**99.00-100.06**	**0.67**	**0.59**
95 % CI	**98.92-99.88**	**99.00 – 100.06**	**99.33 – 100.73**	**98.73 – 99.97**
Absolute Mean Difference	**0.1**		**0.6**	

Table 10: Robustness data for Etifoxine Hydrochloride % assay and system suitability parameters under deliberate variations in flow rate (±0.1 mL/min), detection wavelength (±2 nm), and column temperature (±2°C), with absolute difference from control condition.

Change in parameter	**Condition**	**Area**	**% Assay**	**Absolute difference of % Assay**
Control	As per method	857405	100.1	NA
Change in flow rate1.0 ml/min (±0.1 ml/min)	1.1 ml/min	840274	98.1	2.0
	0.9 ml/min	864365	100.9	0.8
Change in wavelength (±2 nm)	249 nm	867923	101.3	1.2
	245 nm	843315	98.5	1.6
Change in Column Temp (±2 °C)	42°C	850632	99.3	0.8
	38°C	860587	100.5	0.4

Table 11: Robustness data for Alprazolam % assay and system suitability parameters under deliberate variations in flow rate (±0.1 mL/min), detection wavelength (±2 nm), and column temperature (±2°C), with absolute difference from control condition

Change in parameter	**Condition**	**Area**	**% Assay**	**Absolute difference of % Assay**
Control	As per method	3085365	100.1	NA
Change in flow rate1.0 ml/min (±0.1 ml/min)	1.1 ml/min	3051620	99.0	1.1
	0.9 ml/min	3136654	101.8	1.7
Change in wavelength (±2 nm)	249 nm	3116048	101.1	1.0
	245 nm	3069215	99.6	0.5
Change in Column Temp (±2 °C)	42°C	3071362	99.7	0.4
	38°C	3095201	100.5	0.4

Figure 1: FTIR spectrum of Etifoxine Hydrochloride showing characteristic absorption bands at 2938.69 cm^{-1} (C–H stretching), 1682.33 cm^{-1} (C=N stretching), 1328.24 cm^{-1} (C–N stretching), and 1209.63 cm^{-1} (C–O stretching). (At column width)

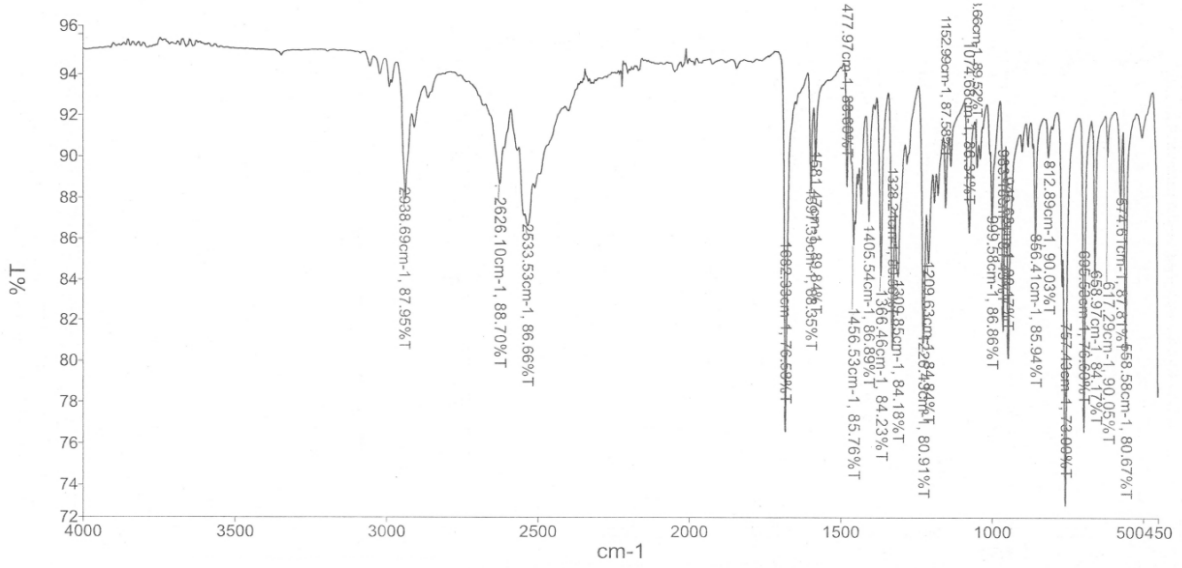


Figure 2: FTIR spectrum of Alprazolam showing characteristic absorption bands at 2924.19 cm^{-1} (C–H stretching), 1644.95 cm^{-1} (C=N stretching), 1587.73 cm^{-1} (C=C stretching), 1376.98 cm^{-1} (C–H bending), and 771.01 cm^{-1} (C–Cl stretching).

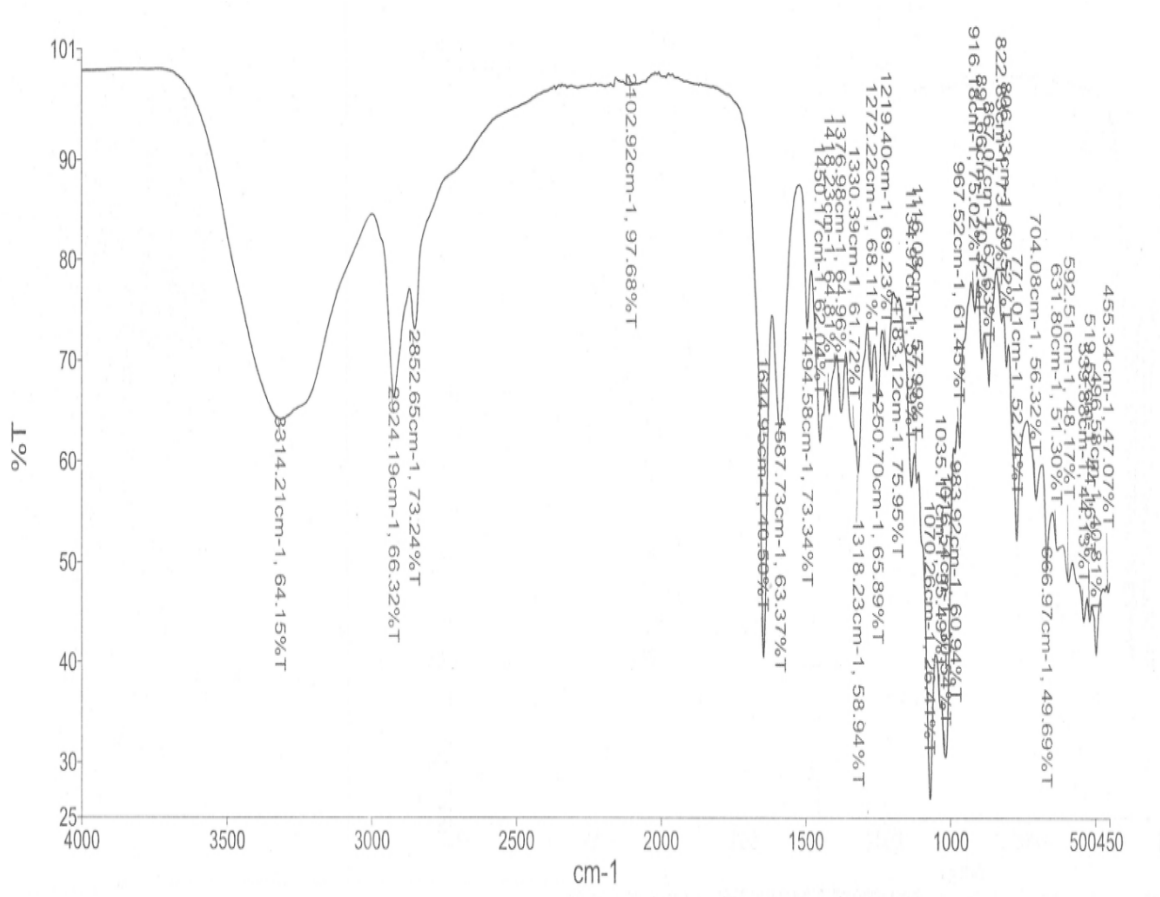


Figure 3: UV absorption spectrum of Etifoxine Hydrochloride (10 µg/mL in Water: Acetonitrile 40:60 v/v) scanned from 200 to 400 nm, showing primary λmax at 285 nm (absorbance 0.4795 AU).

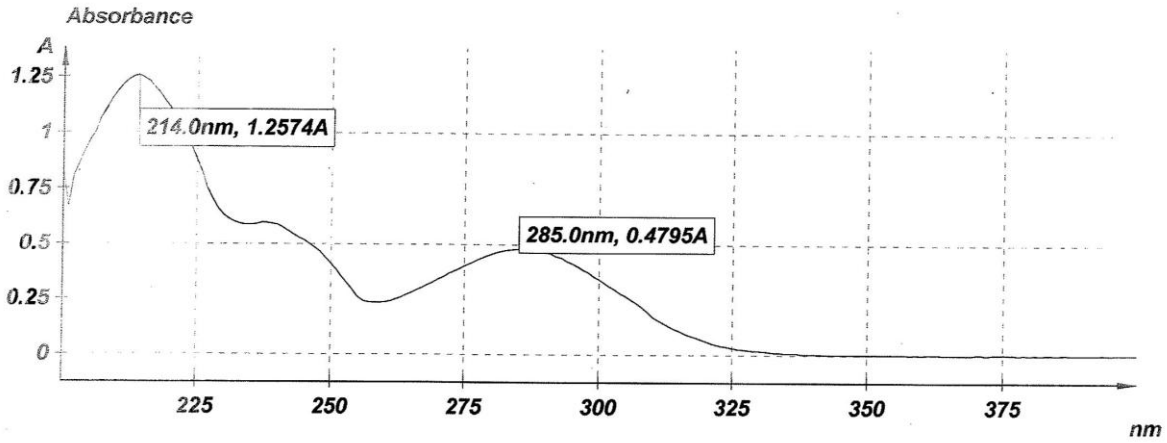


Figure 4: UV absorption spectrum of Alprazolam (10 µg/mL in Water: Acetonitrile 40:60 v/v) scanned from 200 to 400 nm, showing primary λmax at 234 nm (absorbance 0.8409 AU).

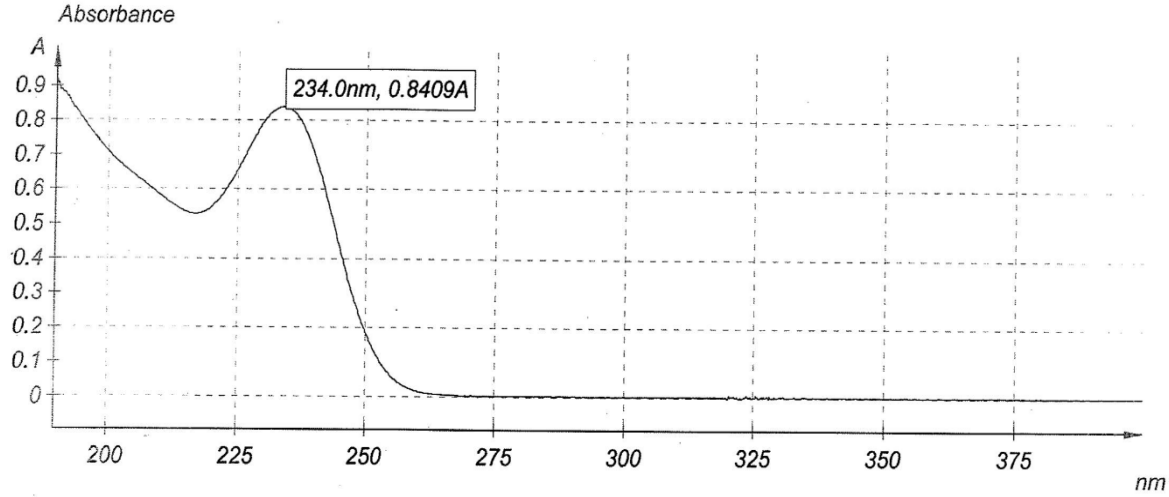


Figure 5: Overlain UV absorption spectra of Etifoxine Hydrochloride and Alprazolam (each 10 µg/mL in Water: Acetonitrile 40:60 v/v) scanned from 200 to 400 nm, illustrating the isoabsorptive (Q-absorption) point at 247 nm (absorbance 0.3494 AU) selected as the HPLC detection wavelength.

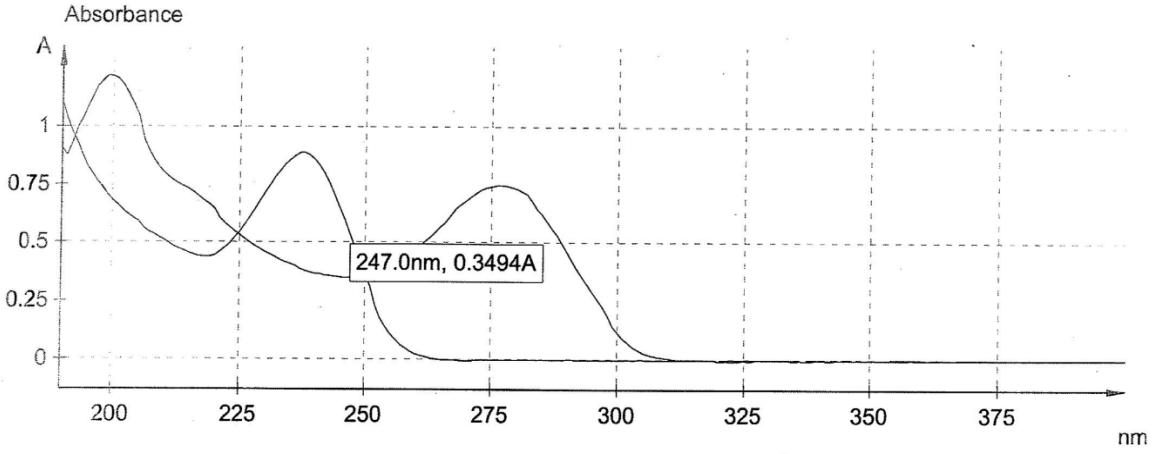


Figure 6: Optimized RP-HPLC chromatogram of Etifoxine Hydrochloride (50 µg/mL, Rt = 6.87 min, tailing factor 1.1, theoretical plates 6354) and Alprazolam (60 µg/mL, Rt = 10.42 min, tailing factor 1.0, theoretical plates 10423) on Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) with phosphate buffer pH 4.0: Acetonitrile (25:75 v/v) at 1.0 mL/min, 40°C, detection at 247 nm. Resolution factor = 3.2.

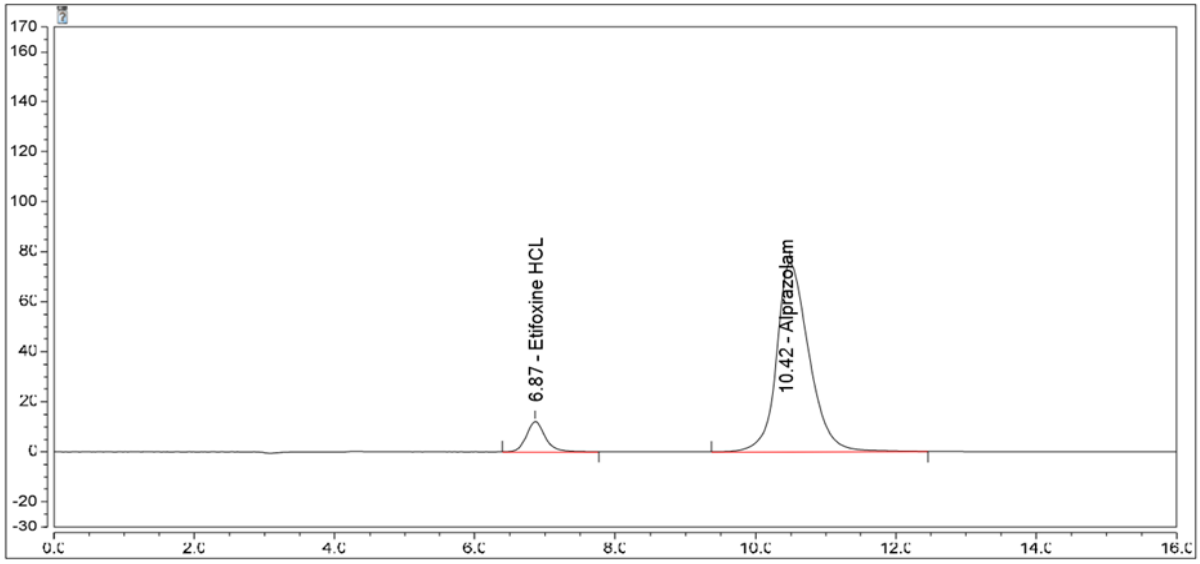


Figure 7: Blank (diluent: Water: Acetonitrile 40:60 v/v) chromatogram confirming absence of interfering peaks at the retention times of Etifoxine Hydrochloride (6.85 min) and Alprazolam (10.44 min).

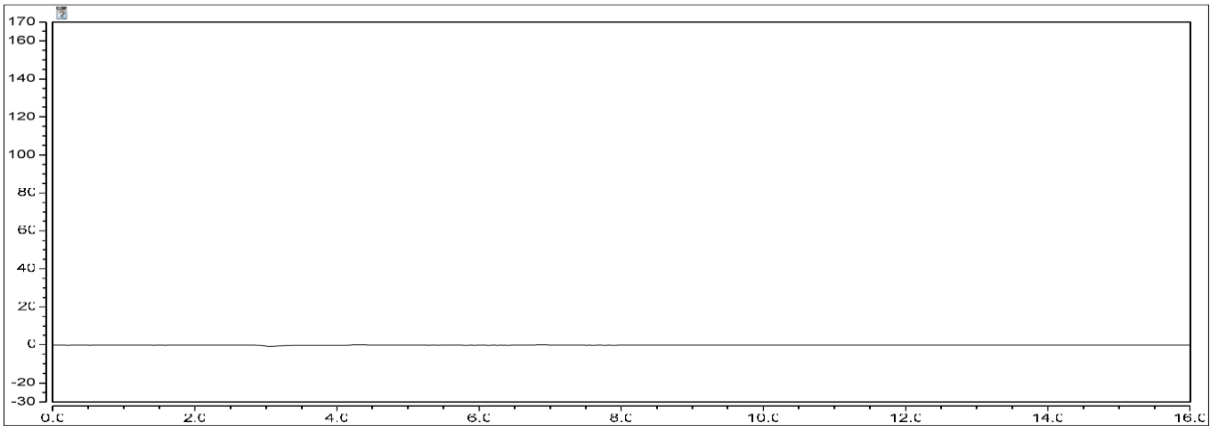


Figure 8: Standard solution chromatogram with PDA peak purity plots for Etifoxine Hydrochloride (purity angle 2.65 < threshold 4.02) and Alprazolam (purity angle 2.13 < threshold 3.96), confirming spectral homogeneity of both peaks.

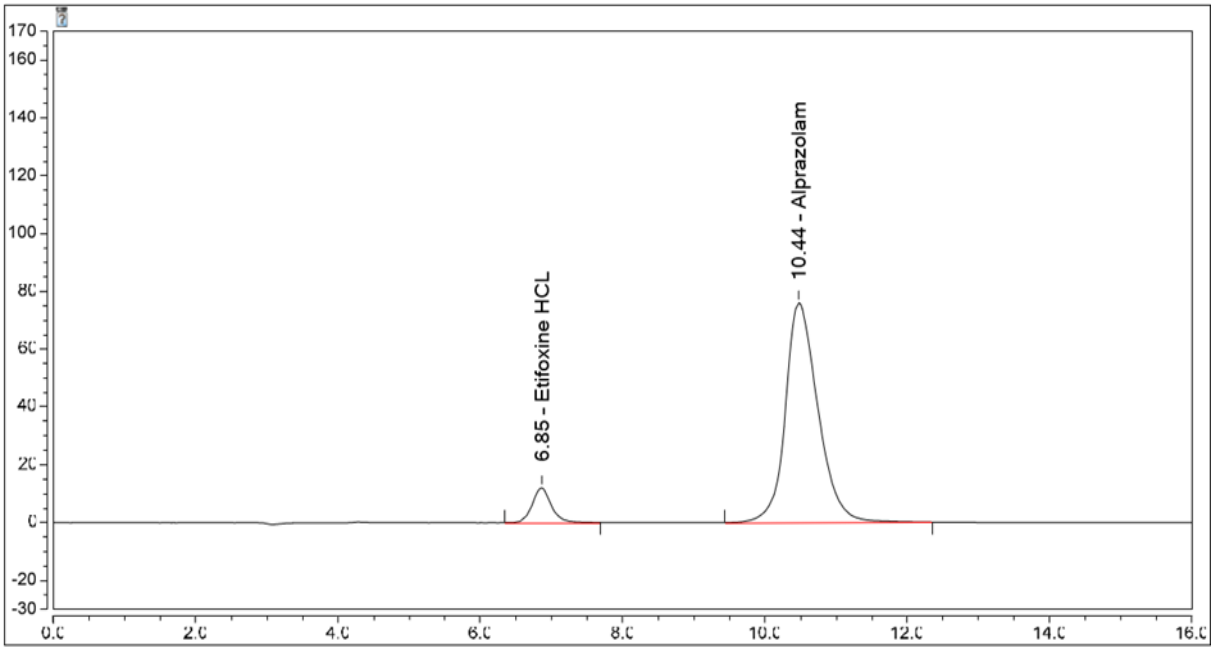


Figure 9: Sample solution chromatogram with PDA peak purity plots for Etifoxine Hydrochloride (purity angle 2.58 < threshold 3.91) and Alprazolam (purity angle 2.08 < threshold 3.71), confirming absence of co-eluting impurities in both peaks.

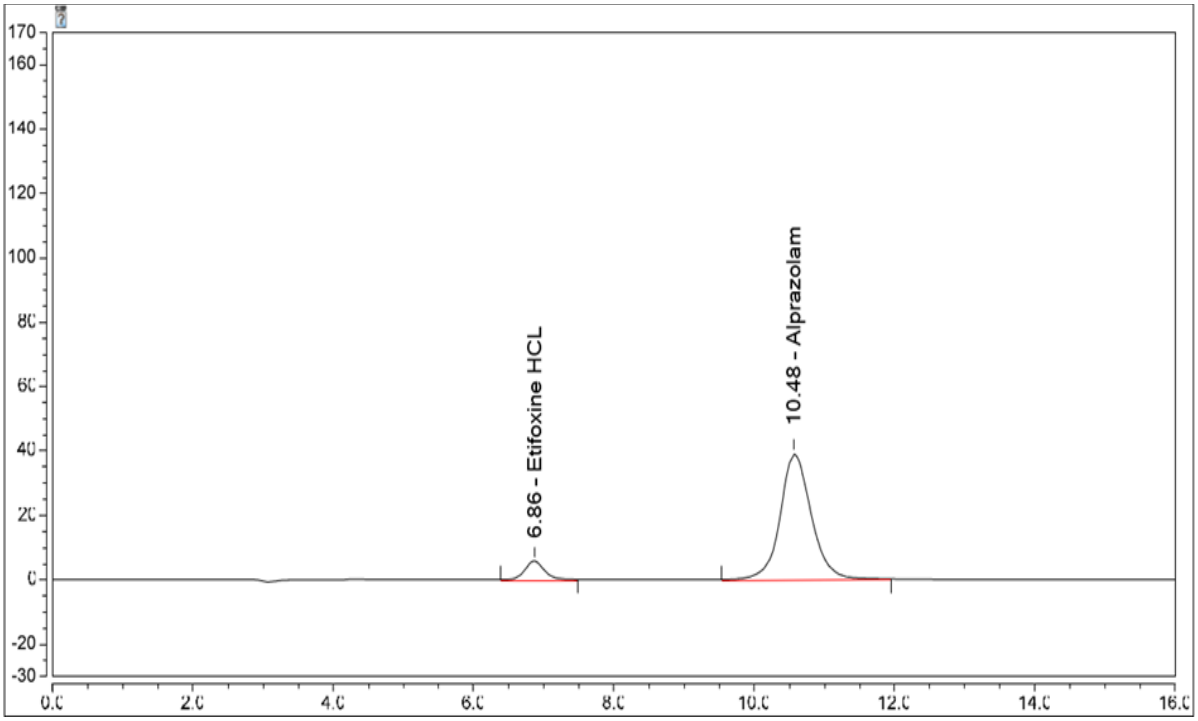


Figure 10: Individual standard chromatogram of Etifoxine Hydrochloride (Rt = 6.89 min) confirming peak identity by retention time match within ±2.0% of the standard solution.

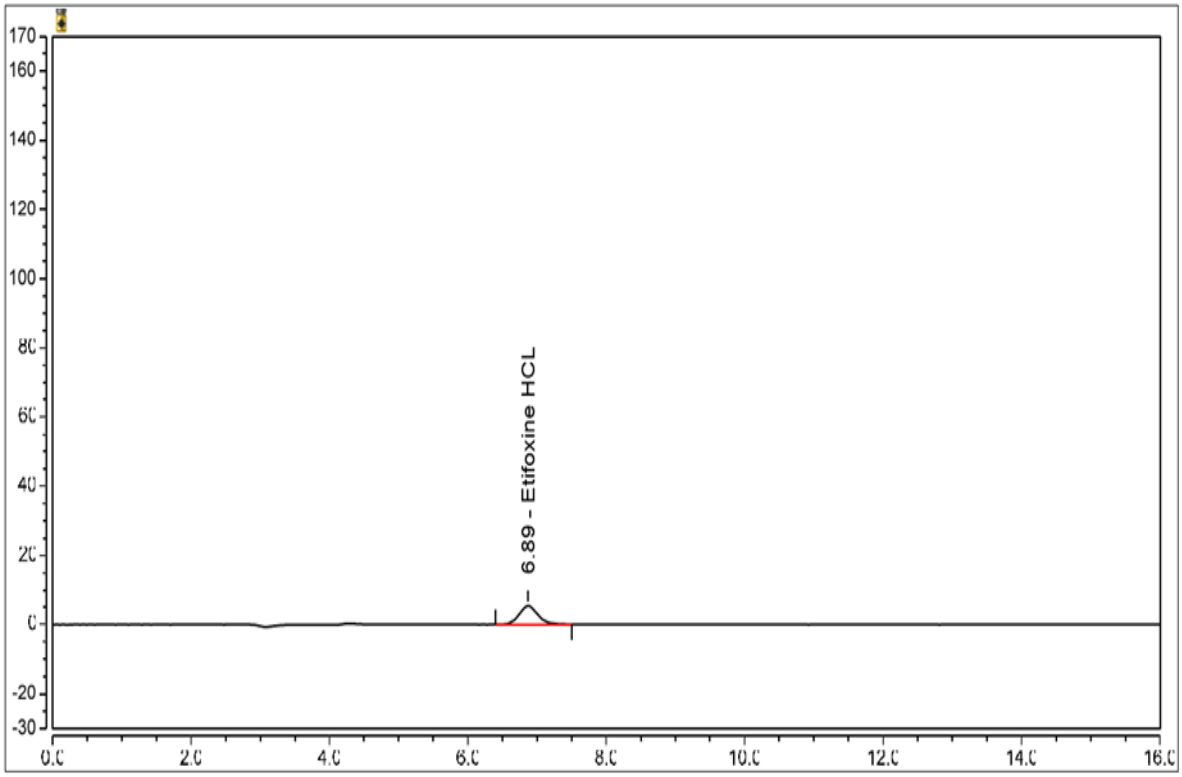


Figure 11: Individual standard chromatogram of Alprazolam (Rt = 10.46 min) confirming peak identity by retention time match within ±2.0% of the standard solution.

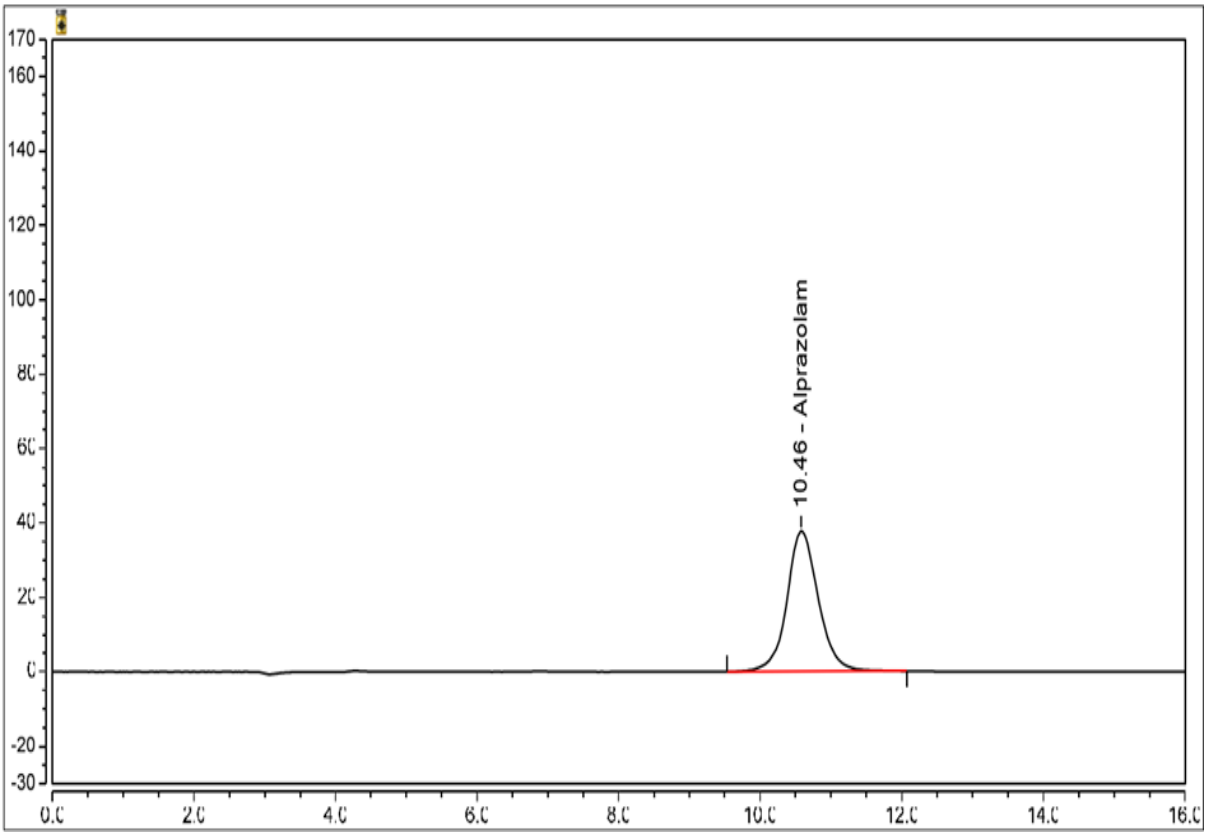


Figure 12: Linearity calibration curve for Etifoxine Hydrochloride over the concentration range 25–75 µg/mL (50–150% of working concentration). Slope = 17,083; Intercept = 670; Correlation coefficient r = 0.99999913; % Y-intercept = 0.08%.

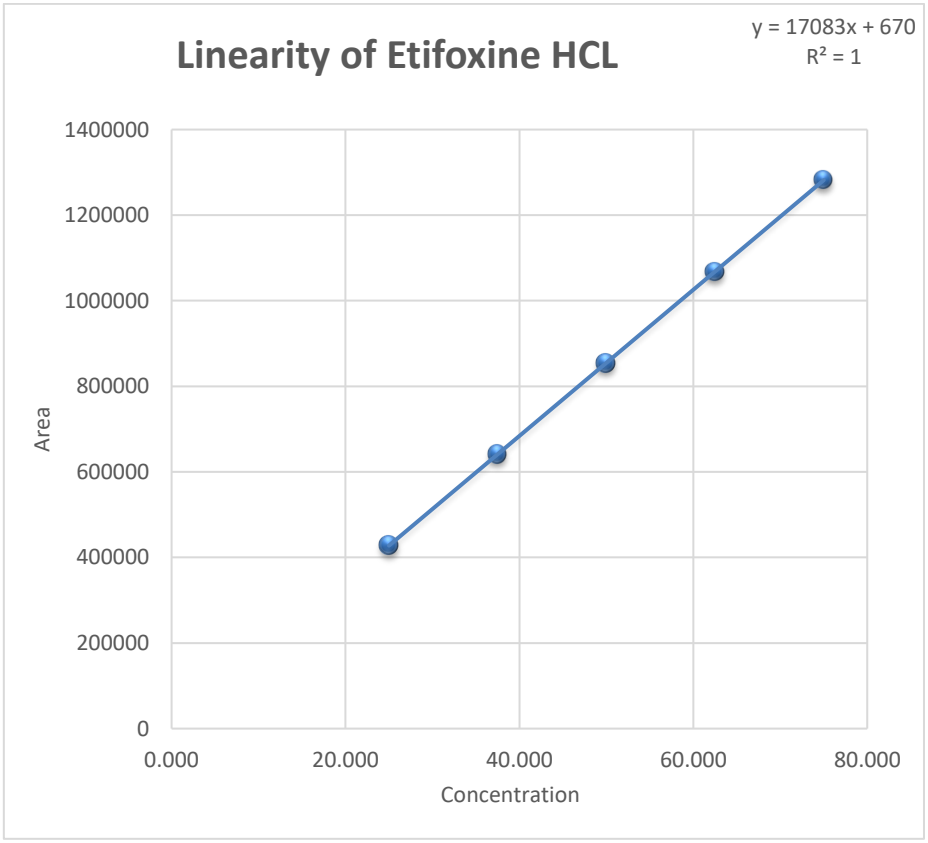
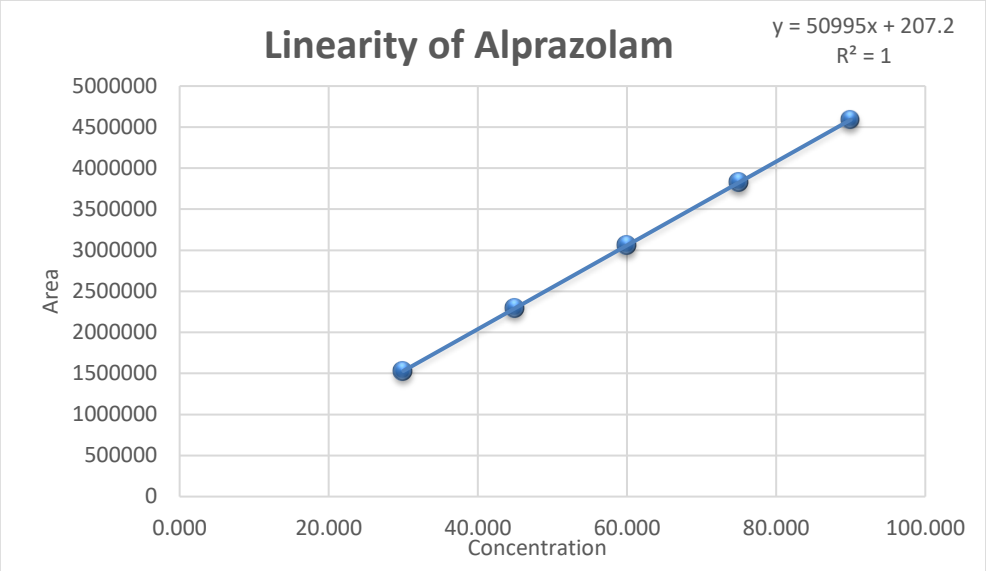


Figure 13: Linearity calibration curve for Alprazolam over the concentration range 30–90 µg/mL (50–150% of working concentration). Slope = 50,995; Intercept = 207.2; Correlation coefficient r = 0.99999996; % Y-intercept = 0.007%.



DISCUSSION

This study developed and validated an isocratic RP-HPLC method for the simultaneous estimation of Etifoxine Hydrochloride and Alprazolam in bulk API form. No prior published method addresses this combination with full ICH Q2(R1) validation the literature contains only independent methods for each drug or pairings with pharmacologically unrelated compounds. That gap is what this work directly addresses.

Method development ran through six successive trials, each targeting a specific deficiency in the previous condition. Early trials on a Zorbax SB-CN column failed to elute Etifoxine HCl or gave Alprazolam peaks with theoretical plates as low as 1635 unacceptable for a validated assay. Switching to a Waters Symmetry column improved elution but produced asymmetric peaks and an unnecessarily long run time. The Hypersil ODS C18 column (150 × 4.6 mm, 5 µm) with phosphate buffer pH 4.0: acetonitrile (25:75 v/v) resolved both issues. Retention times settled at 6.87 min for Etifoxine HCl and 10.42 min for Alprazolam, with tailing factors of 1.1 and 1.0 and theoretical plates of 6354 and 10,423 all within pharmacopoeial limits.

The pH 4.0 phosphate buffer was selected on standard reversed-phase logic. Both drugs are partially ionised at this pH operating 2–3 units below their pKa values (5.18 for Etifoxine HCl; 5.01 for Alprazolam) suppresses ionisation enough to give stable, reproducible retention and reduces silanol-based secondary interactions that broaden peaks on C18 phases. Acetonitrile was preferred over methanol for its lower UV cutoff and reduced viscosity, which sharpen peaks and lower system backpressure at this organic fraction.

Detection at 247 nm was selected because this is the isoabsorptive point where both analytes show proportional UV response (0.3494 AU for the mixture vs. 0.4795 AU and 0.8409 AU at their individual maxima). Working at either drug's primary λmax would have given adequate signal for one and poor signal for the other 247 nm avoids that disparity cleanly.

System suitability was fully compliant across five replicates: peak area %RSD of 0.4% and 0.6%, tailing factors of 1.2 and 1.0, theoretical plates of 6204 and 10,574, and a resolution of 3.2. Despite sharing an anxiolytic pharmacophore and overlapping UV spectra, both compounds are baseline separated under these isocratic conditions a practically important outcome for quality control settings where simplicity and robustness matter.

Linearity correlation coefficients of 0.99999913 and 0.99999996 with %Y-intercepts of 0.08% and 0.007% confirm negligible proportional error in the calibration model across 50–150% of working concentration. Mean recoveries of 99.2–99.5% for Etifoxine HCl and 99.1–99.9% for Alprazolam, with all 95% confidence intervals within 98.0–102.0%, confirm the method is free from systematic error across the assay range.

Precision %RSD values stayed at or below 0.7% for repeatability and 0.6% for intermediate precision.

The absolute mean difference between analysts was 0.1% for Etifoxine HCl and 0.6% for Alprazolam both comfortably within the 2.0% tolerance. Robustness testing identified flow rate as the most critical variable; the largest deviation observed was 2.0% for Etifoxine HCl at 1.1 mL/min, which is expected behaviour for a moderately retained compound on an isocratic C18 system. All other variations remained well within limits.

LOD and LOQ were not evaluated ICH Q2(R1) does not require these for assay procedures, and the method was not designed as an impurity detection system.

Taken together, the validation data confirm a method that meets all applicable ICH Q2(R1) requirements in a single 16-minute isocratic run shorter and less solvent-intensive than the individual methods previously reported for each drug separately.

CONCLUSION

An RP-HPLC method was developed and validated for the simultaneous estimation of Etifoxine Hydrochloride and Alprazolam in an API mixture. The optimised conditions Hypersil ODS C18 column (150 × 4.6 mm, 5 µm), phosphate buffer pH 4.0 and acetonitrile (25:75 v/v) as mobile phase, UV detection at 247 nm, column temperature 40°C, flow rate 1.0 mL/min, 16-minute run time produced well-resolved, symmetric peaks with a resolution factor of 3.2 and theoretical plates exceeding 6000 for both analytes. Validation against all applicable ICH Q2(R1) parameters gave the following results. Linearity held across 50–150% of working concentration, with correlation coefficients of 0.99999913 and 0.99999996 for Etifoxine HCl and Alprazolam. Mean recoveries were 99.2–99.5% (Etifoxine HCl) and 99.1–99.9% (Alprazolam). Precision was %RSD ≤ 0.7% for method precision and ≤ 0.6% for intermediate precision across all concentration levels. Specificity was confirmed through blank interference studies and PDA peak purity assessment. Deliberate changes in flow rate, detection wavelength, and column temperature each produced assay deviations within ±2.0%. LOD and LOQ were not evaluated, as ICH Q2(R1) does not require these for assay procedures.

No previously published RP-HPLC method had combined validated simultaneous determination of both drugs in bulk API form. Existing methods cover each drug independently or pair them with different partners, typically over narrower concentration ranges and without a complete ICH Q2(R1) validation dataset.

The instrumentation Waters e2695 with a 2998 PDA detector and Empower PRO software is standard in pharmaceutical QC laboratories, and the mobile phase uses common, low-cost reagents. The method is therefore directly applicable to routine quality control without specialist infrastructure.

Future work could extend the method in three directions: forced degradation studies under ICH Q1A(R2) conditions to establish stability-indicating capability; bioanalytical adaptation for plasma and urine matrices to support pharmacokinetic studies; and formulation-level assay development for solid and liquid dosage forms incorporating both drug.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The working standards of Etifoxine Hydrochloride and Alprazolam were provided as gift samples by MSN Organic Pvt. Ltd. and Lee Pharma Ltd., respectively. Neither supplier had any role in study design, data collection, analysis, or the decision to publish.

REFERENCES

1. Prabhakar C, Ganesan G, Chaudhari D, Dalaya N, Mohide A, Thaker S, et al. Efficacy and safety of etifoxine versus alprazolam in generalized anxiety disorders with somatic symptoms: a randomized, double-blind, comparative study. J Anxiety Disord. 2026;

2. Wein S, Riebel M, Seidel P, Brunner LM, Wagner V, Nothdurfter C, et al. Local and global effects of sedation in resting-state fMRI: a randomized, placebo-controlled comparison between etifoxine and alprazolam. Neuropsychopharmacology. 2024;49(11):1738–48.

3. Pérez-Lozano P, Suñé-Negre JM, Roig M, García-Montoya E, Miñarro M, Ticó JR. Development and validation of an HPLC method for determination of alprazolam in tablets. *J Pharm Biomed Anal.* 2004;35(5):1005–1011.

4. Nuss P, Ferreri F, Bourin M. An update on the anxiolytic and neuroprotective properties of

etifoxine: from brain GABA modulation to a whole-body mode of action. Neuropsychiatr Dis Treat. 2019;15:1781–95.

5. ICH Harmonised Tripartite Guideline Q2(R1). Validation of analytical procedures: text and methodology. Geneva: IFPMA; 2005.

6. ICH Harmonised Tripartite Guideline Q1A(R2). Stability testing of new drug substances and products. Geneva: IFPMA; 2003.

7. Pang J, Hu Y, Chen X. HPLC determination of etifoxine hydrochloride and related substances in capsules. *Chin J Pharm Anal.* 2008;43(23):1823–25.

8. Djabrouhou N, Guermouche MH. Development of a stability-indicating HPLC method of etifoxine with characterization of degradation products by LC-MS/TOF, ^{1}H and ^{13}C NMR. J Pharm Biomed Anal. 2014;100:11–20.

9. Choudary ML, Srinivas M, Kumari BU. Method development and validation for estimation of etifoxine in capsule dosage form by using RP-HPLC/UV. J Drug Deliv Ther. 2024;14(7):64–70.

10. Shingate SV, Bhosale AV, Kuchekar BS. A simple, cost-effective, stability-indicating HPLC method for the simultaneous determination of alprazolam and propranolol in tablet dosage forms. *World J Pharm Res.* 2019;56(2):47–52.

11. Rele RV, Nikam MA. Development and validation of a novel RP-HPLC method for estimation of alprazolam in tablet dosage forms. *World J Pharm Res.* 2016;5(8).

12. Rupsi, Lakshmana Prabu S, Thirumurugan R. Development and validation of RP-HPLC method for simultaneous estimation of alprazolam and melatonin in combined pharmaceutical dosage forms. Asian J Pharm Clin Res. 2022.

13. Abirami G, Vetrichelvan T, Uma Maheswary S. Analytical method development for the estimation of alprazolam and melatonin by using RP-HPLC in bulk and tablet dosage form. Indo American Journal of Pharmaceutical Research. 2014;4(11):5200–5208.