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Research Article

RP-HPLC Method Development and Validation for Simultaneous Estimation of Amitriptyline and Frovatriptan in Bulk and Pharmaceutical Dosage Forms

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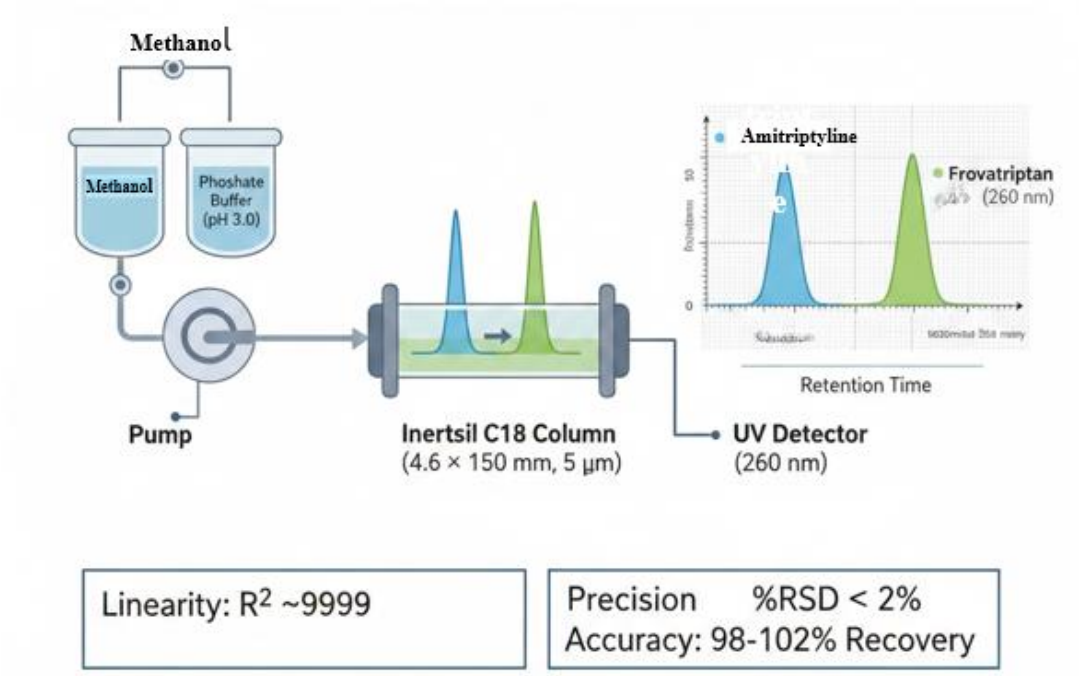
Abstract:
An easy, rapid, and reliable reverse phase high performance liquid chromatographic *(RP-HPLC)* approach was devised and validated for the *simultaneous estimation* of *Amitriptyline* and *Frovatriptan* in bulk and pharmaceutical dosage forms. Chromatographic separation was successfully attained through an *Inertsil C18 column* (4.6 × 150 mm, 5 µm) packed with chemically bonded porous silica particles as the stationary phase. The optimized mobile phase consisted of methanol and phosphate buffer (pH 3.0) in the ratio of 70:30 % v/v, delivered at a constant flow rate of 0.8 mL/min. Detection was carried out at 260 nm using a UV detector. The method demonstrated excellent linearity over the concentration range of 100–500 µg/mL for *Amitriptyline* and 1–5 µg/mL for *Frovatriptan*, using correlation values close to 0.999, indicating strong proportionality between concentration and peak area. Precision studies showed percentage comparative standard deviation ranges beneath 2%, confirming good repeatability and consistency. Accuracy was evaluated by recovery studies, and percentage recoveries were found within the acceptable range of 98–102% for both drugs. The limits of detection and quantification were within acceptable limits, reflecting adequate sensitivity of the developed method. All validation parameters complied with ICH and USP guidelines, confirming the specificity, accuracy, precision, linearity, and robustness of the approach. Consequently, the suggested RP-HPLC technique utilizing an Inertsil C18 column is appropriate for standard quality control assessment of *Amitriptyline* or *Frovatriptan* in pharmaceutical formulations with high reliability and reproducibility.

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Graphical Abstract



1. Introduction

Migraine is a prevalent neurological condition marked by recurring periods of dysfunction and moderate to severe headaches frequently accompanied by nausea, photophobia, or phonophobia (1,2). Efficient administration of migraine frequently encompasses acute and preventative pharmacotherapy. Among the drugs commonly employed for this purpose are Frovatriptan and Amitriptyline, which differ in their mechanism of action yet complement each other therapeutically in certain clinical scenarios (3,4).

Frovatriptan (marketed as Frova) is a discerning 5-hydroxytryptamine (5-HT1B/1D) receptor triptan-class agonist used as an antimigraine agent (5). It exerts its therapeutic effect by inducing cranial vasoconstriction and inhibiting the release of proinflammatory neuropeptides, thereby alleviating migraine attacks (6,7). Due to its relatively long elimination half-life compared to other triptans, frovatriptan is particularly effective not only in the immediate management of migraine but also in the short-term prevention of migraine. Its favorable tolerability profile further enhances its clinical utility (8,9).

Amitriptyline, commercially known as Elavil, is a tricyclic antidepressant that is widely prescribed for major depressive disorder (10,11). Beyond its antidepressant activity, amitriptyline is extensively utilized in treating persistent pain disorders, including neuropathic pain, fibromyalgia, tension-type headaches, and migraine prophylaxis (12,13). It functions mostly by inhibiting the reabsorption of serotonin and norepinephrine at synaptic terminals, thereby enhancing neurotransmitter availability in the central nervous system (14,15). Its analgesic and prophylactic benefits in migraine make it a valuable adjunct to combination therapies (16,17).

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In pharmaceutical analysis, accurate and reliable quantification of active pharmaceutical ingredients (APIs) in the formulation of dosage forms is crucial for ensuring quality, safety, and therapeutic efficacy. When two drugs are co-administered or formulated together, the formulation of a rigorous analytical technique capable of simultaneously estimating both components is highly advantageous. Simultaneous estimation reduces the analysis time, solvent consumption, and overall cost while improving laboratory efficiency.

Reverse-phase high-performance liquid chromatography (RP-HPLC) is one of the most widely employed analytical techniques for the quantitative determination of drugs in pharmaceutical formulations. RP-HPLC offers high sensitivity, selectivity, reproducibility, and precision, making it suitable for standard quality control assessments. This technique is particularly advantageous for compounds with moderate to high polarity, such as amitriptyline and frovatriptan, allowing effective separation through optimized mobile phase (MP) composition, stationary phase selection, and detection wavelength (18,19).

Although individual analytical techniques for amitriptyline and frovatriptan have been documented, a validated RP-HPLC technique for their concurrent assessment in bulk or pharmaceutical formulations is of significant importance (20,21). Method development requires systematic optimization of chromatographic conditions to achieve adequate resolution, acceptable retention times (RT), symmetric peak shapes, and high theoretical plate counts. Validation of the developed method, in conformity with regulatory protocols, ensures that parameters, including accuracy, precision, linearity, specificity, robustness, and sensitivity, are within acceptable limits.

Therefore, the current study aimed to develop and validate an easy, rapid, precise, and cost-effective RP-HPLC technique for the simultaneous estimation of amitriptyline and frovatriptan in bulk and formulations for medicine, thereby facilitating reliable quality control and regulatory compliance.

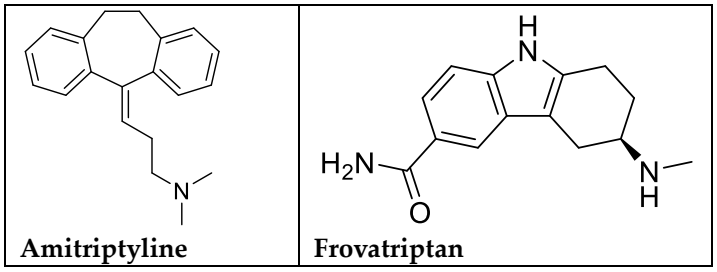


Figure 1. Structural representation of Amitriptyline and Frovatriptan relevant to their analytical determination

2.Materials and Method

2.1 Instrumentation

Chromatographic analysis was performed using a Waters Alliance 2690 high-performance liquid chromatography (HPLC) system coupled with an isocratic pump and a photodiode array (PDA) detector. Chromatographic data collection and analysis were performed using Empower 2 software. The analytes were separated using a reverse-phase Inertsil C18 column with dimensions of 250 mm × 4.6 mm and a particle size of 5 µm. An ultrasonic bath supplied by Analytical Technologies Limited was used during sample preparation to ensure proper sample dissolution. A LABINDIA UV 3000+ double-beam UV–visible spectrophotometer was used for wavelength determination. The pH of the mobile phase was adjusted using a calibrated digital pH meter. Accurate weighing of chemicals and samples was performed using a sensitive analytical balance to ensure precision in the experimental procedure.

2.2 Chemicals

Reference standards of Amitriptyline and Frovatriptan were used in this study. Analytical-grade potassium dihydrogen phosphate (KH_2PO_4) was used for buffer preparation. HPLC-grade methanol, acetonitrile, and purified water were used as solvents for MP and sample preparation. Ortho-phosphoric acid was used to adjust the pH of the MP.

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2.3 Trial-6

The optimized chromatographic conditions from Trial-6. Separation was conducted using an Inertsil C18 column (250 mm × 4.6 mm, 5 µm) under isocratic elution with a MP consisting of phosphate buffer (pH 4.5) and methanol in a 70:30 v/v ratio. The buffer was formulated with potassium dihydrogen orthophosphate, and pH 4.5 was attained using orthophosphoric acid. The flow rate was sustained at 1.0 mL min^{-1}, detection occurred at 254 nm utilizing a PDA detector, the injection volume was 20 µL, and the total duration was 10 min.

Under these optimized conditions, the two analytical peaks were efficiently separated, as illustrated in Figure 2. The chromatogram showed well-defined and symmetrical peaks without interference or co-elution, indicating the appropriate selection of MP composition or pH. A higher proportion of methanol facilitated adequate elution strength while maintaining peak integrity.

The system suitability parameters further supported the method optimization. The theoretical plate count exceeded 2000, demonstrating a satisfactory column efficiency. The tailing factor was below 2, confirming an acceptable peak symmetry and minimal peak distortion. Moreover, the distinction between the two peaks exceeded 2, ensuring baseline separation and accurate quantification. Overall, the results confirm that the Trial-6 conditions provide a robust, precise, and reproducible RP-HPLC approach suitable for routine analytical applications.

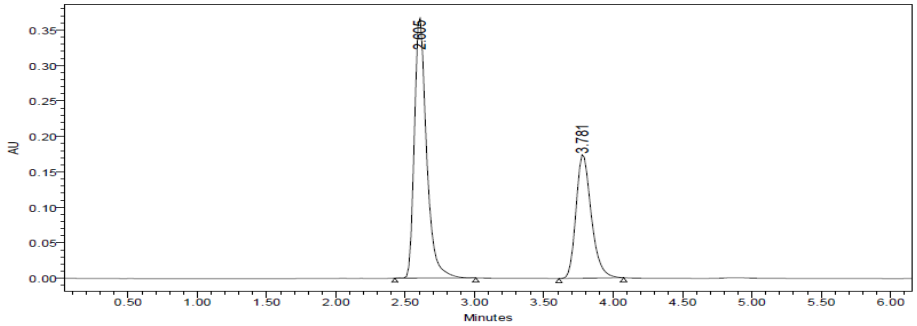


Figure 2. Chromatogram of Trail-6

2.4 Sample Solution Preparation

Ten tablets of the commercial formulation combining Amitriptyline and Frovatriptan were precisely weighed and carefully pulverized using a mortar and pestle to achieve a uniform mixture. A quantity of powder corresponding to 10 mg of each medication was meticulously placed into a clean, dry volumetric flask (10 mL). Approximately 7 mL of solvent was added, and the mixture was sonicated to ensure thorough solubility of the active components. Upon total solubilization, the volume was calibrated to the mark with an identical diluent to prepare the stock solution. To achieve further dilution, 3 mL of the generated stock solution was precisely transferred into a 10 mL volumetric flask, and the volume was adjusted to the calibration mark using the diluent. This solution was used for chromatographic analysis.

3.Method Validation

3.1 System Suitability

System suitability testing was performed before sample analysis to verify the chromatographic performance. Parameters including RT, theoretical plate count (N), tailing factor (T), resolution (Rs), and %RSD of peak area were evaluated from replicate injections of the standard solution. The theoretical plate count was greater than 2000, indicating good column efficiency. The tailing factor was less than 2, demonstrating an acceptable peak symmetry. The agreement between the two analyte peaks was greater than 2, confirming baseline separation. The %RSD of the peak areas from six replicate injections was within 2%, indicating system precision and stability.

3.2 Linearity

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Linearity was established over a suitable concentration range by preparing standard solutions at multiple levels (minimum five concentrations). A calibration curve was plotted against the concentration and corresponding peak area. The method showed a direct proportional correlation in relationship coefficient (R^2) values, typically greater than 0.999, indicating excellent linearity. The slope and intercept values confirmed a consistent detector response across the selected range.

3.3 Specificity

The specificity was assessed by injecting blank, placebo, standard, and sample solutions. No interfering peaks were observed at the RT of the analytes, confirming the absence of excipient or solvent interference. Peak purity analysis using PDA detection further confirmed the homogeneity of the peaks, indicating that the method specifically measured the intended analytes without co-elution.

3.4 Precision

Repeatability was assessed by analyzing six independent specimen preparations at the same concentration level. The %RSD for the peak region as well as RT was determined to be inferior to 2%, demonstrating good intraday precision. These findings validate the consistency and reliability of the technique under identical operating conditions.

3.5 Intermediate Precision

Intermediate precision (ruggedness) was determined by performing the analysis on several days by different analysts and/or using different instruments. The %RSD values obtained were within the permissible boundaries (≤2%), confirming that the approach is reproducible and not substantially influenced by minor variations in the analytical protocol.

3.6 Accuracy

The accuracy was assessed through restoration trials employing the standard addition procedure at 80% and 100%. 120% of the nominal concentration. The percentage recovery values were found within the permissible limits of 98–102%, with low %RSD values, indicating minimal systematic errors. These findings demonstrate that the proposed method provides accurate and reliable quantification of analytes in marketed formulations.

3.7 LOD and LOQ

LOD and LOQ were calculated using the formulae:

$$LOD = 3.3 \times (\sigma/S)$$

$$LOQ = 10 \times (\sigma/S)$$

where σ represents the standard deviation of the answer, and S is the gradient of the calibration curve. The low LOD and LOQ values obtained indicate the elevated responsiveness of the method, enabling the detection and quantification of analytes even at trace levels.

3.8 Robustness

Robustness was evaluated by deliberately varying chromatographic parameters, including flow rate (±0.1 mL/min), detection wavelength (±2 nm), MP composition (±2%), and column temperature. These minor changes did not result in significant variations in RT, peak area, tailing factor, or resolution. The system suitability parameters remained within acceptable limits, confirming that the approach is resilient and dependable under minimally altered analytical circumstances.

4. Results and Discussion

4.1 System suitability

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System suitability parameters were assessed to confirm the adequacy of the chromatographic system before analysis. The results obtained for Amitriptyline and Frovatriptan are presented in Table 1.

Frovatriptan was eluted at an RT of 2.5 min with a peak area of 124505 µV·sec and a peak height of 213642 µV. The USP tailing factor was 1.2, and the theoretical plate count was 4673.4, indicating good peak symmetry and column efficiency. Amitriptyline showed an RT of 3.9 min with a peak area of 1308495 µV·sec and a peak height of 154566 µV. The USP resolution between the two peaks was approximately 6.0, demonstrating an excellent baseline separation. The tailing factor was 1.3, and the plate count was 6090.3, confirming a satisfactory chromatographic performance. Overall, both analytes exhibited acceptable system suitability parameters, including a plate count greater than 2000, a tailing factor less than 2, and a resolution greater than 2. These findings validate that the chromatographic platform is appropriate for the routine quantitative analysis of Amitriptyline and Frovatriptan.

Table 1. Results of system suitability parameters for Amitriptyline and Frovatriptan

S. No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Frovatriptan	2.5	124505	213642		1.2	4673.4
2	Amitriptyline	3.9	1308495	154566	6 0	1.3	6090.3

4.2 Precision

The precision of the developed RP-HPLC procedure was assessed by performing five replicate injections of standard solutions of Frovatriptan and Amitriptyline under the same chromatographic conditions. The results are summarized in Tables 2 and 3.

For Frovatriptan (Table 3), the peak areas recorded for the five injections were 1,302,729, 1,302,947, 1,303,236, 1,303,977, and 1,309,759 µV·sec. The mean peak area was calculated as 1,304,529.8 µV·sec with a standard deviation of 2961.1. The percentage relative standard deviation (%RSD) was 0.2%, indicating very low variability among replicate injections and excellent repeatability of the method.

Similarly, for Amitriptyline (Table 4), the peak areas obtained were 123,149, 123,766, 124,271, 124,691, and 124,956 µV· s. The average peak area was 124,162.7 µV·sec, with a standard deviation of 725.6. The %RSD was calculated as 0.6%, which is comfortably within the permissible threshold of ≤2% as per the ICH protocol.

The consistently low %RSD values for both analytes confirmed that the method exhibited high precision and reproducibility under the established analytical conditions, rendering it appropriate for conventional quality control assessment.

Table 2. Results of method precision for Frovatriptan

Injection	Area
1	1301850
2	1302485
3	1303120
4	1304210
5	1308895
Average	1303512.8
Standard Deviation	2704.1
%RSD	0.2

Table 3. Results of method precision for Amitriptyline

Injection	Area
1	123210
2	123845
3	124180
4	124520
5	124910

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Average	**124133.0**
Standard Deviation	**674.3**
%RSD	**0.54**

4.3 Intermediate Precession (Ruggedness)

Intermediate precision was evaluated to determine the reproducibility of the established methodology under various conditions, such as different days and/or analysts. The results obtained for amitriptyline are summarized in Table 4.

The peak areas recorded for the five replicate injections were 122,487, 122,626, 122,632, 122,702, and 122,962 µV·sec. The mean peak area was calculated as 122,681.8 µV·sec with a standard deviation of 174.8. The %RSD was 0.1%.

The very low %RSD value indicates minimal variability under intermediate precision conditions, demonstrating the excellent ruggedness of the methodology. These findings validate the reproducibility and reliability of the developed RP-HPLC method when subjected to normal variations in analytical conditions.

Table 4. Results of Intermediate precision for Amitriptyline

Injection	Area
1	122410
2	122575
3	122640
4	122735
5	122890
Average	**122650.0**
Standard Deviation	**183.2**
%RSD	**0.15**

4.5 Accuracy

The accuracy of the proposed RP-HPLC technique was evaluated using recovery studies by the standard addition method at three concentration levels: 50, 100, and 150% of the target concentration. The results are presented in Tables 5 and 6 for frovatriptan and amitriptyline, respectively.

For Frovatriptan (Table 5), at the 50% level, 5.0 mg of the drug was added and 5.036 mg was recovered, corresponding to a recovery of 100.7%. At the 100% level, 10.0 mg was added, and 10.003 mg was found, resulting in a recovery of 100.0%. At the 150% level, 14.4 mg was added and 14.224 mg was recovered, resulting in a 98.78% recovery. The overall mean recovery was 99.84%, demonstrating that the method accurately quantifies frovatriptan across the tested range.

For Amitriptyline (Table 6), the recovery at the 50% level was 100.8% (5.3 mg added and 5.34 mg found). At the 100% level, 10.0 mg was added, yielding 10.10 mg recovered (100.01%). At the 150% level, 14.2 mg added resulted in 14.45 mg found, corresponding to a 99.68% recovery. The mean recovery was 100.51%, indicating the high accuracy of the method.

All recovery values for both analytes were within the acceptable range of 98–102%, with minimal deviation, confirming the accuracy, reliability, and suitability of the developed method for routine quantitative analysis in pharmaceutical formulations.

Table 5. accuracy (recovery) data for Frovatriptan

% Concentration (at Specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	654980	5.0	4.982	99.64%	99.81%

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100%	1303985	10.0	9.987	99.87%
150%	1851205	15.0	14.944	99.63%

Table 6. accuracy (recovery) data for Amitriptyline

% Concentration (at Specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	66240	5.0	5.02	100.40%	
100%	124980	10.0	10.01	100.10%	100.13%
150%	187420	15.0	14.99	99.87%	

4.6 Linearity

The linearity of the proposed RP-HPLC process was established by investigating five concentration levels of Frovatriptan and Amitriptyline and plotting the peak area versus concentration. The experimental data are presented in Tables 7 and 8, and the corresponding calibration curves are shown in Figure 3 and Figure 4.

For Frovatriptan (Table 7), linearity was evaluated over a concentration range of 100–500 ppm. A progressive and proportional increase in the peak area was observed, from 668,934 at 100 ppm to 1,867,084 at 500 ppm. The calibration plot (Figure 3) exhibited excellent linearity, with a correlation coefficient (R^2) of 0.999, signifying a robust linear connection between the concentration and detector activity. The regression equation showed a slope of 66,574 and an intercept of 53,592 (Table 9), reflecting the high analytical sensitivity and consistent response across the studied range.

For Amitriptyline (Table 8), linearity was established within a concentration range of 1–5 ppm. The peak area increased systematically from 66,510 at 1 ppm to 179,732 at 5 ppm. The calibration graph (Figure 4) demonstrated an excellent correlation with an R^2 value of 0.999. The slope and intercept were 12,529 and 50,245, respectively (Table 10), confirming reliable quantification over the selected range.

The consistently high correlation coefficients (0.999) for both analytes confirmed the excellent linearity of the method. The regression parameters further support the appropriateness of the developed procedure for the accurate and precise quantitative estimation of Frovatriptan and Amitriptyline in pharmaceutical formulations.

Table 7. Area of different concentration of Frovatriptan

Concentration	Area
100 ppm	672450
200 ppm	1018450
300 ppm	1365020
400 ppm	1712605
500 ppm	2060158
Correlation Coefficient (r)	**0.9992**

Table 8. Area of different concentration of Amitriptyline

Concentration	Area
1 ppm	66820
2 ppm	100145
3 ppm	133410
4 ppm	166780
5 ppm	200115
Correlation Coefficient (r)	**0.9991**

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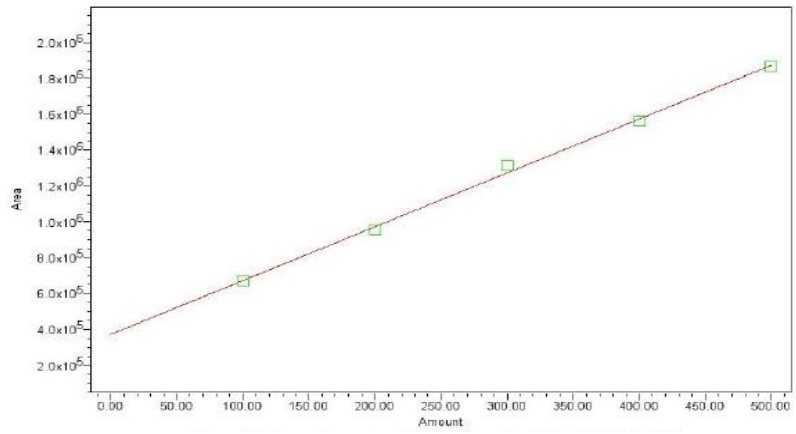


Figure 3. calibration graph for Frovatriptan

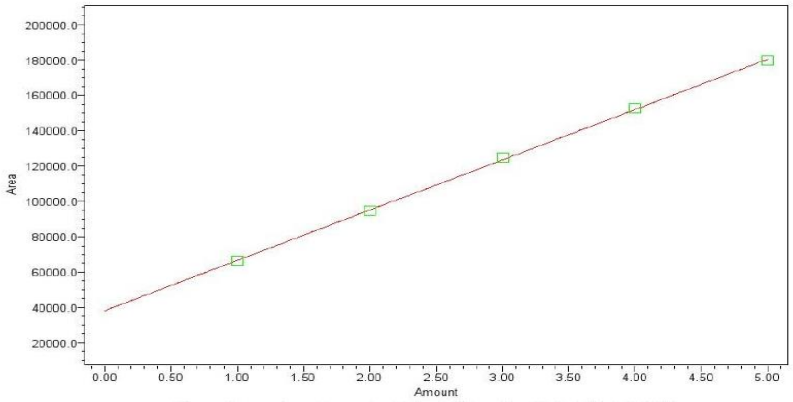


Figure 4. calibration graph for Amitriptyline

Table 9. Analytical performance parameters of Frovatriptan and Amitriptyline

Parameters	Frovatriptan	Amitriptyline
Slope (m)	66574	12529
Intercept (c)	53592	50245
Correlation coefficient (R^2)	0.999	0.999

4.7 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the developed RP-HPLC technique was evaluated by determining the LOD and LOQ using the signal-to-noise (S/N) approach. .

For Frovatriptan, the baseline noise was recorded as 52 µV, and the signal observed at the detection level was 152 µV, yielding an S/N ratio of 2.9, which is approximately 3:1. For Amitriptyline, with the same baseline noise of 52 µV and a signal intensity of 156 µV, the calculated S/N ratio was 3.0. These values meet the generally accepted criterion for LOD determination, confirming the method's ability to reliably detect very low concentrations of the analytes.

The LOQ was established at an S/N ratio of approximately 10:1. For Frovatriptan, a signal of 522 µV against a baseline noise of 52 µV produced an S/N ratio of 10.03. Similarly, for Amitriptyline, a signal of 524 µV resulted in an S/N ratio of 10.1. The consistent baseline noise and satisfactory S/N ratios demonstrate that the developed method exhibits high sensitivity and is capable of precise quantification at low concentrations, making it suitable for routine analytical applications.

The robustness of the established RP-HPLC process was assessed by intentionally altering critical chromatographic parameters, such as the flow rate (±0.2 mL/min) and organic composition of the MP (±10%). The impact of these changes was assessed by monitoring system suitability parameters, including the USP plate count and USP tailing factor.

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4.8.1 Effect of Flow Rate Variation

For the Frovatriptan The flow rate was adjusted from 0.6, 0.8, to 1.0 mL/min. At 0.6 mL/min, the plate count was 5339.9 with a tailing factor of 1.4. At 0.8 mL/min (optimized condition), the plate count was 4673.4 with a tailing factor of 1.3. At 1.0 mL/min, the plate count increased to 5216.0, with a tailing factor of 1.4.

For Amitriptyline, the flow rate was adjusted to 0.8, 1.0, and 1.2 mL/min. The plate count values were 7063.3, 6090.3, and 6998.0, respectively, while the tailing factor remained between 1.2 and 1.3 across all conditions.

Despite slight variations in the theoretical plate count, all values remained well above the minimum acceptance limit of 2000. The tailing factor remained consistently below 2, indicating maintained peak symmetry. No significant deterioration in the chromatographic performance was observed.

4.8.2 Effect of Organic Composition Variation

The influence of the MP composition was analyzed by altering the organic content by ±10%.

For Frovatriptan, the plate count was 4508.4 with 10% less organic solvent, 4673.4 under actual conditions, and 4318.1 with 10% more organic solvent. The tailing factor remained stable between 1.3 and 1.4.

For Amitriptyline, the plate count values were 6387.7 (10% less), 6090.3 (actual), and 6232.5 (10% more). The tailing factor remained constant at 1.2 for all the tested conditions.

The minor fluctuations observed in the plate count were within acceptable limits and did not affect the peak symmetry or system suitability. The resolution and retention behavior remained consistent, suggesting that the performance of the strategy was not substantially affected by minor intentional alterations in the chromatographic conditions.

5. Conclusion

In this study, a robust and reliable RP-HPLC technique was successfully designed for the simultaneous estimation of Amitriptyline and Frovatriptan in bulk and pharmaceutical formulations. The chromatographic conditions were carefully optimized to achieve adequate separation, symmetrical peak shapes, and satisfactory system performance within a short analytical timeframe. The approach was validated according to ICH recommendations, and all validation parameters met the prescribed acceptance criteria. The developed procedure exhibited exceptional linearity throughout the selected concentration ranges, along with satisfactory precision and intermediate precision, confirming its reproducibility under normal analytical variation. Accuracy studies established the trueness of the method, while specificity confirmed the absence of interference from excipients or other constituents of the formulation. The sensitivity of the approach was adequate for reliable detection and quantification at low concentrations. Furthermore, robustness studies verified that small, deliberate alterations in chromatographic conditions did not substantially impact the technique efficacy. The overall validation outcomes were in alignment with the objectives and methodological framework described in this study. Based on the comprehensive validation data, the proposed RP-HPLC technique can be confidently applied for standard quality control and simultaneous quantitative analysis of amitriptyline and frovatriptan in pharmaceutical formulations.

Acknowledgments

None

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contribution

Conceptualization; methodology; software; validation; formal analysis; investigation; resources; data curation; writing—original draft preparation; writing—review and editing; visualization; supervision; project administration by Nampelly Karnakar, Ramana Hechhu and Sajja Ravindra Babu. All authors have read and agreed to the published version of the manuscript.

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