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Original Article

# DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE DETERMINATION OF BAMIFYLLINE HYDROCHLORIDE IN TABLET DOSAGE FORM

**PANCHUMARTHY RAVISANKAR[1*], SHAHEEM SULTHANA[1], INTURI. MARY THANUJA[1], A. DIHITHA CHOWDARY[1], J. VYSHNAVI[1]**

**Vignan Pharmacy College, Vadlamudi, Guntur, Andhra Pradesh, India**
**Email: banuman35@gmail.com**

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**ABSTRACT**

**Objective:** The objective of the current study was to develop and validate a novel RP-HPLC method for determination of bamifylline hydrochloride in pharmaceutical dosage form.

**Methods:** Chromatographic separation was conducted on Agilent technologies-1260 series with the G1311C quaternary pump, eclipse XDB $C_{18}$ column (4.6 mm i.d. X 250 mm, 5 µm particle sizes) and equipped with photodiode array detector G1315D. Mobile phase consisted of methanol and acetonitrile were mixed in the ratio of 90:10 v/v, was used at a flow rate of 1 ml/min and detection wavelength was set at 263 nm.

**Results:** The retention time for bamifylline hydrochloride was found to be 2.913 min. The calibration was linear ($r^2$= 0.9996) in the concentration range of 2-10 µg/ml. The limit of detection and the limit of quantitation were found to be 0.4825 µg/ml and 1.4621 µg/ml respectively. Recovery of bamifylline hydrochloride in tablet formulation was observed in the range of 99.6-99.8 %. Percentage assay of bamifylline hydrochloride (Bamifix) was found to be 99.4 % w/w.

**Conclusion:** Thus the novel proposed method for bamifylline hydrochloride was found to be feasible for the estimation of bamifylline hydrochloride in bulk as well as a pharmaceutical dosage form.

**Keywords:** Bamifylline hydrochloride, RP-HPLC, Validation, ICH guidelines


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## INTRODUCTION

The chemical name of bamifylline hydrochloride is 8-Benzyl-7-[2-[N-ethyl-N-2-hydroxyethyl amino) ethyl] theophylline hydrochloride (fig. 1). Bamifylline hydrochloride is a theophylline derivative and is used as Anti-asthmatic agent [1], bronchodilator [2], a Non-selective phosphodiesterase inhibitor and chronic obstructive pulmonary disease (COPD). Mechanism of action of bamifylline hydrochloride is an adenosine $A_1$ receptor antagonist [3]. Generally, bamifylline hydrochloride is not converted to theophylline in the body. Bamifylline and theophylline drugs are bronchodilators and their synthetic origin and belong to Xanthine. Theophylline and the physical mixtures TA-NSD [4] and compatibility studies with different polymers [5] were investigated by using a polarizing microscope, FTIR, DSC, PXRD and KFT. A thorough literature survey of bamifylline hydrochloride revealed that very few analytical methods have been reported for estimation of bamifylline hydrochloride hitherto. The majority of methods for determination of bamifylline hydrochloride in biological fluids and pharmaceutical dosage forms include liquid chromatography with ultraviolet detection [6] HPLC [7-11], HPTLC [12, 13]. This novel proposed method contributes quick estimation, correct peak shape, precise, simple, and quick, use of smaller sample volumes and utilizing methanol as a mobile phase which is economical when compared with other existing methods. So it is necessary to develop a simple, precise and rapid RP-HPLC method for quantitative determination of bamifylline hydrochloride. This work describes the validation parameters stated by the International Conference on Harmonization [ICH] guidelines Q2 (R1) [14, 15].

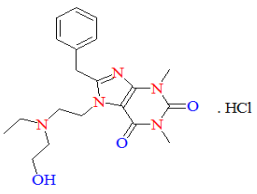


**Fig. 1: Chemical structure of bamifylline hydrochloride from pubchem**

## MATERIALS AND METHODS

### Chemicals and reagents

The above said the standard drug was gifted from Hetero Labs Ltd., Hyderabad, India. All the chemicals used in this method were of high-grade purity and purchased from Merck Chemical Division Ltd., Mumbai. HPLC grade acetonitrile, water, methanol and triethylamine were obtained from Merck Pharmaceuticals private Ltd., Mumbai, India. Commercial tablets of the above said formulation was obtained from a local pharmacy.

### Instrumentation and conditions

The high pressure liquid chromatographic system utilized was an Agilent high-pressure liquid chromatograph 1260 series with the GI311C quaternary pump, eclipse XDB-$C_{18}$ column (5 µm particle size x 4.6 × 250 mm) (made in USA) and a diode array detector G1315D was utilized. Ezchrome elite software was used for chromatography data acquisition, processing and control of HPLC chromatograph. Digital pH meter (systronics model-802), an electronic balance (Shimadzu TX223L), a sonicator (spectral lab, model UCB 40) and UV-Visible spectrophotometer (systronics model-2203) were used in this study.

### Preparation of mobile phase

To prepare mobile phase HPLC grade methanol and acetonitrile were mixed in the ratio of 90:10 % v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

### Preparation of stock and working standard solutions

Accurately 10 mg of pure bamifylline hydrochloride was weighed and transferred in 10 ml clean volumetric flask and 5 ml mobile phase was added, if necessary sonicate to dissolve. The volume was adjusted up to the mark with the mobile phase. This is the primary stock solution of bamifylline hydrochloride with a concentration of 1000 µg/ml. The secondary stock solution is prepared by adding 1 ml of primary stock solution in 10 ml volumetric flask and made up the volume with a mobile phase having the concentration range 100

µg/ml. five working standard solutions were prepared for calibration graph by adding defined volumes of the secondary stock solution and diluting with the mobile phase. The concentrations of bamifylline hydrochloride are 2, 4, 6, 8 and 10 µg/ml respectively.

**Sample preparation for tablets**

Accurately weighed twenty bamifylline hydrochloride tablets and average weight was calculated. Accurately weighed a portion of tablet, powder equivalent to 100 mg of bamifylline hydrochloride and transfer into a 100 ml volumetric flask to this 50 ml mobile phase was added and sonicated for 15 min. Mobile phase was adjusted up to the mark. The solution was filtered using 0.45 µm nylon filter. From the above solution pipette out 1.0 ml into a 100 ml volumetric flask and dilute with mobile phase up to the mark and mix well. Further diluted to get desired concentration. The amount present in the tablet was calculated from plotted calibration graph or utilizing regression equation.

After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the sample solution was loaded in the 20 µl fixed sample loop of the injection port.

**Analytical method validation**

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters such as specificity, system suitability, linearity, precision, accuracy, robustness, limit of detection (LOD), limit of quantitation (LOQ) as per ICH Q2 (R1) guidelines.

**System suitability parameters**

The chromatographic systems used for analysis must pass system suitability before going to start the experiment. At first HPLC system is stabilized for forty minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and retention time. The % RSD for the peak area of six replicate injections of Sofosbuvir standard NMT 2.0. The parameters such as tailing factor, % RSD and theoretical plates were studied.

**Linearity**

A standard stock solution of the bamifylline hydrochloride (1 mg/ml) was prepared with the mobile phase. To study the linearity range of drugs, serial dilutions were made from standard stock solution in the range of 2-10 µg/ml.

**Specificity**

Specificity of an analytical method is its ability to measure accurately and specifically the analyte of interest without interference from placebo and degradation products. The specificity of the method was established by injecting blank, placebo and standard solution in triplicate and recording the chromatograms.

**Precision**

The precision of the method was determined by repeatability (intraday) and intermediate precision (interday). Repeatability was determined by performing six repeated analysis of the same working solution of bamifylline hydrochloride on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on different days and also by another analyst performing the analysis in the same laboratory (between-analysts).

**Accuracy**

The accuracy of a method is defined as the closeness of a measured value to the true value. The recovery studies were carried out at 50 %, 100 %, and 150 % of the target level in the tablet in triplicate each in the presence of placebo.

**Robustness**

The robustness was determined by analyzing the same sample under a variety of conditions. The factors considered to be: variations in the flow rate, the organic ratio of mobile phase and pH. There were no significant changes in the chromatographic pattern when the above modifications were made in the experimental conditions, showing thus that the method is robust. The % RSD of bamifylline hydrochloride should be not more than 2.0 %.

**LOD and LOQ**

Limit of detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated based on using following formulas, LOD = 3.3 x σ/S and LOQ = 10 x σ/S, where σ is the deviation response. S is the slope of the calibration curve.

**RESULTS AND DISCUSSION**

**Method development and optimization**

The current study was aimed at developing a sensitive, rapid and accurate reversed-phase HPLC method for the analysis of bamifylline hydrochloride in bulk drug and in pharmaceutical dosage form. In order to get decorous retention time, sharp and well-resolved peak, the parameters such as different flow rates, detection wavelength, a choice of mobile phases containing acetonitrile, methanol, and HPLC grade water were studied. Good quality symmetrical sharp peak, minimum tailing factor in short run time was obtained with $C_{18}$ column and mobile phase composed of methanol: acetonitrile in the ratio of 90:10 v/v, at a flow rate of 1 ml/minute with maximum lambda max at 263 nm. All the system suitability parameters were computed at the optimised chromatographic conditions. The retention time of 2.913, plate number of 12215 and a tailing factor of 1.112 were obtained for bamifylline hydrochloride. The obtained values of the entire system suitability parameters are within the limits of the agreeable range, which shows that the proposed method is fit for detection of bamifylline hydrochloride in the tablet form. The optimum chromatographic conditions and system suitability parameters are tabulated in table 1.

**Table 1: Optimized chromatographic conditions and system suitability parameters**

| Parameter | Chromatographic conditions |
|---|---|
| Instrument | Agilent Technologies 1260 series with the G1311C quaternary pump. |
| Column | Eclipse XDB $C_{18}$ column (4.6 mm i.d. X 250 mm, 5 µm particle size) |
| Detector | 1260 series DAD VL photo diode array detector G1315D |
| Mobile phase | Methanol: acetonitrile (90: 10 % v/v) |
| Flow rate | 1 ml/minute |
| Detection wavelength | UV at 263 nm |
| Runtime | 10 min |
| Temperature | Room temperature (25 °C) |
| Volume of injection loop | 20 µl |
| Retention time * | 2.913 min |
| Theoretical plates [th. pl]* | 12215 |
| Tailing factor* | 1.112 |

*= number of six determinations.

**Linearity**

The calibration curve was constructed between concentrations versus peak area by the prepared in the concentration range of 2-10 µg/ml of stock solution. The linearity range was found to be 2-10 µg/ml and the results are tabulated in table 2. The calibration graph of bamifylline hydrochloride is presented in fig. 2. The regression equation was found to be Y = 60359x+2678.1. The correlation coefficient of bamifylline hydrochloride $r^2$ was noted as 0.9996 which states that the method was good linear to the concentration versus peak area responses. The results show that a phenomenal relationship between peak area and concentration of the drug in the calibration curve. The standard chromatograms of bamifylline hydrochloride are presented in fig. 3 to fig. 7.

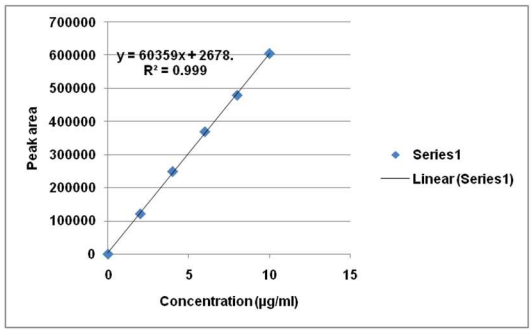


**Fig. 2: Calibration graph of bamifylline hydrochloride**

**Table 2: Calibration data of bamifylline hydrochloride**

| S. No. | Concentration (µg/ml) | Peak area, (mAu) |
|---|---|---|
| 1 | 2 | 121573 |
| 2 | 4 | 249585 |
| 3 | 6 | 369733 |
| 4 | 8 | 480018 |
| 5 | 10 | 605006 |

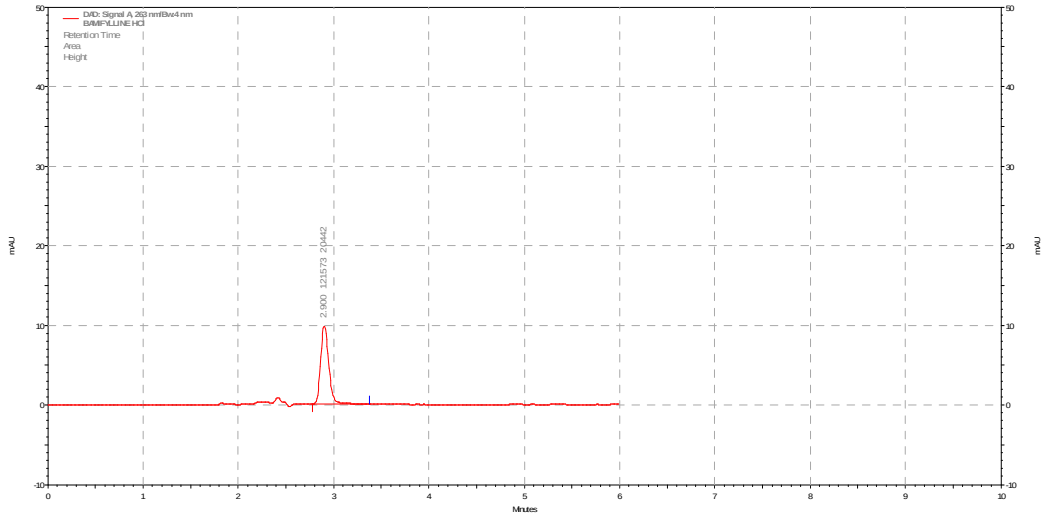


**Fig. 3: Standard chromatogram bamifylline hydrochloride (2 µg/ml)**

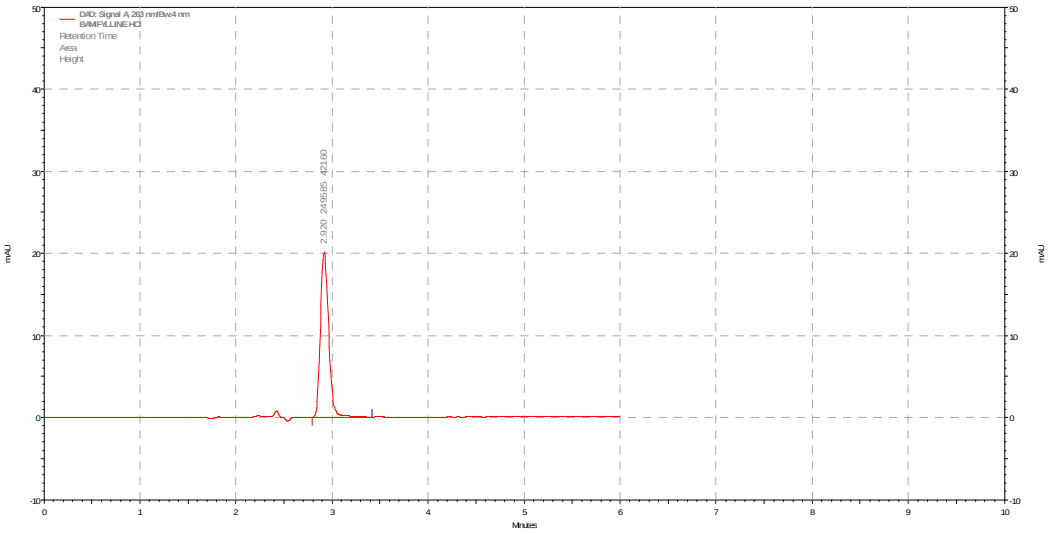


**Fig. 4: Standard chromatogram bamifylline hydrochloride (4 µg/ml)**

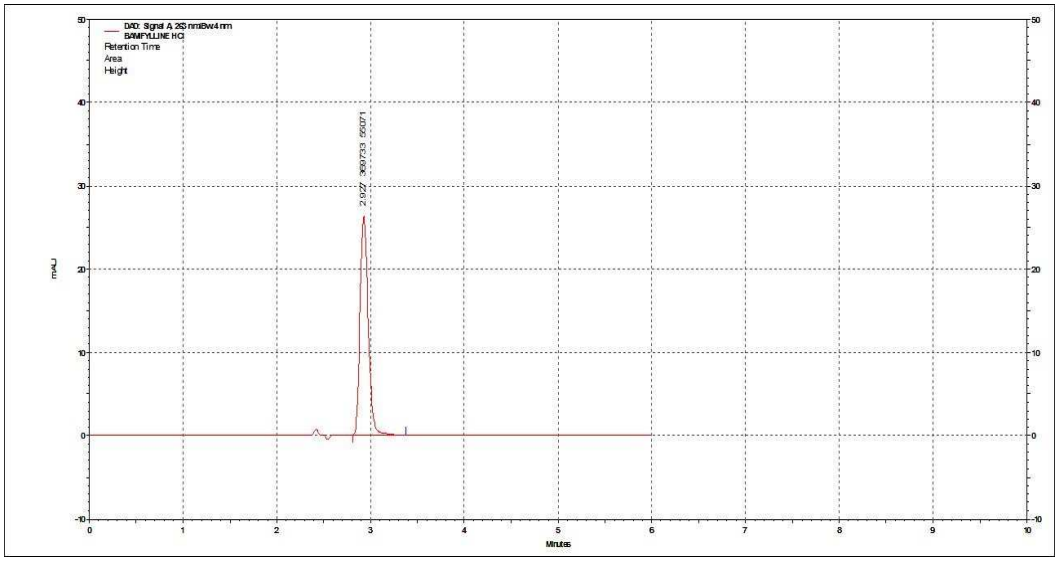


**Fig. 5: Standard chromatogram bamifylline hydrochloride (6 µg/ml)**

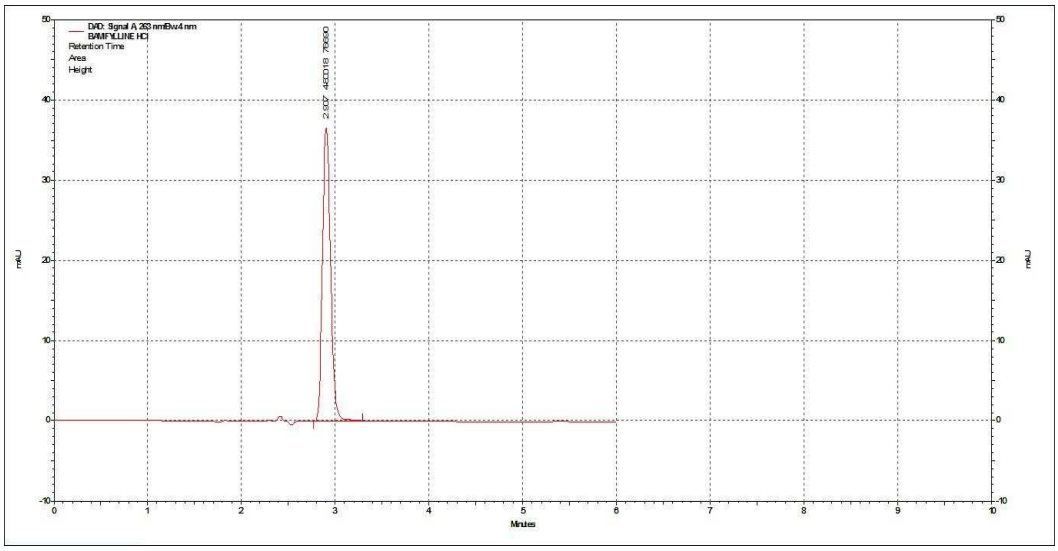


**Fig. 6: Standard chromatogram bamifylline hydrochloride (8 µg/ml)**

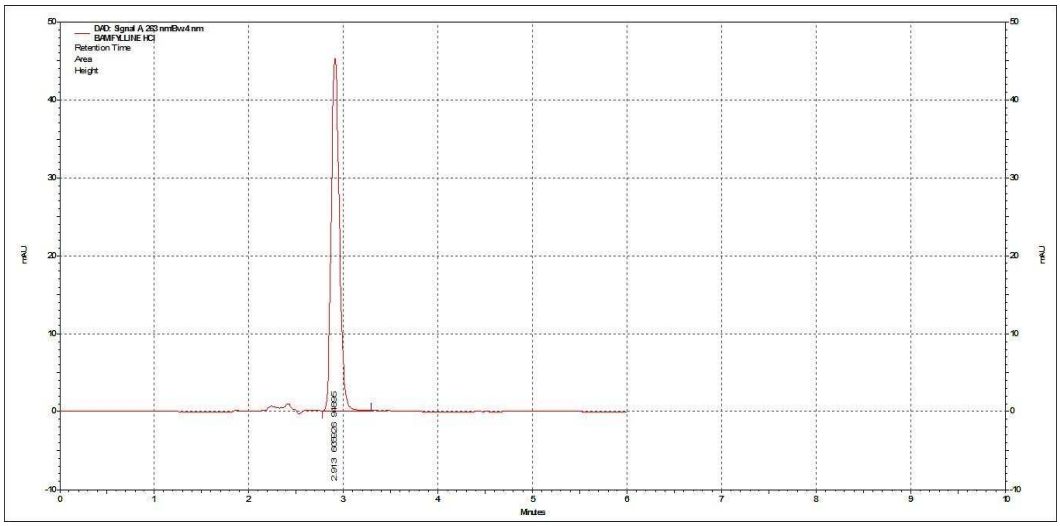


**Fig. 7: Standard chromatogram bamifylline hydrochloride (10 µg/ml)**

**Specificity**

Commonly used tablet excipients did not interfere with this method. It shows that the method is specific. Furthermore, the well-shaped peaks also indicate the specificity of the method. The specificity results are tabulated in table 3.

**Precision**

System precision is shown in table 4. It was noted that the percentage RSD values of precision for Intra-day and inter-day (table 5) precision was 0.0008 and 0.0007 respectively. Intra-day and inter-day % RSD values lower than 2% clearly assuring that this method was found to be fairly precise and reproducible.

**Accuracy**

Regarding accuracy, a known amount of the standard drug was added to the fixed amount of preanalyzed sample solution. % recovery was calculated by comparing the area before and after addition of the standard drug. The standard addition method was performed at 50 %, 100 % and 150 % levels. The high value of recoveries obtained for bamifylline hydrochloride indicates that the proposed method was found to be accurate. The recovery results are presented in table 6.

**Table 3: Specificity study**

| Name of the solution | Retention time ($t_R$) min. |
|---|---|
| Mobile phase | No peaks |
| Placebo | No peaks |
| BMF 10 µg/ml | 2.913 min. |

**Table 4: Results of system precision**

| Sample | Concentration (µg/ml) | Injection no. | Peak area (mAU) | mean±SD* | % RSD# |
|---|---|---|---|---|---|
| Bamifylline | 10 | 1 | 605006 | | |
| | | 2 | 605009 | 6053.7±472.3 | 0.07 |
| | | 3 | 605200 | | |
| | | 4 | 605098 | | |
| | | 5 | 605980 | | |
| | | 6 | 605985 | | |

*Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

**Table 5: Intra-day and Inter-day precision data of bamifylline**

| Concentration (µg/ml) | Intra-day precision | Inter-day precision | | |
|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 |
| 6 | 369733 | 369732 | 369731 | 369732 |
| | 369731 | 369733 | 369734 | 369738 |
| | 369737 | 369734 | 369727 | 369726 |
| | 369735 | 369730 | 369729 | 369729 |
| | 369738 | 369735 | 369730 | 369741 |
| | 369730 | 369738 | 369736 | 369735 |
| mean±SD* | 3.224±3697 | 2.732±3697 | 3.311±3697 | 5.612±3697 |
| % RSD# | 0.000872 | 0.000739 | 0.000896 | 0.001518 |

*Each value is represented as a mean±SD of 5 observations (n=5), SD: Standard Deviation, RSD: Relative Standard Deviation, #Acceptance criteria<2.0.

**Table 6: Accuracy study**

| The amount of the sample added (µg/ml) | The amount of standard drug solution added (µg/ml) | Percentage of standard added | Mean percent recovery | %RSD* |
|---|---|---|---|---|
| 6 | 3 | 50 % | 99.7 | 0.11 |
| 6 | 6 | 100 % | 99.8 | 0.09 |
| 6 | 9 | 150 % | 99.6 | 0.13 |

*Average of triplicate injections

**Table 7: Robustness results of bamifylline hydrochloride**

| Parameter | Optimized | Used | Retention time (min) | Plate count$ | Peak asymmetry # | Remark |
|---|---|---|---|---|---|---|
| Flow rate | 1.0 | 0.8 ml/min | 2.917 | 12,200 | 1.190 | *Robust |
| (±0.2 ml/min) | ml/min | 1.0 ml/min | 2.913 | 12,215 | 1.25 | *Robust |
| | | 1.2 ml/min | 2.911 | 12,300 | 1.21 | *Robust |
| Detection wavelength | 263 nm | 258 nm | 2.912 | 12,245 | 1.113 | Robust |
| (±5 nm) | | 263 nm | 2.913 | 12,215 | 1.112 | Robust |
| | | 268 nm | 2.913 | 12,225 | 1.113 | Robust |
| Mobile phase composition | 90:10 v/v | 95:05 v/v | 2.911 | 12,649 | 1.202 | *Robust |
| (Methanol: Acetonitrile) (±0.5%) | | 90:10 v/v | 2.913 | 12,215 | 1.25 | *Robust |
| | | 85:15 v/v | 2.919 | 12,440 | 1.102 | *Robust |

Acceptance criteria (Limits): #Peak Asymmetry<1.5, $Plate count>2000, * Significant change in Retention time.

**Robustness**

The robustness of the developed method was evaluated by small deliberate changes in method parameters such as flow rate (±0.2 ml/min), detection wavelength (±5 nm) and mobile phase composition (±0.5 %). The % RSD values of robustness which is less than 2 % reveals that the proposed method is robust. The results of robustness study results are shown in table 7. Even though the small changes in the

conditions did not significantly effect on the peak asymmetry, plate count and retention time of bamifylline hydrochloride.

**LOD and LOQ**

The developed method found to be high sensitivity with LOD of 0.4825 µg/ml and LOQ of 1.4621 µg/ml. The LOD and LOQ values are presented in table 8. The results of LOD and LOQ supported the sensitivity of the developed method.

**Analysis of tablet formulation**

The developed, validated method was successfully applied for the determination of bamifylline hydrochloride in their tablet dosage form. The assay result (table 9) shows that the amount of the drug was in excellent agreement with the labelled value of the formulation. The representative sample chromatogram of bamifylline hydrochloride is shown in fig. 8. Eventually, summary of validation parameters is shown in table 10.

**Table 8: LOD and LOQ results of bamifylline hydrochloride**

| | |
|---|---|
| Limit of Detection (LOD) | 0.4825 µg/ml |
| Limit of Quantitation (LOQ) | 1.5922 µg/ml |

**Table 9: Results of analysis of bamifylline hydrochloride**

| S. No | Formulation | Labelled amount mg/tablet | Amount found mg/tablet | Mean % assay±SD | % RSD* |
|---|---|---|---|---|---|
| 1 | Bamifix tablets | 600 | 595.01 | 99.4±1 | 0.100 |

*Average of six determinations; SD: standard deviation; RSD: relative standard deviation.

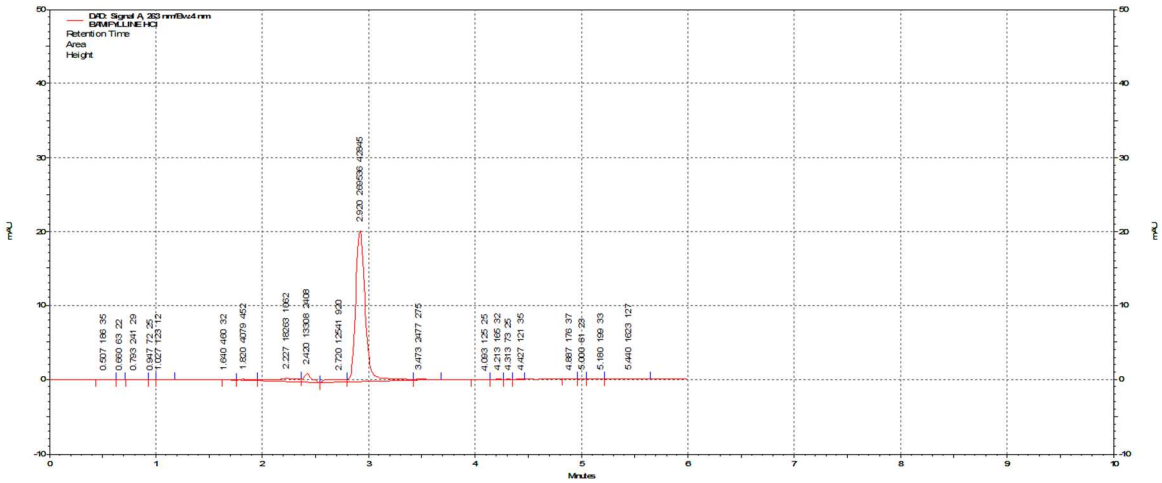


**Fig. 8: Bamifylline hydrochloride sample chromatogram**

**Table 10: Summary of validation parameters**

| Validation parameters | Results |
|---|---|
| Detection wavelength ($\lambda_{max}$) | 263 nm |
| Beer's law limits (µg/ml) | 2–10 µg/ml |
| Regression equation | Y = 60359x+2678.1 |
| Correlation coefficient ($r^2$) | 0.9996 |
| Flow rate | 1 ml/minute |
| Retention time ($R_t$) | 2.913 min |
| Intra-day Precision (% RSD) | 0.0008 |
| Inter-day Precision (% RSD) | 0.0007 |
| Accuracy (% recovery) | 99.6-99.8 % w/w |
| Limit of Detection (µg/ml) | 0.4825 µg/ml |
| Limit of Quantitation (µg/ml) | 1.4621 µg/ml |
| Assay (% w/w) | 99.4 % w/w |

**CONCLUSION**

In conclusion, a simple, accurate, sensitive, rapid and precise RP-HPLC method was developed and validated for the estimation of bamifylline hydrochloride in pharmaceutical dosage form. Statistical analysis for the above said results obviously demonstrates that the method is fit for the estimation of bamifylline hydrochloride in tablet forms without any interference. This method can help research studies, quality control and routine analysis with lesser resources available. The results of the assay of pharmaceutical formulation of the developed method are highly reliable and reproducible and is in good agreement with the label claim of the drug. Hence the method can be used for the regular analysis of bamifylline hydrochloride in tablet dosage form without any interference of excipients.

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**CONFLICT OF INTERESTS**

Declared none

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