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Stability indicating RP-HPLC method for estimation of bamifylline hydrochloride in tablet formulation: Development and validation consideration

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Abstract

A simple, specific and robust stability indicating high performance liquid chromatographic (HPLC) method for estimation of bamifylline hydrochloride was developed and validated. Bamifylline hydrochloride was separated and quantitated on Inertsil ODS-3V column (150 mm length, 4.6 mm id, 5 µm particle size) using a blend of methanol-water [0.5 % triethylamine & OPA to adjust pH 7] (60 : 40 v/v) as a mobile phase and at a flow rate of 1.5 mL/min. Quantification was achieved with UV detector at 277 nm over the concentration range 10 - 150 µg/mL. The developed HPLC method allowed separation and quantification of bamifylline hydrochloride with good linearity (r^2 – 0.999) in the studied concentration range. Limit of detection and limit of quantification were found to be 0.34 µg/mL and 1.04 µg/mL, respectively. The method was validated as per the International Conference on Harmonization (ICH) guidelines. Bamifylline hydrochloride stock solution was subjected to different stress conditions. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. Stressed samples were assayed using the developed HPLC method. The validation data showed that the method is precise, accurate, reproducible, and selective for the analysis of bamifylline hydrochloride. The method was successfully applied to the estimation of bamifylline hydrochloride in tablet dosage form.

Keywords: Bamifylline hydrochloride, RP-HPLC, Stability indicating method, Validation

Introduction

Bamifylline hydrochloride (BMFH) is a stimulant drug of the xanthine chemical class which acts as a selective adenosine A_1 receptor antagonist, used in the systemic treatment of obstructive airway diseases and asthma. BMFH is soluble in water, methanol, ethanol, HCl and NaOH. Chemically bamifylline is 8-benzyl-7-[2-[ethyl(2-hydroxyethyl)amino]ethyl]-1,3-dimethylpurine-2,6-dione [1].

No official method for the estimation of BMFH is available in literature. Papadoyannis et al., reported reverse phase high performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of BMFH and major metabolite AC-119 [1]. Gerlo et al., developed RP-HPLC method for the determination of BMFH in human plasma of neonates [2]. Belliardo et al., developed reverse phase ion pair chromatography method for the determination of BMFH and its major metabolite in human plasma [3]. Nicot et al. reported HPLC method

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for determination of BMFH and its three major metabolites in human plasma [4]. Carlucci et al., reported determination of BMFH impurities in bulk material and pharmaceutical forms using liquid chromatography with ultraviolet detection [5]. Patel et al., reported high performance thin layer chromatographic method for estimation of BMFH in bulk and tablet formulation [6].

Most of the methods reported were proposed for the analysis of biological samples and require tiresome procedures for sample pretreatment. Further, the lack of an official pharmacopoeial method for estimation of BMFH provoked the authors to develop stability indicating HPLC method for estimation of BMFH which is simple, rapid, less expensive and environment friendly.

The current study describes the development and validation of a stability-indicating RP-HPLC method for estimation of BMFH in the presence of its degradation products according to ICH guideline [7]. The developed method is applied for routine analysis of BMFH in pharmaceutical tablet dosage form.

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Materials and Methods

The pure BMFH powder was provided form Cadila Healthcare Ltd., Ahmadabad, Gujarat. Acetonitrile, methanol, hydrochloric acid, sodium hydroxide, ortho phosphoric acid (OPA) & triethyl amine were of HPLC grade purchased from Spectrochem Pvt. Ltd.-Fine Chemicals, Mumbai, India. HPLC grade water was obtained In-house using a Millipore Milli Q Gradient Water Purification System (Molsheim, France).

Instrumentation: Analysis was performed on a Shimadzu HPLC system, Model: 2010 A HT liquid chromatography Manufacturer: Shimadzu, Japan. Consisted of a system controller (SCL-10AVP), on-line degasser (DGU-14A), low pressure gradient flow control valve (FCV-10ALVP), solvent delivery module (LC-10ADVP), auto injector (SIL-10 ADVP), column oven (CTO-10AVP), UV – VIS and PDA detector (SPD-10AVP) and CLASS – VP software version 6.14 SP1 & Agilent HPLC system Model: 1200 series with Inertsil ODS3V C18 column (150 mm x 4.6 mm i.d., 5 µm) and PDA detector with Chemstation software version.

Chromatographic condition: Chromatographic separation achieved using an analytical column, Inertsil ODS3V C18 column (150 mm x 4.6 mm i.d., 5 µm). Mobile phase was consisted of methanol : water [0.5 % Triethylamine & OPA to adjust (pH 7)] (60 : 40). The elution was achieved isocratically at a flow rate of 1.5 mL/min with injection volume of 20 µL. Column temperature was maintained at 45 °C and chromatograph was recorded at wavelength 277 nm.

Preparation of bamifylline hydrochloride standard stock solution: A 50 mg of standard bamifylline hydrochloride was accurately weighed and transferred to a 100 mL volumetric flask and dissolved in 50 mL water. The flask was sonicated for 10 min. The flask was shaken and volume was made up to the mark with water to give a solution containing 500 µg/mL of BMFH. 5 mL of this stock solution was transfer to 50 mL volumetric flask and made up to the mark with water to give a solution containing 50 µg/mL.

Sample preparation for determination of Bamifylline hydrochloride in tablet dosage form: Twenty tablets (300 mg/tablet) were weighed. Powder equivalent to 50 mg of BMFH was transferred to 100 mL of volumetric flask containing 50 mL water, sonicated for 20 min. The flask was shaken and volume was made up to the mark with water. The above solution was filtered through Nylon filter (0.45 µm). 5 mL of aliquot was taken and transferred to volumetric flask of 50 mL capacity and volume was made up to the mark with the water to give a solution containing 50 µg/mL of bamifylline hydrochloride. This solution was used for the estimation of bamifylline hydrochloride in tablet dosage form.

Method validation: Validation of the developed HPLC method was carried out as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) [7].

Specificity: Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. Commonly used excipients (starch, microcrystalline cellulose and magnesium stearate) were spiked into a preweighed quantity of drugs. The chromatogram was taken by appropriate dilutions and the quantity of drug was determined.

Specificity was also studied by performing forced degradation study using acid and alkali hydrolysis, chemical oxidation, dry heat, photochemical, and moise degradation studies and interference of the degradation products were investigated [7].

- Acid hydrolysis: 50 mg of standard bamifylline hydrochloride was accurately weighed and transferred to a 100 mL volumetric flask. 5 mL water and 5 mL of 5 M HCl were added, kept at 80 ºC for 3 hrs on water bath. After treatment, solution was neutralized with 5 mL of 5 M NaOH to pH 7 and volume was made up to the mark with water. 5 mL of the above solution was transfered to 50 mL volumetric flask and made up to the mark with water. The resulted solution was subjected to chromatograph to check the specificity.

- Alkali hydrolysis: Alkali hydrolysis was carried out as per the procedure described for the acid degradation using 1 M NaOH instead of 5 M HCl. (1 M HCl was used for the neutralization purpose to pH 7)

- Oxidative stress degradation : Peroxide degradation was carried out as per the procedure described for the acid degradation using 5 % v/v H_2O_2 instead of 5 M HCl. There was no neutralization after the degradation.

- Thermal (Dry heat) degradation: BMFH API and powder of tablets were heated at 100 ˚C for 48 hrs and from this 50 µg/mL solution containing BMFH was prepared. This solution was chromatographed to check the specificity.

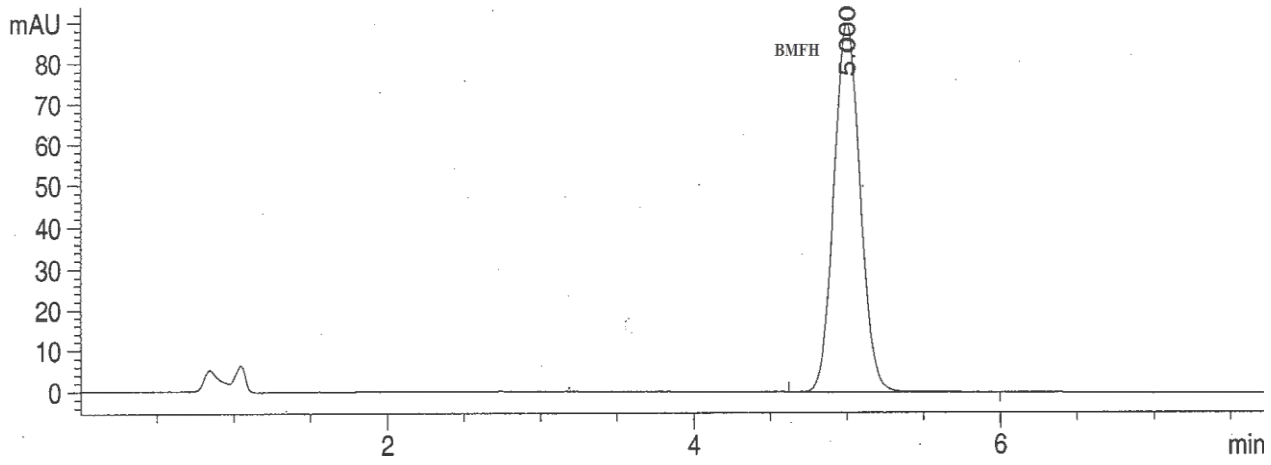

Figure 1 Chromatogram of standard solution containing 20 µg/mL bamifylline hydrochloride

- Photochemical degradation: BMFH API and powder of tablets were exposed to UV light for 72 hrs and from this 50 µg/mL solution containing BMFH was prepared. This solution was chromatographed to check the specificity.

- Moist degradation: BMFH API and powder of tablets were exposed to 40 ˚C and 75 % relative humidity for 48 hrs, and from this 50 µg/mL solution containing BMFH was prepared. This solution was chromatographed to check the specificity.

All the solutions were passed through Whatman filter no. 41 before injection. For each degradation study, blank solutions were also prepared without taking API or tablet powder as per the same procedure described above. All the blank solutions were passed through Whatman filter no. 41 before injection. Each blank was injected separately and chromatoghraphed. Chromatograms of blank solutions were compared with respective sample chromatograms to check the interference of water, HCl, NaOH and H_2O_2.

Linearity of the method: Calibration curve was constructed by plotting peak area vs. concentrations of BMFH, and the regression equations were calculated (n = 6). The calibration curve was plotted over the different concentrations 10, 20, 25, 50, 70, 100 and 150 µg/mL of BMFH. Aliquots of standard working solution were transferred to a series of 10 mL volumetric flasks and diluted to the mark with mobile phase. Aliquots (20 µL) of each solution were injected under the operating chromatographic condition described above (n = 6).

Precision:

Repeatability: Method precision for assay was established by determining the assay of six sample preparations of BMFH (50 µg/mL) under same conditions. Six replicates of sample were prepared at the sample concentration and analyzed on same day, and relative standard deviation (% RSD) was reported.

Intermediate precision (Reproducibility): The intraday and interday precisions of the proposed method responses 3 times on the same day (intraday) and 3 times on different days (interday) for 3 different concentrations were determined by analyzing the corresponding of standard solutions of BMFH (40, 50 and 60 µg/mL). The results were reported in terms of % RSD.

Detection limit and quantitation limit: The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be reliably detected from the background level. Limit of quantification (LOQ) of an analytical procedure is the lowest amount of an analyte that can be quantitatively determined with appropriate precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times \sigma / S; \quad LOQ = 10 \times \sigma / S$$

Table 1 Degradation study of bamifylline hydrochloride at different stress conditions using the proposed RP-HPLC method

Stress condition	% Assay		% Degradation	
	API	TABLET	API	TABLET
Acidic	99.65 ± 0.26	98.79 ± 0.18	-	-
Alkaline	86.10 ± 0.22	89.91 ± 0.25	13.90 ± 0.19	10.09 ± 0.31
Oxidative	85.97 ± 0.29	88.03 ± 0.41	14.03 ± 0.23	11.97 ± 0.36
Thermal	98.77 ± 0.47	98.57 ± 0.26	-	-
Photo	98.75 ± 0.30	98.60 ± 0.31	-	-
Moist	98.80 ± 0.42	98.56 ± 0.62	-	-

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve [7]. Accuracy (% Recovery)Accuracy was determined over the range 80 % to 120 % of the sample concentration [7]. Calculated amount of BMFH was added in placebo, containing commonly used excipients hypromellose, hydroxypropyl methylcellulose acetate succinate, sodium lauryl sulfate, sucrose, talc, titanium dioxide, and triethyl citrate to attain 80 %, 100 % and 120 % of sample concentration. Each sample was prepared in triplicate at each level and injected. The chromatograms were recorded and from the peak area of drug, % recovery was calculated from regression equation of the calibration curve.

Robustness: Robustness of method was studied by changing value the parameters as follows.

a) Mobile phase ratio (± 2% absolute) as Methanol : buffer (62 : 38), Methanol : buffer (58 : 42).

b) Flow rate of the mobile phase as 1.3 and 1.7 mL/min of the mobile phase.

System suitability test: The system suitability test was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed, using five replicate injections of a reference solution containing 50 µg/mL of BMFH. The parameters measured were peak area with % RSD, retention time, theoretical plates, and tailing factor (peak symmetry) [6, 7].

Results and Discussion

HPLC method development and optimization: A validated stability-indicating analytical method demonstrates the capability of the method to quantify the active pharmaceutical ingredient and to determine possible degradation products without any interference. To obtain the best chromatographic conditions, the mobile

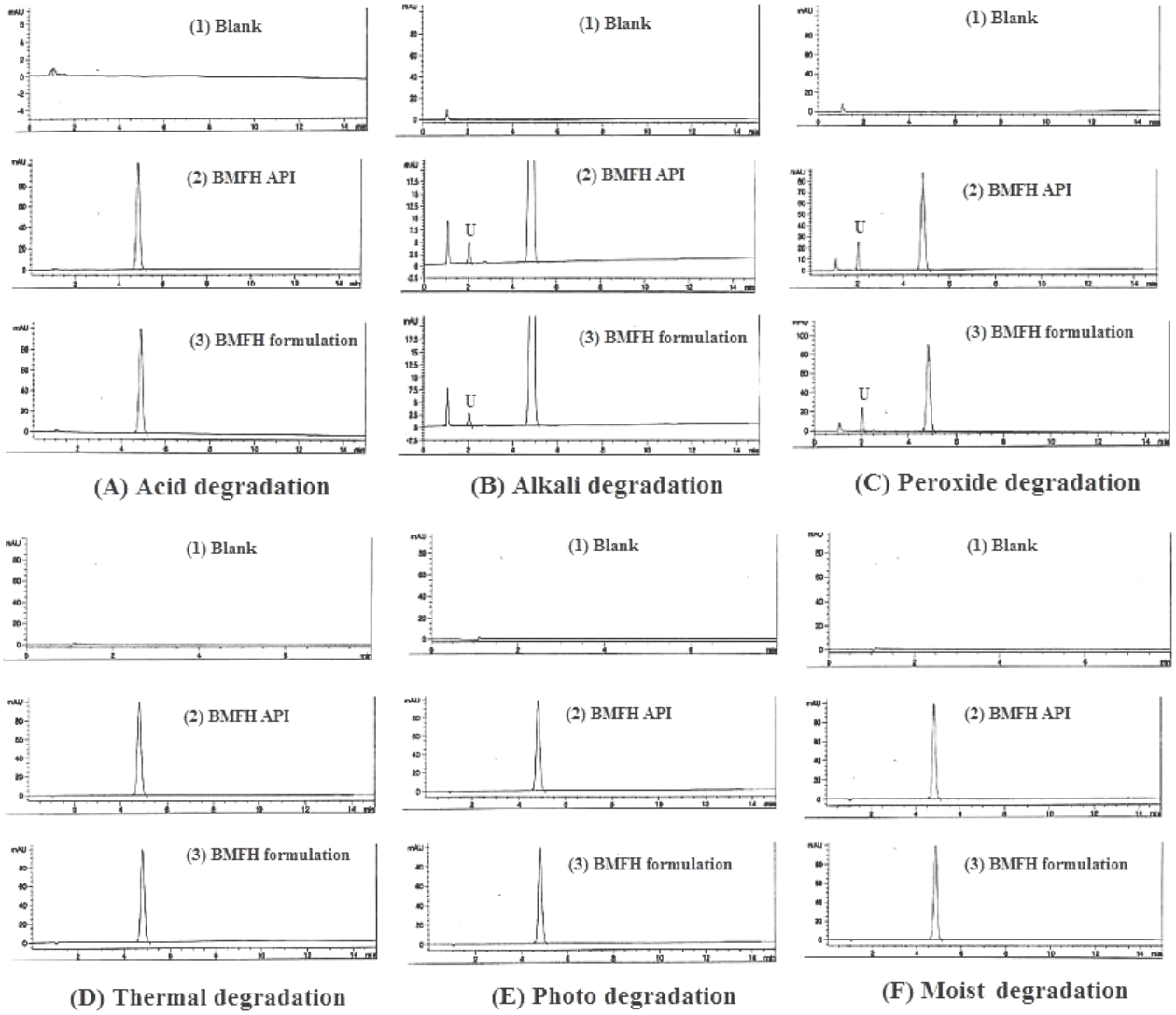

Figure 2 Chromatograms for (1) blank, (2) bamifylline hydrochloride API and (3) bamifylline hydrochloride tablet formulation after forced degradation study using (A) Acid (B) Alkali (C) Peroxide (D) Thermal (E) Photo and (F) Moisture, respectively

Table 2 Regression analysis of calibration curve for bamifylline hydrochloride of the proposed RP-HPLC method

Linearity level	Bamifylline hydrochloride	
	Conc (µg/mL)	Mean area ± SD (n=3)
1	10	217155 ± 563.51
2	20	436188 ± 2451.40
3	25	541174 ± 930.37
4	50	1077475 ± 892.08
5	70	1542151 ± 739.52
6	100	2191028 ± 5076.50
7	150	3248088 ± 813.03
Correlation coffecient	0.9999	
Slop of regression line	21730	
Y- intercept	2579	

phase was optimized to provide sufficient selectivity and sensitivity in a short separation time. The use of methanol resulted in better sensitivity and short analysis time against the acetonitrile as an organic solvent. Various proportions of methanol and water were tried. Methanol : water (60 : 40 v/v) resulted in the shortest analysis time. 0.5 % triethylamine and ortho-phosphoric were used to adjust pH 7 resulted better symmetry of peak. Different columns such as Purosphere star (250 mm x 4.6 mm i.d., 5 µm), Inertsil C8 (250 mm x 4.6 mm i.d., 5 µm), Symmetry C8 column (250 mm x 4.6 mm i.d., 5 µm) and Inertsil ODS3V C18 column (250 mm x 4.6 mm i.d., 5 µm) were evaluated. The Inertsil ODS-3V analytical column was selected, as it provided the best chromatographic performance and acceptable peak characteristics, including tailing factor and number of theoretical plates. Moreover, the acceptable resolution of BMFH and the degradation products was obtained, confirming the stability-indicating capability of the proposed method.

A satisfactory separation with good peak symmetry and steady baseline was achieved with Inertsil ODS-3V column and methanol-water (0.5 % Triethylamine & OPA to adjust pH 7) 60 : 40 as a mobile phase at flow rate of 1.5 mL/min. The quantitation of BMFH was achieved at 277 nm. The optimized conditions of the HPLC method were validated for the analysis of BMFH in tablet formulations and application for quality control. Figure 1 shows a typical chromatogram obtained by the proposed RP-HPLC method, demonstrating the resolution of the symmetrical peak corresponding to BMFH. The retention time observed (5.0 min) allows a fast determination of the drug, which is suitable for QC laboratories.

Method validation: Forced degradations were performed to provide the indication of the stability-indicating properties of an analytical method, particularly when there was no information available about the potential degradation products.

The stress testing studies resulted that the method was highly specific for BMFH from its potential degradation products. The drug was found to be degraded in basic and peroxide medium. The degradation products were completely distinguishable from the parent compound (BMFH). Alkali stress led to 86.10 % recovery with unknown degradation peak at 2.1 min, and peroxide stress led to 85.97 % recovery with one unknown degradation peak at 2.1 min (Figure 2). Prominent peak of BMFH was stable at 5.0 min. The force degradation studies in acid, thermal, UV and moisture degradation conditions of BMFH resulted in an insignificant decrease of the peak area and no any detectable degradation products (Figure 2A-F). Table 1 outlines the results of degradation study of BMFH at each stress condition.

Table 3 Precision study data of the proposed RP-HPLC method

	Concentration (µg/mL)	Peak Area at 277 nm Mean ± SD	%RSD
Intraday Precision (n = 3)	40	868486 ± 6447.33	0.74
	50	1077606 ± 6877.60	0.64
	60	1328596 ± 6916.16	0.52
Interday Precision (n = 3)	40	877473 ± 7248.88	0.83
	50	1075720 ± 9634.23	0.90
	60	1334976 ± 9782.93	0.73
Repeatability	50	1076952 ± 2254.56	0.21

Specificity is a measure of the degree of interference from other active ingredients, excipients, impurities, and degradation products. Specificity in a method ensures that a peak response is due to a single component only. In the present study, the ability of the method to separate the drug from its degradation products and the non – interference of the excipients indicate the specificity of the method. Values of peak purity index were higher than 0.9999. These results indicated that the proposed method is specific and stability-indicating, and can be applied for stability studies and QC analysis of BMFH in pharmaceutical products [7].

The linearity of a method is defined as its ability to provide measurement results that are directly proportional to the concentration of the analyte. The linearity of the detector was obtained by diluting the analyte stock solution and measuring the associated responses, while the linearity of the analytical method was determined by making a series of concentrations of the analyte from independent sample preparations (weighing and spiking). The linearity data described in the present study demonstrated the acceptable linearity for BMFH over the range of 80 to 120 % of the target concentration (Figure 3). Linear correlation was obtained between peak areas and concentrations of BMFH in the range of 10 – 150 µg/mL. The following regression equation was found by plotting the peak area (y) versus the BMFH concentration (x) expressed in µg/mL: y = 21730x + 2579. The correlation coefficient (r^2 : 0.999) obtained for the regression line demonstrated the excellent relationship between peak area and concentration of BMFH. Data of regression analysis were summarized in Table 2.

The precision, evaluated as the repeatability of the method, was studied by calculating the RSD for six determinations of the 50 µg/mL sample of BMFH performed on the same day and under the same experimental conditions. The obtained RSD value was 0.21 %. The RSD value for repeatability study was found to be < 1 %, which indicated that the proposed method was repeatable [7].

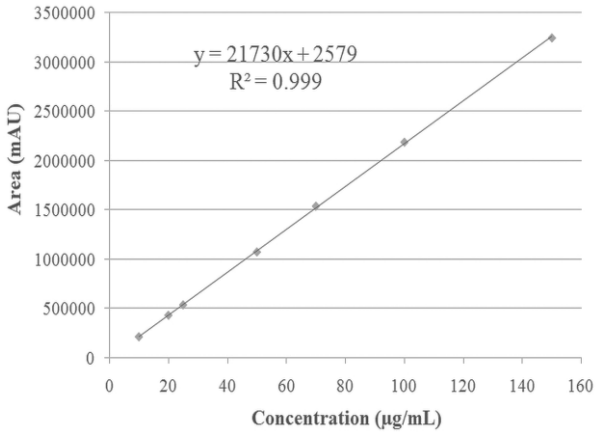

Figure 3 Calibration curve of bamifylline hydrochloride using proposed RP-HPLC method

The intermediate precision was assessed by analyzing three different concentrations with three replicates on same day (intraday) and 3 different days (interday); the mean values obtained were % RSDs of 0.52 - 0.74 and 0.73 - 0.90 %, respectively. (Table 3) The RSD values for intermediate precision was found to be < 2 %, which indicated that the proposed methods were reproducible [7].

The accuracy was assessed by the standard addition method for three replicate determinations of three different solutions containing 40, 50 and 60 µg/mL BMFH. The recoveries were obtained in a range of 97.48

Table 4 Precision study data of the proposed RP-HPLC method

Level	Amount of drug added (µg/mL)	Amount of drug Recovered (µg/mL)	% Recovery	RSD
80 %	40	39.22 ± 0.30	98.05 ± 0.74	0.76
100 %	50	49.24 ± 0.31	98.49 ± 0.63	0.64
120 %	60	59.80 ± 0.15	99.67 ± 0.18	0.18

Table 5 Robustness study (n = 3)

	Parameters	% RSD
Mobile phase composition (Methanol : water)	60 : 40	0.38
	62 : 38	0.48
	58 : 42	0.42
Flow rate (mL/min)	1.5	0.41
	1.3	0.39
	1.7	0.36

Table 6 System suitability test parameters of bamifylline hydrochloride for the proposed RP-HPLC method (n = 5)

Sr. No.	Parameters	Mean ± SD	% RSD
1	Peak area	1078752 ± 431.5	0.04
2	No. of theoretical plates	4986 ± 53.35	1.07
3	Retention time (min)	4.856 ± 0.01	0.22
4	Asymmetry	0.96 ± 0.01	0.84

Table 7 Assay results for the tablet dosage form using the proposed RP-HPLC method

Formulation	Amount of powder Equivalent to 50 mg taken from Tablet (mg)	Amount of drug found in Tablet (mg)	%Assay (n=3)
Batch 1 (45/F013)	50	49.74	99.48 ± 0.263
Batch 2 (45/F014)	50	50.07	100.14 ± 0.010

to 99.88 % for BMFH using the proposed HPLC method (Table 4). The high values of recovery indicated that the proposed HPLC method was accurate [6, 7].

The LOD and LOQ were determined from standard deviation of the intercepts and slope of linear regression curves. The limit of detection (LOD) and limit of quantification (LOQ) for BMFH were found to be 0.34 and 1.04 µg/mL, respectively.

The results and the experimental range of the selected variables evaluated in the robustness study are given in Table 5. There were no significant changes in the chromatographic pattern when the modifications were made in the experimental conditions, thus showing the method to be robust.

System suitability study requires asymmetry of analyte peak should not be more than 1.5, theoretical plates of analyte peak should not be less than 2000, and relative standard deviation for five replicated injections of standard preparation should not be more than 2.0 % [6, 7]. As shown in Table 6, the results indicated that the system was suitable for the analysis intended.

Method application: The planned RP-HPLC method was applied for the estimation of BMFH in a tablet dosage form. The results in Table 7 revealed the quality of the analyzed pharmaceutical samples and the applicability of the method for routine analysis of BMFH in tablet dosage form.

Conclusion

The results of stress degradation studies according to ICH guidelines demonstrated that the method was specific and stability indicating. Based on the results obtained from the analysis of forced degradation samples, it can be accomplished that there is no other coeluting peak with the main peaks, and the method is specific for the estimation of BMFH in the presence of degradation products.

A simple and rapid isocratic stability-indicating RP-HPLC method has been developed and validated for estimation of BMFH in pharmaceutical tablet dosage form. The results of the validation studies confirmed that the RP-HPLC method was precise, accurate, specific and robust. It possesses significant linearity (r^2 = 0.9999), precision with a mean RSD of 0.82 %. The proposed method was successfully applied and is suggested for the quantitative analysis of BMFH in pharmaceutical formulations for QC, where economy and time are essential.

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