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Stability-Indicating Liquid Chromatographic Method for the Determination of Bendamustine Hydrochloride in Parenterals

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Abstract

A stability-indicating high-performance liquid chromatographic method was developed and validated for the determination of Bendamustine hydrochloride in injectables. Reversed-phase chromatography was performed on Shimadzu Model CBM-20A/20 Alite, equipped with SPD M20A prominence photodiode array detector using C18 (250 mm × 4.6 mm, 5 µm particle size) column with acetonitrile: tetra butyl ammonium hydrogen sulphate (80:20, V/V) as mobile phase at a flow rate of 0.8 mL/min. with UV detection at 233 nm. Linearity was observed in the concentration range of 1.0–200 µg/mL (R^2 = 0.999) with regression equation y = 10691 x + 98237. The limit of quantitation (LOQ) and limit of detection (LOD) were found to be 0.812 and 0.268 µg/mL respectively. The forced degradation studies were performed by using HCl, NaOH, H_2O_2 etc. Bendamustine hydrochloride is more sensitive towards elevated temperatures (80ºC) in comparison to acidic and oxidative conditions but very much resistant towards alkaline conditions. The method was validated as per ICH guidelines. The RSD for intra-day (0.14-0.32) and inter-day (0.47-0.66) precision were found to be less than 1 %. The percentage recovery was in good agreement with the labeled amount in the pharmaceutical formulations and the method is simple, specific, precise and accurate for the determination of Bendamustine hydrochloride in pharmaceutical formulations.

Keywords: Bendamustine hydrochloride, nitrogen mustard, liquid chromatography, stability-indicating.

Introduction

Bendamustine hydrochloride (BMH), (figure 1) chemically known as (4-{5-[bis-(2-chloroethyl) amino]-1-methyl-1*H*benzimidazol-2-yl} butanoic acid) is an active nitrogen mustard[1]. It is used for the treatment of patients with chronic lymphocytic leukemia[2]. It contains a mechlorethamine group and a benzimidazole heterocyclic ring with a butyric acid substituent. Mechlorethamine and its derivatives form electrophilic alkyl groups. These groups form covalent bonds with electron-rich nucleophilic moieties, resulting in interstrand DNA crosslinks. The bifunctional covalent linkage can lead to cell death via several pathways[3]. Bendamustine is active against both quiescent and dividing cells. Besides biotransformation[4-7], Bendamustine, similar to other nitrogen mustards, undergoes degradation by hydrolysis. Two hydrolysis products of Bendamustine have been detected, namely monohydroxy and dihydroxy derivatives (4-{5-[(2-chloroethyl)-(2-hydroxyethyl) amino]-1-methyl-1*H*-benzimidazol-2-yl} butanoic acid and 4-{5-[bis-(2-hydroxyethyl)amino]-1-methyl-1*H*benzimidazol- 2-yl}butanoic acid)[8]. Because of the hydrolytic degradation in aqueous solutions, nitrogen mustards are often supplied for administration in a lyophilized form that requires reconstitution, usually in water.

Literature review revealed that there is only one HPLC method for the determination of stability of Bendamustine hydrochloride immobilized onto polyphosphoesters[9] and only one spectrophotometric method[10]. There is not even a single stability indicating liquid chromatographic method for the determination of BMH in pharmaceutical dosage forms. In the present work a simple stability indicating reverse phase liquid chromatographic method has been developed for the determination of BMH for parenterals and validated as per ICH guidelines[11]. In the present work we developed simple, rapid and accurate reverse phase liquid chromatographic method for the determination of Bendamustine hydrochloride injections. Apart from this, it can be used for assays of Bendamustine hydrochloride in biological fluids or in pharmacokinetic investigations.

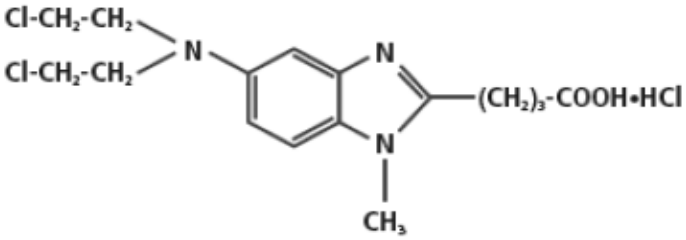


Figure-1
Chemical Structure of Bendamustine hydrochloride (BMH)

Material and Methods

Chemicals and solutions: Bendamustine hydrochloride standard (purity 99.0%) was obtained from White Pharmaceuticals (India). It is available as injection in a single dose vial containing 100 mg of BMH as white to off-white

lyophilized powder. It is available commercially with brand names BENDIT® (White Pharmaceuticals, India) and PURPLZ® (India) as lyophilized powder for injection with a label claim of 100 mg of bendamustine hydrochloride in a single dose vial. Acetonitrile (HPLC grade), tetra butyl ammonium hydrogen sulphate (TBAHS), sodium hydroxide (NaOH) and hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were obtained from Merck (India). All chemicals were of an analytical grade and used as received.

HPLC instrumentation and conditions: Chromatographic separation was achieved by using a Shimadzu Model CBM-20A/20 Alite, equipped with SPD M20A prominence photodiode array detector using C18 (250 mm × 4.6 mm, 5 µm particle size) column maintained at 25 ºC. Isocratic elution was performed using acetonitrile and 10 mM TBAHS (80:20, v/v). The overall run time was 10 min. and the flow rate was 0.8 mL/min. 20 µl of sample was injected into the HPLC system.

The mobile phase was prepared by accurately weighing and transferring 3.3954 grams of tetra butyl ammonium hydrogen sulphate (TBAHS) (10mM) (pH 3.4) in to a 1000 mL volumetric flask, dissolving and diluting to volume with HPLC grade water.

Bendamustine hydrochloride stock solution (1000 µg/mL) was prepared by accurately weighing 25 mg of bendamustin hydrochloride in a 25 mL amber volumetric flask and making up to volume with mobile phase. Working solutions for HPLC injections were prepared on a daily basis from the stock solution in a solvent mixture of acetonitrile and 10 mM TBAHS (80:20, v/v) (mobile phase). Solutions were filtered through a 0.45 µm membrane filter prior to injection. Single dose vials containing 100 mg of BMH as lyophilized powder were procured from the local market, and powder equivalent to the weight of 25 mg bendamustine hydrochloride was accurately weighed into a 25 mL volumetric flask and made up to volume with acetonitrile. The volumetric flask was sonicated for 30 min to enable complete dissolution of bendamustine hydrochloride. The solution was filtered and aliquots of filtrate were transferred using a pipette into 5 mL volumetric flasks and made up to volume with mobile phase to yield a concentration of 10 µg/mL. These solutions were filtered through a 0.45 µm nylon filter before injections.

Forced degradation studies/specificity: Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method[12]. All solutions for use in stress studies were prepared at an initial concentration of 1 mg/mL of bendamustine hydrochloride and refluxed for 30 min at 80ºC. All samples were then diluted in mobile phase to give a final concentration of 10 µg/mL and filtered before injection.

Acid decomposition was carried out in 0.1 M HCl at a concentration of 1.0 mg/mL Bendamustine hydrochloride and after refluxation for 30 min at 80ºC the stressed sample was cooled, neutralized and diluted with mobile phase. Similarly stress studies in alkaline conditions were conducted using a concentration of 1.0 mg/mL in 0.1 M NaOH and refluxed for 30 min at 80ºC. After cooling the solution was neutralized and diluted with mobile phase. Solutions for oxidative stress studies were prepared using 3% H_2O_2 at a concentration of 1 mg/mL of Bendamustine hydrochloride and after refluxation for 30 min at 80ºC on the thermostat the sample solution was cooled and diluted accordingly with the mobile phase. For thermal stress testing, the drug solution (1 mg/mL) was heated in thermostat at 80ºC for 30 min, cooled and used.

Method validation: The method was validated for the following parameters: system suitability, linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity and robustness [11]. The intra-day precision of the assay method was evaluated by carrying out assay of bendamustine hydrochloride at three concentration levels (10, 20 and 50 µg/mL) (n=3) against a qualified reference standard. The % RSD of three obtained assay values at three different concentration levels was calculated. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (10, 20 and 50 µg/mL) and each value is the average of three determinations (n=3). The % RSD of three obtained assay values on three different days was calculated.

The limit of quantification (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1)[11]. Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels of the assay analyte concentration (1, 2, 5, 10, 20, 50, 80, 100, 120 150 and 200 µg/mL). 20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The solutions extracted from the marketed formulations were injected in to the HPLC system and the peak area of the chromatograms was noted. The analytical curve was evaluated on three different days. The peak area vs. concentration data was analyzed with least squares linear regression. The slope and y-intercept of the calibration curve was reported.

The accuracy of the assay method was evaluated in triplicate at three concentration levels (80, 100 and 120%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of bendamustine hydrochloride in the drug product. The study was carried out in triplicate at 36, 40 and 44 µg/mL. The percentage recovery in each case was calculated. The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength (231 and 235 nm), percentage of acetonitrile in the mobile phase (82 and 78%), flow rate (0.7 and 0.9 mL/min) and pH (3.3 and 3.5).

Robustness of the method was studied using six replicates at a concentration level of 10 µg/mL of bendamustine hydrochloride.

Results and Discussion

The present proposed RP-HPLC method is very simple, precise, robust and accurate for the determination of bendamustine hydrochloride and no method was reported till now in the literature. The complete separation of the analytes was accomplished in less than 10 min and the method has been successfully used to perform long-term and accelerate stability studies of bendamustine hydrochloride formulations. Overall, the data demonstrated that the excipients and the degradation products did not interfere with the bendamustine hydrochloride peak, indicating the selectivity of the method.

Initially the stressed samples were analyzed using a mobile phase consisting of acetonitrile: TBAHS (70:30, v/v) at a flow rate of 1.0 mL/min. Under these conditions, peak tailing was observed and the drug was eluted in less than 2 min. So the ratio of mobile phase was changed (80:20, v/v) and the flow rate was maintained as 0.8 mL/min by which the drug was eluted at about 2.719 min. Using these experimental conditions all peaks were well resolved with good symmetry. Therefore, a mobile phase of acetonitrile: TBAHS (80:20, v/v) provided the best chromatographic response and was used for further studies.

Method validation: The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. The parameters measured were peak area, retention time, tailing factor, capacity factor and theoretical plates. In all measurements the peak area varied less than 2.0%, the average retention time was 2.72 minutes (relative standard deviation (% RSD) = 0.23 %), the capacity factor was more than 2, theoretical plates were 7652 (more than 2000) and tailing factor was 1.21 (less than 2) for the bendamustine hydrochloride peak. The proposed method offers high sensitivity and bendamustine hydrochloride can be detected accurately. In all the cases, the bendamustine hydrochloride peak was well separated from the degradation products.

The calibration curve for bendamustine hydrochloride was linear over the concentration range of 1.0–200 µg/mL (table 1).

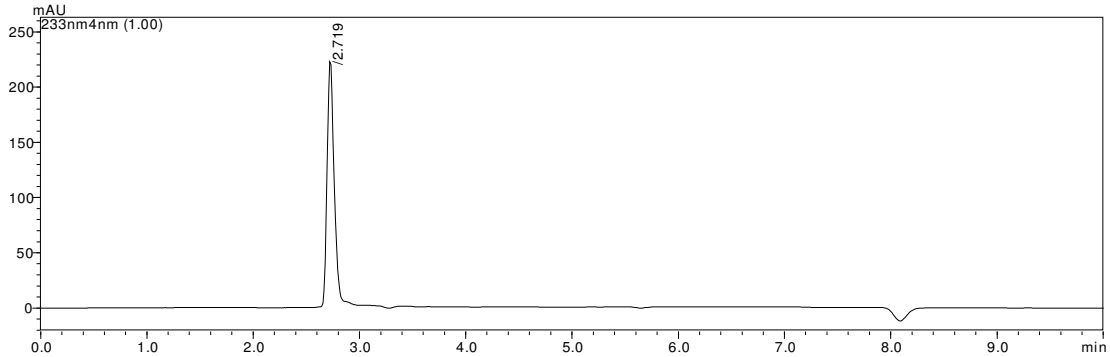
Table-1
Linearity of Bendamustine hydrochloride

Conc. (µg/mL)	*Mean area ± SD (n = 3)	*RSD (%)
1	179312 ± 573.79	0.32
2	358624 ± 1362.771	0.38
5	582197 ± 2503.45	0.43
10	1109823 ± 5327.15	0.48
20	2320443 ± 11834.26	0.51
50	5296410 ± 28600.61	0.54
80	8626676 ± 56073.39	0.65
100	10800132 ± 74520.91	0.69
120	13262732 ± 85491.67	0.65
150	16502142 ± 140268.2	0.85
200	21040176 ± 157777.7	0.75

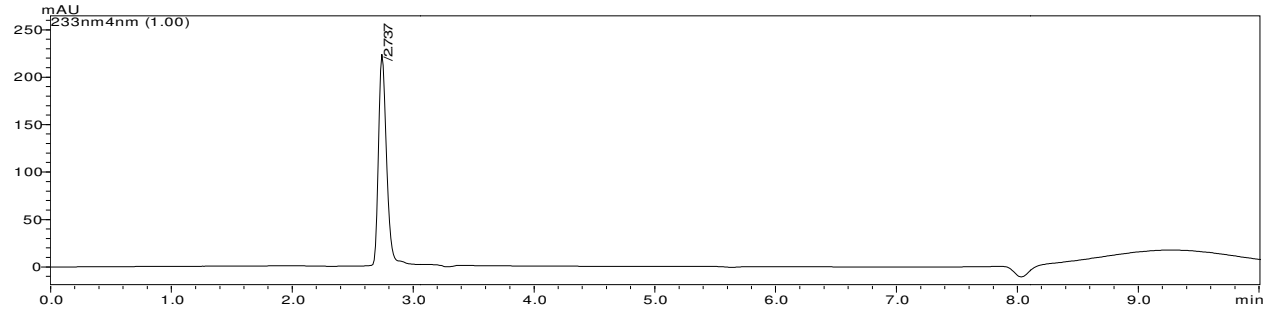
*Mean of three replicates

The representative chromatogram for bendamustine hydrochloride was shown in figure 2A and the chromatograms obtained from the extracted marketed formulations were shown in figure 2 B and 2 C.

The data for the peak area of bendamustine hydrochloride versus bendamustine hydrochloride concentration were treated by linear regression analysis and the correlation coefficient (R^2) of 0.999 was obtained. The regression equation for the calibration curve (figure 3) was found to be y = 10691 x + 98237. The LOQ and LOD were determined based on the 10 and 3.3 times the standard deviation of the response, respectively, divided by the slope of the calibration curve. The LOQ was found to be 0.812 µg/mL and the LOD was found to be 0.268 µg/mL.



[A]



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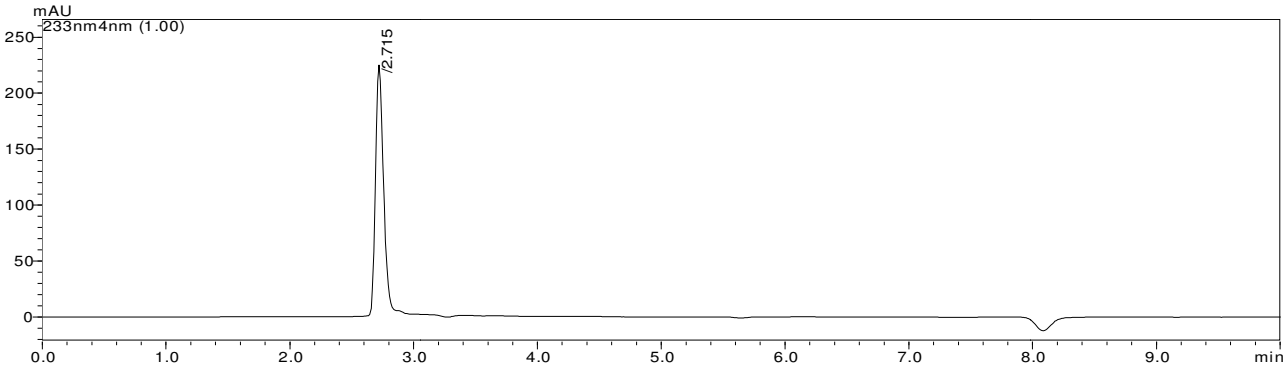


[C]

Figure-2
Representative chromatograms of Bendamustine hydrochloride (10 µg/mL) [A], BENDIT® (100 mg) [B] and PURPLZ® (100 mg) [C]

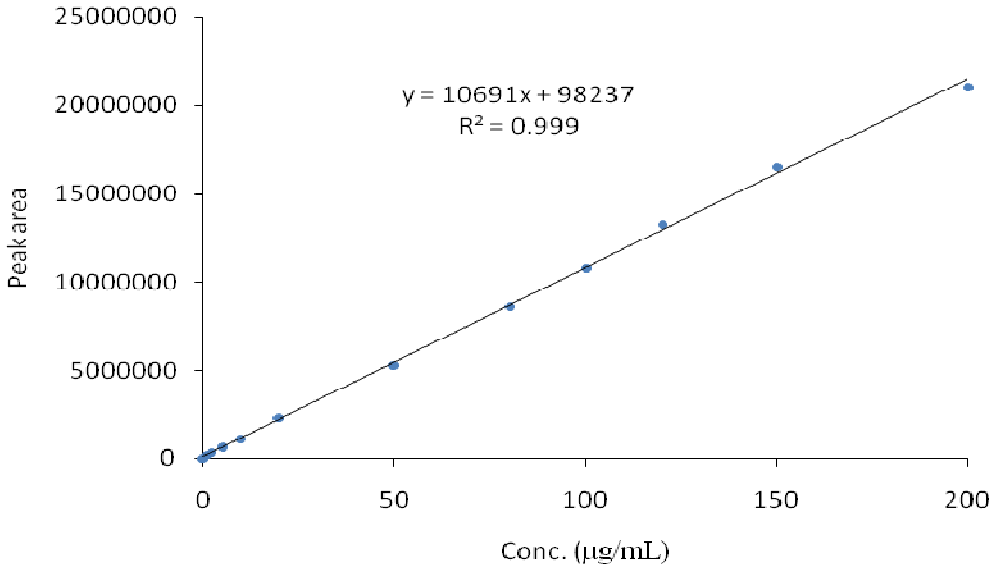


Figure-3
Calibration curve of Bendamustine hydrochloride

The precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) of the bendamustine hydrochloride standard solutions. Repeatability was calculated by assaying three samples of each at three different concentration levels (10, 20 and 50 µg/mL) on the same day. The inter-day precision was calculated by assaying three samples of each at three different concentration levels (10, 20 and 50 µg/mL) on three different days. The % RSD range was obtained as 0.14-0.32 and 0.47-0.66 for intra-day and inter-day precision studies respectively (table 2).

Table-2
Precision Study of Bendamustine hydrochloride

S. No.	Conc. (µg/mL)	Inter-day precision		Intra-day precision	
		Mean* ± SD	RSD (%)	Mean* ± SD	RSD (%)
1	10	1115833 ± 5291.50	0.47	1111156.33 ± 1527.33	0.14
2	20	2334109.67 ± 11846.24	0.51	327109.67 ± 5859.47	0.25
3	50	5333076.67 ± 35118.85	0.66	5310743.33 ± 16921.39	0.32

*Mean of three replicates

Table-3
Accuracy - recovery study of Bendamustine hydrochloride by standard-addition method

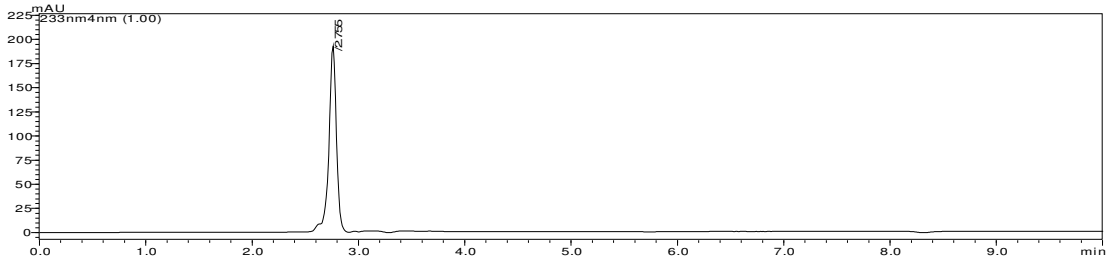
Sample No.	Spiked concentration (µg/mL)	*Measured concentration (µg/mL)	Recovery* (%)	*RSD (%)
1	16 (80 %)	15.83	98.94	0.42
2	20 (100 %)	19.91	99.55	
3	24 (120 %)	23.94	99.75	

*Mean of three replicates

The method accuracy was proven by the recovery test. A known amount of bendamustine hydrochloride standard (20 µg/mL) was added to aliquots of samples solutions (16, 20 and 24 µg/mL), and then diluted to yield total concentrations as 36, 40 and 44 µg/mL as described in table 3. The assay was repeated (n = 9) over 3 consecutive days. The resultant % RSD for this study was found to be 0.42 % with a corresponding percentage recovery 98.94-99.75%.

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis[12]. The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changing from the original condition. The detection wavelength was set at 231 and 235 nm (± 2 nm), the ratio of percentage of acetonitrile; TBAHS in the mobile phase was applied as 78:22 and 82:18 (v/v) (± 2 %), the flow rate was set at 0.7 and 0.9 mL/min (± 0.1 mL/min) and the pH was 3.3 and 3.5 (± 0.1). The results obtained from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The % RSD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% indicating that the developed method was robust.

The specificity of the developed method was determined by injecting sample solutions (10 µg/mL) which were prepared by forcibly degrading under such stress conditions as heat, oxidative agent, acid and base under the proposed chromatographic conditions. The stability indicating capability of the method was established from the separation of bendamustine hydrochloride peak from the degraded samples derived from software. The degradation of bendamustine hydrochloride was found to be very similar for both powder (for injection) and standard. Typical chromatograms obtained following the assay of stressed samples are shown in figure (4A-4D).



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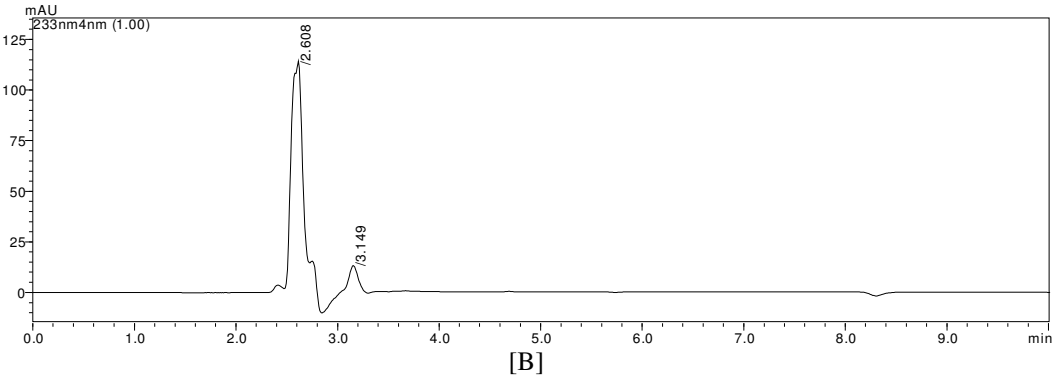

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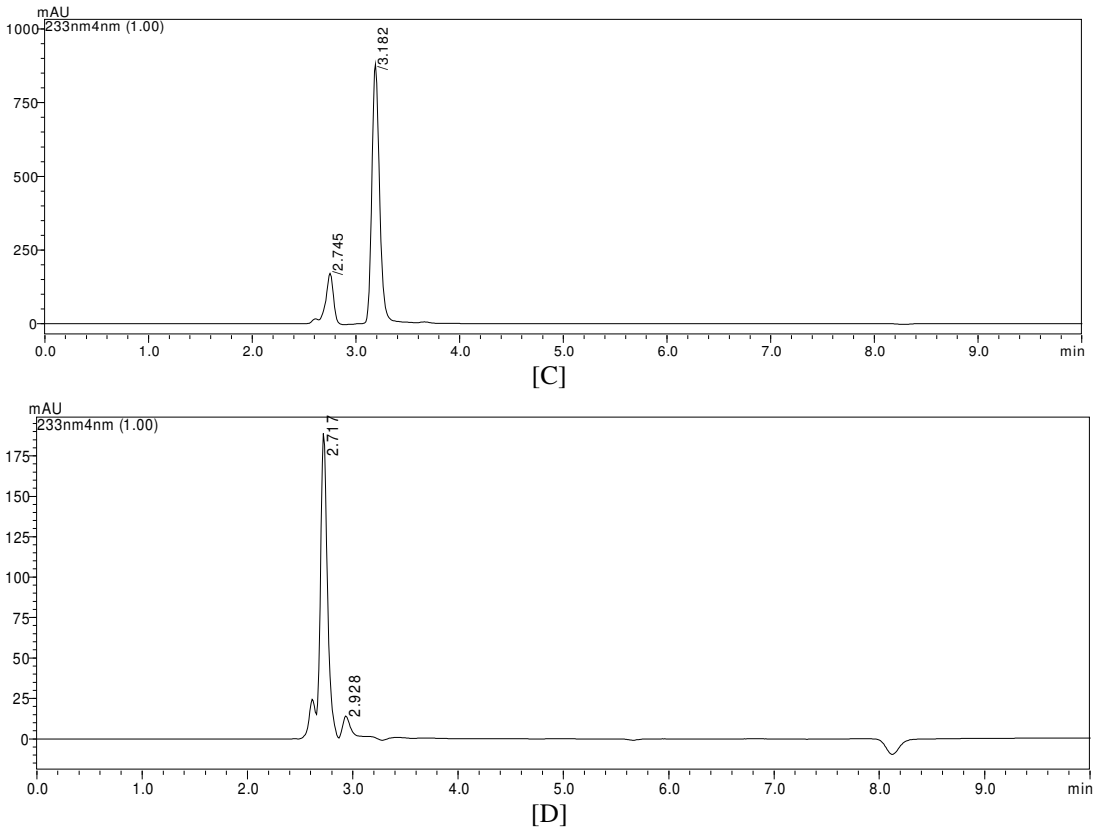

Figure-4
Representative chromatograms of Bendamustine hydrochloride (10 µg/mL) on acidic [A], alkaline [B], oxidative [C], thermal [D] degradations

The proposed method was applied to the determination of Bendamustine hydrochloride injections BENDIT® and PURPLZ® and the result of these assays yielded 98.92% and 99.87% respectively (RSD is < 2.0 %). The result of the assay (table 4) indicates that the method is selective for the assay of Bendamustine hydrochloride without interference from the excipients used in these injections (figure 2B and 2C).

Table-4
Analysis of Bendamustine hydrochloride commercial formulation (Injections)

Sample No.	Formulation	Labeled claim (mg)	*Amount found (mg)	*Recovery (%)
1	BENDIT®	100	98.92	98.92 ± 0.02
2	PURPLZ®	100	99.87	99.87 ± 0.01

*Mean of three replicates

Forced degradation studies: Bendamustine hydrochloride standard and lyophilized powder for injection were found to be quite stable under dry heat conditions. Bendamustine hydrochloride undergoes alkaline hydrolysis (3.79 %) and the butanoic acid moiety present in the chemical structure may be responsible for this. An extra peak was eluted at 3.143 min without interfering with the drug peak. Less than 15% decomposition was observed in acidic and oxidation conditions. 21.78% of drug was decomposed on exposure to high temperature i.e. 80 ± 5ºC indicating that the drug is more sensitive towards elevated temperatures and the drug is quite stable at room temperature (25ºC). It can be concluded that Bendamustine hydrochloride is more resistant towards alkaline and less resistant towards acidic and oxidative conditions but sensitive to elevated temperature conditions (table 5).

Table-5
Forced degradation studies of Bendamustine hydrochloride

Stress Conditions	*Drug recovered (%)	*Drug decomposed (%)
Standard Drug	100	-
Acidic Hydrolysis	86.60	13.40
Alkaline Hydrolysis	96.21	3.79
Oxidative degradation	87.94	12.06
Thermal degradation	78.22	21.78

*Mean of three replicates

Conclusion

A stability-indicating RP-HPLC method was developed, validated and applied for the determination of bendamustine hydrochloride in pharmaceutical dosage forms. The developed method was validated and as per ICH guidelines and was found to be accurate, precise, robust and specific. The chromatographic elution step is undertaken in a short time (< 4 min). No interference from any components of pharmaceutical dosage form or degradation products was observed and the method has been successfully used to perform long-term and accelerate stability studies of bendamustine hydrochloride formulations.

Acknowledgements

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