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Stability- Indicating Novel HPLC Method for Estimation of Bendamustine in Pharmaceutical Forms

Gade Srinivas Reddy [1]*, S.L.N.Prasad Reddy[2], L.Shiva kumar Reddy[3]
1.Research Scholar, Department of Pharmaceutical sciences, Jawaharlal Nehru Technical University, Hyderabad, Telangana, INDIA
2.Principal, Sanskruthi College of pharmacy, Ghatkesar, Hyderabad, Telangana.

ABSTRACT

A simple, precise, novel stability-indicating method was developed and validated for the estimation of Bendamustine in pharmaceutical forms. The Assay method was developed by using a detector set at 235nm and reverse phase c18 column (Inertsil ODS-2,150mmx4.6mm, 5 µm).The mobile phase consisted of 0.1%Trifluoroacetic acid: Acetonitrile 70:30 (v/v) in isocratic flow rate at 1.5mL/min for 10minutes.Sample cooler temperature and Column oven temperature were monitored in the method at 5°c and 25°c Respectively. The retention time of bendamustine was 4.8min.The proposed method was achieved and validated with respect to precision, specificity, accuracy, linearity, range, Analyte Stability, Filter variability, Robustness. The %Recovery of Bendamustine at Each level in formulations was found to be in a range of 98-102%. The linearity of the assay was established in the range of 5.053 to 75.793 µg/mL with correlation coefficient (R^2) > 0.9999. The limits of detection and quantification were 0.03 and 0.05mg/mL, respectively. Specificity of the assay demonstrates no interference from and degrading products formed by alkaline, acidic, oxidative, UV light and high temperature (Thermal) conditions. This method showed more accurate, rugged and reliable determination of Bendamustine for drug stability assay in pharmaceutical studies.

Keywords: Bendamustine, Liquid chromatography, Method validation, Stability indicating method.

*Corresponding Author Email: srinupharma@gmail.com
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INTRODUCTION

Bendamustine hydrochloride is belongs to a class of cancer chemotherapy, drugs known as alkylating agents. The chemical name of bendamustine hydrochloride is 1Hbenzimidazole-2-butanoic acid, 5-[bis(2-chloroethyl)amino]-1 methyl-, monohydrochloride. Its empirical molecular formula is $C_{16}H_{21}Cl_2N_3O_2$ • HCl and the molecular weight is 394.7. Chemistry of Bendamustine hydrochloride contains a mechlorethamine group and a benzimidazole heterocyclic ring with a butyric acid substituent (figure 1). Bendamustine is a bifunctional mechlorethamine derivative containing a purine-like benzimidazole ring. 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. Bendamustine is active against both quiescent and dividing cells. The exact mechanism of action of bendamustine remains unknown.

TREANDA® is Brand name of Bendamustine used for the treatment of patients with chronic lymphocytic leukemia and also for the treatment of patients with indolent B-cell non-Hodgkin lymphoma.

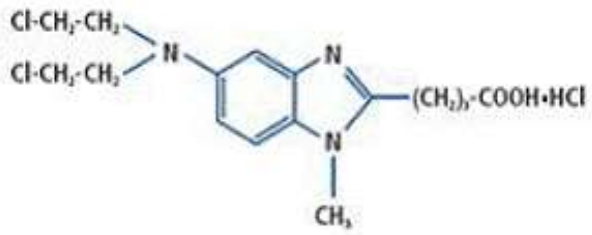

Figure 1: Structure of Bendamustine HCl

MATERIALS AND METHOD

Chemicals and Reagents

Bendamustine active pharmaceutical ingredient (API) and lyophilized formulation of bendamustine were procured from Biophore, Hyderabad, and Telangana, India. Analytical grade trifluoroacetic acid was purchased from Merck, Mumbai, HPLC grade acetonitrile and methanol was procured form rankem. 0.45µm membrane filter was purchased from Millipore. The entire experiment was performed using "class A" volumetric glassware and HPLC grade water.

Instrumentation

Determination of Bendamustine was performed by using Waters HPLC (Milford, MA, USA) PDA system consisting of a quaternary solvent manager, a sample manager, column-heating compartment, and photodiode array detector. This system was controlled and output signal was

monitored by Waters empower software3.0. Sartorius semi micro balance was used for all weighing and Thermo scientific pH meter was used for buffer pH adjustment and sonication was carried out by using fast clean ultrasonic bath. All samples were filtered through 0.45 µm membrane Millipore filters.

Chromatographic conditions

The analysis was carried out by using high performance liquid chromatography (HPLC). The Bendamustine was separated on Inertsil ODS-2 column, 150mm length, 4.6mm internal diameter packed with porous silica of particle size 5µm, at 25°C column oven temperature. The flow rate was maintained at 1.5 mL minute−1. The separation was achieved by isocratic elution with a run time of 10 minutes. The mobile phase was filtered through a 0.45µm Millipore filter, before use. UV detection was performed at 235nm. The sample injection volume was 10 µL. The degassed composition of 0.1%TFA: Acetonitrile (70:30 v/v) was used as the mobile phase.

Standard solution preparation

The standard solution of Bendamustine Hydrochloride monohydrate was prepared by dissolving an accurately weighed 26 mg of Bendamustine Hydrochloride monohydrate working standard in to a 50 ml volumetric flask , added 10ml of diluents sonicate to dissolved and diluted to volume with diluents and mixed well. Further dilute 5ml of the above solution into 50ml volumetric flask and diluted to volume with diluents and mixed well.

PREPARATION OF TEST SOLUTION:

For 25mg/vial:

Reconstitute 4 Sample vials with diluents, pooled together into 200ml volumetric flask, diluted to volume with diluent and mixed well and Filtered the solution through0.22µm PVDF filter.(sample stock solution).Further dilute 5ml of the Filtered solution into 50ml volumetric flask and diluted to volume with diluents and mixed well.

For 100mg/vial:

Reconstitute 1 Sample vial with diluents, pooled together into 200ml volumetric flask, diluted to volume with diluent and mixed well and Filtered the solution through0.22µm PVDF filter.(sample stock solution).Further dilute 5ml of the Filtered solution into 50ml volumetric flask and diluted to volume with diluents and mixed well.

Analytical Method Validation*:*

Validation studies were performed according to the ICH guidelines[10]. The method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, robustness, and the stability-indicating capability. The specificity was determined by

analyzing the chromatograms of Lyophiled sample in comparison with those obtained for Bendamustine standard solution aiming at confirming that none of the ingredients interfere with the quantitation of the drug.

The linearity was determined by a least-square linear regression routine using the compound peak area and concentration of the working standard solutions prepared at six concentration levels (5.053, 10.106, 25.264, 40.423, 45.476, 50.528, 63.161and 75.793µg/mL).The method was evaluated by determination of the correlation coefficient and intercept values according to the ICH guidelines.

LOD and LOQ were determined by calibration curve method. Solutions of Bendamustine were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were expressed as 3.3 × Syx/*b* and 10.0 × Styx/*b*, respectively, where Styx is residual variance due to regression and *b* is the mean slope of the linear regression curves. The precision was assessed at intraday and interday precision. The intraday precision was determined by Diluted standard solution, injected six times on the same day.

The Intermediate precision was estimated by injecting Diluted standard solution prepared at the same concentrations on three different days by different operators. Results were reported in terms of relative standard deviation (RSD). The accuracy was investigated by using the standard addition method at different levels: 50, 100, and 150%.The mean recovery of Bendamustine of the target concentration was calculated and accepted with 100 ± 2%.To determine the robustness, experimental HPLC conditions were purposely modified to check the reproducibility of the method. The evaluation of robustness was based on RSD values obtained by changing analytical setting such as isocratic Flow rate (0.8 to 1.2 mL/min), temperature of analytical column (38 to 40°C), and variation in organic composition (68:32 and 72:28).

FORCED DEGRADATION STUDY

Forced degradation studies were carried out to provide some information about the drug Stability and to validate the specificity of the Bendamustine, quantification of the assay.Demonsrated this by carrying out forced degradation of sample with HCl, NaoH, H_2O_2, Thermal and Photolytic. Prepared samples as per Test preparation and injected into HPLC with photodiode array detector.

i) As such sample

Reconstituted 2 sample vials with diluents, pooled together in 100ml volumetric flask, Diluted to volume with diluents and mixed well. And filter the solution through 0.22µm PVDF filter.further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

ii) Acid degradation

Taken one sample vial and added 1ml of 1N HCl solution, kept the sample solution in water bath at 60°c for 30minutes then added 1ml of 1N NaoH solution to neutralize the solution, transferred whole solution in to a50ml volumetric flask make up to the volume with diluents and mixed well and filtered the solution through 0.22µm PVDF filter. Further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

iii) Alkali degradation

Taken one sample vial and added 1ml of 0.1N NaoH solution, kept the sample solution in room temperature for 15 minutes then added 1ml of 0.1N HCl solution to neutralize the solution, transferred whole solution in to a50ml volumetric flask make up to the volume with diluents and mixed well and filtered the solution through 0.22µm PVDF filter. Further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

iv) Peroxide degradation

Taken one sample vial and added 1ml of 30% H_2O_2 solution, kept the sample solution in water bath at 40°c for 5 minutes then transferred whole solution in to a50ml volumetric flask make up to the volume with diluents and mixed well and filtered the solution through 0.22µm PVDF filter. Further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

v) Photolytic degradation

Taken one sample vial (previously which are exposed to UV light)and reconstituted the vial with 20ml of diluents then transferred whole solution in to a50ml volumetric flask make up to the volume with diluents and mixed well and filtered the solution through 0.22µm PVDF filter. Further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

vi)Thermal degradation

Taken one sample vial (previously which are exposed to Heat at 50°c for 7days) and reconstituted the vial with 20ml of diluents then transferred whole solution in to a50ml volumetric flask make up to the volume with diluents and mixed well and filtered the solution through 0.22µm PVDF filter. Further diluted 5ml of filtered solution to 50ml with diluents and mixed well.

RESULTS AND DISCUSSION

Method Development

The analytical method of Bendamustine was developed with nature of bendamustine molecule. Based on the literature, Bendamustine has one –COOH group it is polar in nature. Methanol was used as solvent for the preparation of sample solutions based on solubility studies .Hence, non

polar C18 column was used for developing reverse phase high performance liquid chromatogram. From the molecular structure and chemical composition, Bendamustine has chromophore group. Hence there are chances for its UV–Visible detection. The UV experiment was showed maximum absorbance at 235nm for bendamustine.

The analytical methodology for the estimation of Bendamustine in marketed parenterals formulation was optimized with to develop efficient, a precise and accurate assay method. Zorbax SB-C18, (4.6 mm x 25 cm) 5µm particle size and Inertsil-ODS (4.6*250mm*5µm), Inertsil-ODS (4.6*150mm*5µm) were used but appropriate chromatographic separation was achieved on Inertstil-ODS (4.6*150mm*5µm). Various mobile phase compositions were prepared and used but the proposed mobile phase containing 0.1% Triflouro Acetic acid: Acetonitrile in the ratio of 70:30 (v/v) to provide an efficient chromatographic separation. Using UV-visible PDA detector, the optimum drug absorbance was obtained at 235 nm, as there was no interference from excipients present in formulation. The several flow rates were tested, the flow rate of 1.5 ml/min was the best suited. The retention time of Bendamustine is 4.0 minutes. The chromatograms of standard and sample solution of Bendamustine were shown in Fig. 2. Column temperature was 25°c; it is not a critical factor of the Analysis. Sample cooler temperature was 5°c; it is critical factor of the analysis because of diluents is methanol it is evaporated at elevated temperature.

METHOD VALIDATION

The proposed method was used for validated by determining its performance characteristics regarding System suitability/ system precision, specificity (Interference study and Forced degradation study), linearity, Method precision, intermediate precision, accuracy, Standard & solution stability, filter variability and Robustness.

System precision/System Suitability

System precision/system suitability is assessed from the five replicate injections of the standard preparation from the same vial. The results of system precision/system suitability are summarized in Table 1. The analytical procedure is precise with respect to chromatographic system. Atypical chromatogram obtained with the present method is shown in Figure 1 & 2.

Table 1: Results of System Suitability/System precision

Injection#	**Bendamustine Peak**	
	Retention time	Area
1	4.734	1439882
2	4.73	1420316
3	4.729	1426127
4	4.726	1427917

5	4.726	1418839
Mean	4.729	1426616
SD	0.0030	8338.02
%RSD	0.1	0.6
Tailing factor	1.2	
Theoretical plate	5299	

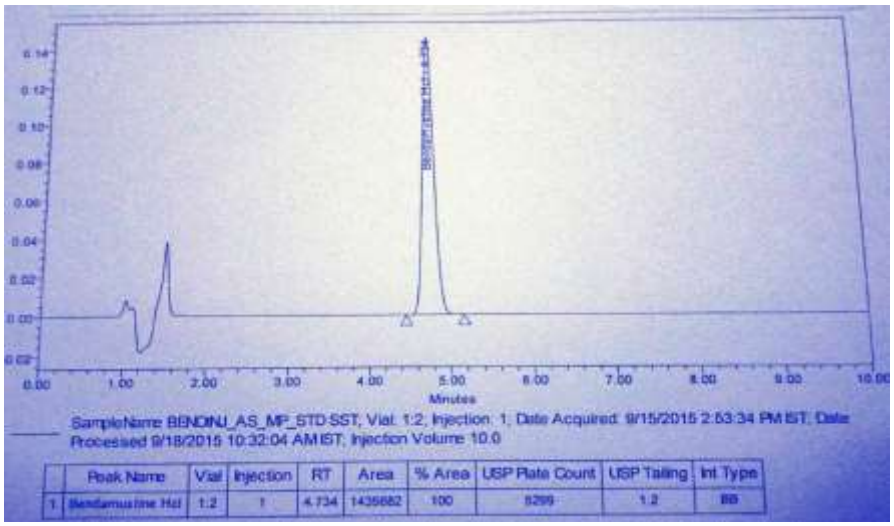

Figure 1: Representative HPLC chromatogram of Bendamustine HCl standard in methanol

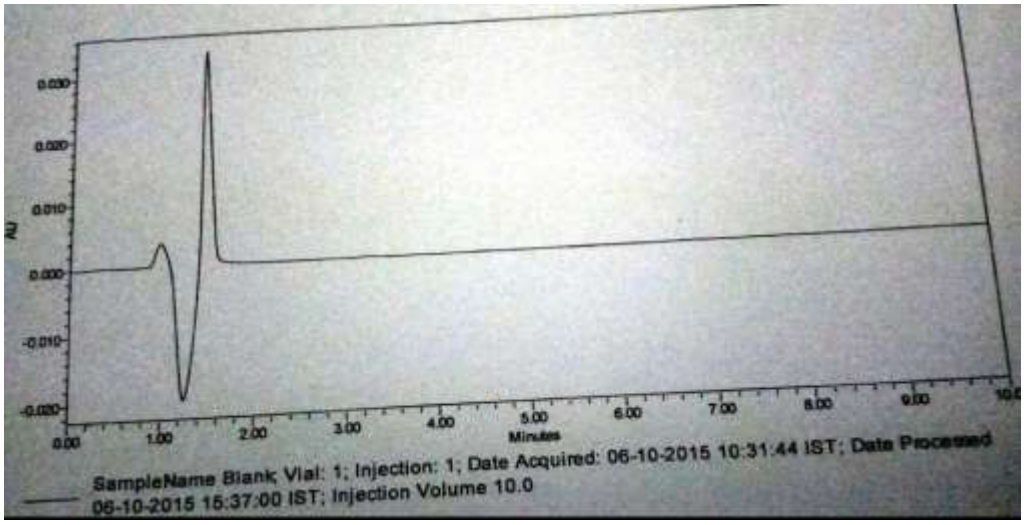

Figure 2: Representative HPLC chromatogram of Blank

Method precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same sample under the prescribed condition.

To evaluate the method precision, six individual samples were prepared for assay and analyzed the samples as per the analytical procedure. Intermediate precision expresses with in- laboratories variations such as Different days, Different analysts, Different columns, Different equipment's etc.

Intermediate precision is established by doing same exercise as system and method precision by different analyst on different day using different column and different equipment. The same lot/Batch of Standard and sample were used within the laboratory. The results of intraday/ interday precision for assay are tabulated in Table 2 and 3.the results found comparable indicates the method is precise and rugged with respect to variation analyst to analyst, day to day, column to column and equipment to equipment for its intended use.

Table 2. Results of Intraday precision

Injection#	Bendamustine Peak	
	Retention time	%ASSAY
1	4.723	101.4
2	4.722	101.9
3	4.721	102.3
4	4.719	100.9
5	4.717	101.2
6	4.716	101.5
Mean		101.5
SD		0.50
%RSD		0.5

Table 3. Results of Interday precision

Injection#	Bendamustine Peak	
	Retention time	%ASSAY
1	4.715	101.6
2	4.715	101.9
3	4.714	100.8
4	4.714	101.3
5	4.713	101.4
6	4.713	101.5
Mean		101.4
SD		0.37
%RSD		0.4

SPECIFICITY

Specificity by interference study

Specificity has been evaluated by assuring no interference observed at the retention time of main analyte in the chromatograms obtained from the diluents, placebo and Known impurities by injecting diluents, placebo solution, Standard solution, sample solution and spiked solution as per the Analytical procedure. All peaks are well separated from Bendamustine peak. Atypical chromatogram obtained with the present method is shown in Figure 1 & 2.

Specificity by Forced degradation

As shown in Table 4, alkaline degradation led to the faster effect on Bendamustine degradation with about 89.8% Bendamustine remaining only after 15 minutes. After exposure to acid Degradation, about 97.0% of Bendamustine was degraded showing less pH effects on bendamustine stability (Table 4). In oxidative condition, the drug was not degraded whereas in high temperature and direct sunlight conditions, bendamustine was found more stable with degradation. At ambient temperature, the drug was stable for at least 2 days. Each Degradation sample Purity angle is less than the purity threshold for bendamustine peak.

Table 4: Results of Specificity by Forced degradation

Stress condition	%assay	%total degradation (with respect to as such)	Purity angle	Purity threshold
As such(unstressed)	102.6	NA	0.107	0.218
alkali	89.8	12.8	0.112	0.228
acid	97	5.6	0.113	0.215
peroxide	102	0.6	0.106	0.218
thermal	101.3	1.3	0.115	0.231
photolytic	102.2	0.4	0.101	0.225

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in sample. The linearity of Bendamustine HCl is established by analyzing Linearity solutions of Different concentrations from 10% to 150% of working concentration of method for assay. The Linearity curve is plotted for area versus concentration. The results summarized in Table 5. Hence the analytical procedure is linear within the concentration range from 10% to 150% (5.053µg/mL to 75.793µg/mL) for Bendamustine HCl.

Table 5: Linearity Results of Bendamustine HCl

Linearity Level	Bendamustine (µg/ml)	Area
10%	5.053	142361
20%	10.106	286356
50%	25.264	711354
80%	40.423	1137043
90%	45.476	1281833
100%	50.528	1426716
125%	63.161	1751978
150%	75.793	2111795
Correlation coefficient(R): 0.9999		Y-intercept: 7932.2959
Slope : 27821		Y-intercept bias at 100% level: 0.6

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found. To demonstrate the accuracy of assay test method, drug substance is spiked quantitatively into placebo from 50% to 150% of working concentration of test concentration at each level with triplicate preparation and analyzed using the test method. The results for bendamustine HCl is tabulated in Table 6. The results are well within the acceptance criteria; hence the method is accurate for its intended use.

Table 6: Results of Accuracy for Bendamustine

Accuracy Level	Sample#	Amount added(mg)	Amount founded(mg)	%Recovery	Average % Recovery	%RSD
50%	1	47.235	47.515	100.6	100.5	0.1
	2	47.329	47.578	100.5		
	3	47.207	47.399	100.4		
100%	1	94.479	94.468	100	99.4	0.9
	2	94.329	94.114	99.8		
	3	94.404	92.88	98.4		
150%	1	141.367	139.762	98.9	99.1	0.3
	2	141.432	140.186	99.1		
	3	141.301	140.407	99.4		
Overall %Recovery						99.7
Overall %RSD						0.8

Range

The range of the analytical method is considered from Linearity, Precision and Accuracy of the method. Based on the Linearity, Method Precision and Accuracy data, Range of the method is 50% to 150% of test concentration.

Robustness

The Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study is performed by analyzing the standard at different conditions. The results obtained with altered conditions are compared against results obtained under normal chromatographic conditions.

Variation in Flow rate (±0.2ml/min)

The Standard was carried out by varying the flow rate of mobile phase to 1.3ml/min and 1.7ml/min. in place of actual flow rate 1.5ml/min. The results are summarized in Table 7.

Table 7. Results of Robustness –Variation in flow rate for Bendamustine

injection#	Flow Rate 1.3ml/min		Flow Rate 1.5ml/min		Flow Rate 1.7ml/min	
	RT	Area	RT	Area	RT	Area
1	5.417	1604526	4.734	1439882	4.170	1245237
2	5.417	1608707	4.730	1420316	4.169	1243739
3	5.417	1610521	4.729	1426127	4.169	1245069
4	5.417	1601094	4.726	1427917	4.169	1249676
5	5.418	1608861	4.726	1418839	4.169	1242068
Mean	NA	1606742	NA	1426616	NA	1245158
%RSD	NA	0.2	NA	0.6	NA	0.2
Tailing Factor	1.2		1.2		1.1	
Theoretical Plates	5601		5299		4842	

Variation in Column Oven Temperature (±2°C)

The Standard was carried out by varying the column oven temperature of 23°C and 27°C. In place of actual the column oven temperature 25°C. The results are summarized in Table 8.

Table 8. Results of Robustness –Variation in Column Oven Temperature

injection#	C O T 23°c		Actual C O T 25°c		C O T 27°c	
	RT	Area	RT	Area	RT	Area
1	4.857	1412117	4.734	1439882	4.571	1421139
2	4.857	1405844	4.730	1420316	4.57	1422116
3	4.857	1392908	4.729	1426127	4.568	1423122
4	4.857	1403670	4.726	1427917	4.567	1421483
5	4.857	1406250	4.726	1418839	4.567	1420494
Mean	NA	1404158	NA	1426616	NA	1421671
%RSD	NA	0.5	NA	0.6	NA	0.1
Tailing Factor	1.2		1.2		1.2	
Theoretical Plates	4990		5299		5347	

*COT means column oven temperature

Variation in Organic composition (±2% of absolute)

The Standard was carried out by varying the mobile phase organic composition of 68:32 and 72:28. In place of actual the mobile phase organic composition 70:30. The results are summarized in Table 9.

Table 9. Results of Robustness –Variation in Organic composition

injection#	M.P composition(68:32)		Actual M.P composition(70:30)		M.P composition(72:28)	
	RT	Area	RT	Area	RT	Area
1	3.731	1436831	4.734	1439882	6.454	1329300
2	3.731	1413689	4.730	1420316	6.454	1386460
3	3.731	1409539	4.729	1426127	6.455	1390738

4	3.731	1415929	4.726	1427917	6.456	1387169
5	3.732	1423610	4.726	1418839	6.456	1391037
Mean	NA	1420460	NA	1426616	NA	1389541
%RSD	NA	0.7	NA	0.6	NA	0.1
Tailing Factor	1.2		1.2		1.2	
Theoretical Plates	4568		5299		5600	

Stability of analyte in solution

Stability of Analyte in solution is evaluated for the Standard and Sample solutions. The Standard and Sample Solution are prepared and analyzed as per analytical procedure. A portion of these solutions were preserved at room temperature and Refrigerator (2-8°C), analyzed at Different time intervals from the time of preparations. The results are calculated from initial versus over a period of time. The results are summarized in Table 10 and 11. The Data indicates that the Standard and Sample Solutions are stable up to 48 hours at room temperature and refrigerator (2-8°c) conditions.

Table 10: Stability of Standard and sample solution at Room temperature

Time Interval	Room Temperature			
	Standard		Sample	
	%Assay	%Difference	%Assay	%Difference
Initial	94.8	NA	101.6	NA
24Hours	95.1	0.3	103.1	1.5
48 hours	95.9	1.1	102.8	1.2

Table 11: Stability of Standard and sample solution at Refrigerator (2-8°C)

Time Interval	Refrigerator(2-8°c)			
	Standard		Sample	
	%Assay	%Difference	%Assay	%Difference
Initial	94.8	NA	101.6	NA
24Hours	93.7	-1.4	100.8	-0.8
48 hours	93.6	-1.2	102.3	0.7

Filter Variability

Filter variability is evaluated for sample solution. The sample solution was prepared and analyzed as per the analytical procedure. A portion of these solutions were filtered through 0.22µm PVDF Filter and 0.22µm nylon Filter and analyzed as per methodology. The results are summarized in Table 12.

Table 12: Filter Variability Results

Type of filter	%Assay	%Difference
Unfiltered	100.8	NA
0.22µm PVDF filter	99.8	1.0
0.22µm nylon filter	99.9	0.9

CONCLUSION

A simple and rapid stability-indicating high-performance liquid chromatographic method was developed and validated for the determination of Bendamustine in pharmaceutical dosage forms. The analytical method is specific, linear, precise, accurate, and robust for a rapid determination of this drug and can be used for studying the stability and routine analysis of Bendamusine.

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