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Analytical Method Development and Validation of Bendamustine in Bulk Using RP-HPLC

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Abstract

A simple, sensitive, precise, accurate and economical method was developed and validated for the estimation of Bendamustine in bulk or formulation by RP-HPLC method using Inertsil column ODS of dimensions ODS -2, (150 x 4.6) mm, 5 µm. The mobile phase (trifluoroacetic acid and acetonitrile) was pumped at a flow rate of 1.5ml/min in the ratio of 68:32 and the eluents were monitored at 230 nm. Linearity was obtained in the concentration range of 10%-150% with R^2 0.999. LOD and LOQ were found to be 2.9 µg/ml and 8.75 µg/ml. The method was statistically validated according to ICH guidelines. RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. The percentage recovery was in good agreement with the labeled amount in the pharmaceutical formulations.

Keywords: Treanda; HPLC; Trifluoroacetic acid

Introduction

Bendamustine bearing the name Treanda is a chemotherapic medication used in the treatment of chronic lymphocytic leukemia, multiple myeloma, and non-hodgkins lymphoma. Bendamustine is a white, water-soluble microcrystalline powder with amphoteric properties. It acts as an alkylating agent causing intra-strand and inter-strand cross-links between DNA bases. After intravenous infusion it is extensively metabolised in the liver by cytochrome p450. More than 95% of the drug is bound to protein – primarily albumin. Only free bendamustine is active. Elimination is biphasic with a half-life of 6–10 minutes and a terminal half-life of approximately 30 minutes. It is eliminated primarily through the kidneys. Combination therapy with bendamustine and rituximab has demonstrated superior efficacy to a standard rituximab-containing chemotherapy regimen in patients with previously untreated indolent B-cell non-Hodgkin lymphoma, and it is currently being compared against the standard first-line regimen in CLL: fludarabine, cyclophosphamide, and rituximab. Ongoing and planned studies are evaluating new strategies in which bendamustine is being combined with existing agents and with novel therapies to optimize use in different clinical settings [1-15].

Materials and Methods

Instrument name	Manufacturer
HPLC	Shimadzu LC 20 10 CHT pump, PDA Detector
Column	Inertsil ODS -2, (150 x 4.6) mm, 5 µm
UV spectrophotometer	Shimadzu, Thermo electron corporation
Electronic balance	Sartorious
Ultra sonicator	Spectral lab-model UCB50
Pipettes, burettes, beakers	Borosil, class-B

Table 1: Instruments used in the present work.

Chemicals	Manufacturer
Bendamustine	Hetero laboratories
Acetonitrile	Merck, Hyderabad
Trifluoroacetic acid	Startech labs, Hyderabad

Table 2: Chemicals and Reagents used in the present work.

Method Development and Validation of Bendamustine

Selection of Wavelength for Detection

Selection of solvent: The solubility of bendamustine was determined in a variety of solvents as per Pharmacopoeial standards. Solubility test was carried out in different solvents like distilled water, methanol, acetonitrile, dilute ethanol. From the solubility studies, it was found that bendamustine was soluble in methanol. Methanol was selected as suitable solvent as there will be no solvent interference while scanning in UV.

Determination of wave length Maxima: UV spectrum of bendamustine in diluent (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 230 nm. At this wavelength bendamustine shows good absorbance.

Preparation of mobile phase: The mobile phase is composed of a mixture of trifluoroacetic acid and acetonitrile in the ratio of 68:32(*v/v*). Prior to use, the mobile phase was degassed and filtered via 0.45 *µ*m membrane filter.

Preparation of Buffer: Add 1.0 mL of Trifluoroacetic acid in 1000 mL water, sonicate for 10 minutes and filter using 0.45 µ filters.

Chromatographic Conditions

Instrument	Shimadzu LC 20 10 CHT pump, PDA Detector
Injection Volume	10 µl
Mobile Phase	Trifluoroacetic acid : Acetonitrile (68:32)
Column	Inertsil ODS -2, (150 x 4.6) mm, 5 µm
Wavelength	230 nm
Flow Rate	1.5 ml/mim
Runtime	10 min

Table 3: Variables in HPLC.

Validation of Rp-Hplc Method

Validation is a key process for effective quality assurance. "Validation" is established documented evidence, which provides a high degree of assurance that a specific process or equipment will consistently produce a product or result meeting its predetermined specification and quality attributes. The Validation parameters are:

- Specificity
- Linearity
- System Suitability Parameters
- Precision
- Accuracy or Recovery
- Assay
- Ruggedness
- Robustness
- LOD and LOQ

Specificity: Specificity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample. It is the measure of degree of interference (or absence thereof) in the complex sample mixtures such as the analyte mixed with the formulation excipients, known impurities and degradation product.

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.

System suitability: The System suitability is an integral part of analytical procedure. The tests are based on the concept that the equipment, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

Precision: Precision is the degree of closeness of agreement among individual test results when the method is applied to multiple samplings of a homogenous sample. It is a measure of either the degree of reproducibility (agreement under different conditions) or repeatability (agreement under same conditions) of the method.

Ruggedness: Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions.

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. 2 mL/min): The standard was carried out by varying the flow rate of mobile phase to 1.3 mL/min. and 1.7 mL/min. in place of actual flow rate 1.5 mL/min.

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 column oven temperature 25°C.

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 Mobile phase organic composition 70:30.

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 in to placebo from 50% to 150% of working concentration of test concentration at each level with triplicate preparation and analyzed using the test method. Typical chromatogram of Accuracy at 100 % level for is shown in figure 4.

Results and Discussion

Selection of Chromatographic Method

Proper selection of the method depends on the nature of the sample (ionic or ionisable or neutral molecules), its molecular weight, pka value and stability. The drug selected in the present study is polar and so reversed phase or ion exchange chromatography can be used. The reversed phase HPLC was selected for the initial separation because of its simplicity and suitability. From the literature survey and with the knowledge of properties of the selected drug, ODS column was tried. ODS 150 X 4.5 mm 5µcolumn was chosen as stationary phase and mobile phase with different compositions such as methanol and water was used. The separations were not observed, so the combination of buffer and methanol was finalized. The buffer used was trifluoroacetic acid buffer.

Effect of Ratio of Mobile Phase

Under the chromatographic conditions mentioned above, the different ratios of buffer and acetonitrile were tried i.e. for trifluoroacetic acid buffer and acetonitrile 68:32 ratios were tried at which they gave good peaks and minimum retention time and good chromatogram with proper resolution.

System Suitability

Injection #	Bendamustine Peak	
	Retention Time	Area
1	4.734	1439882
2	4.730	1420316
3	4.729	1426127
4	4.726	1427917
5	4.726	1418839
Mean		1426616
% RSD		0.6
Tailing factor	1.2	
Theoretical Plate	5299	

Table 4: Results of system suitability for bendamustine

Acceptance Criteria

- The Tailing factor for Bendamustine peak from first injection of standard solution should be not more than 2.0.
- Theoretical Plates for Bendamustine peak from first injection of standard solution should be not less than 2000.
- The relative standard deviation for Bendamustine peak from five replicate injections of standard solution should be not more than 2.0%.

Conclusion: The results met the acceptance criteria; hence the method is system suitable for its intended use.

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 Hydrochloride 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 are summarized in table 5. Typical chromatogram of Linearity at 100 % level is shown in figure1. The linearity graph of is shown in figure 1. Typical chromatogram of Linearity at 100 % level is shown in figure 2.

Linearity Level	Bendamustine HCl (µg/mL)	Area
10 %	5	142361
20 %	10	286356
50%	25	711354
80 %	40	1137043
90 %	45	1281833
100 %	50	1426716
125 %	63	1751978
150 %	75	2111795
Correlation coefficient (R) : 0.9999		Y-intercept: 7930.9
Slope : 27800		Y-intercept bias at 100% level :0.6

Table 5: Results for linearity of bendamustine.

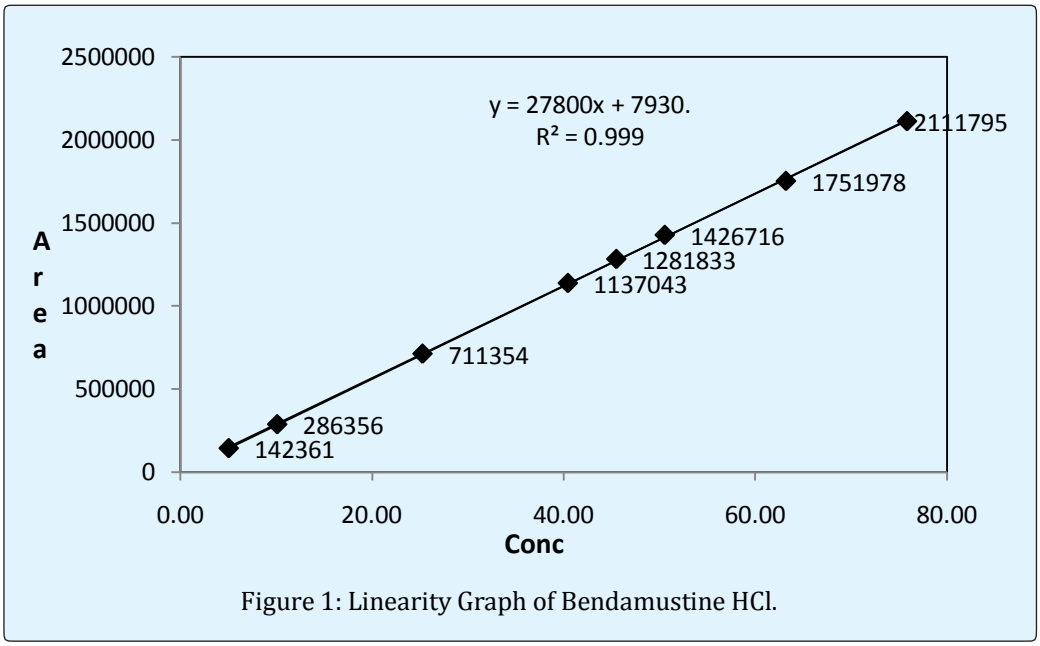

Figure 1: Linearity Graph of Bendamustine HCl.

Acceptance Criteria:

- Correlation coefficient should not be less than 0.999 for Bendamustine Hydrochloride.
- Report the slope of regression line.
- Report the Y-intercept of regression line.
- Y-intercept bias at 100 % level should be between ± 5.0 % for Bendamustine Hydrochloride.

Conclusion

The results are within the acceptance criteria; hence the analytical procedure is linear within the concentration range from 10 % to 150 % (5.057µg/mL to 75.849µg/mL) for Bendamustine Hydrochloride.

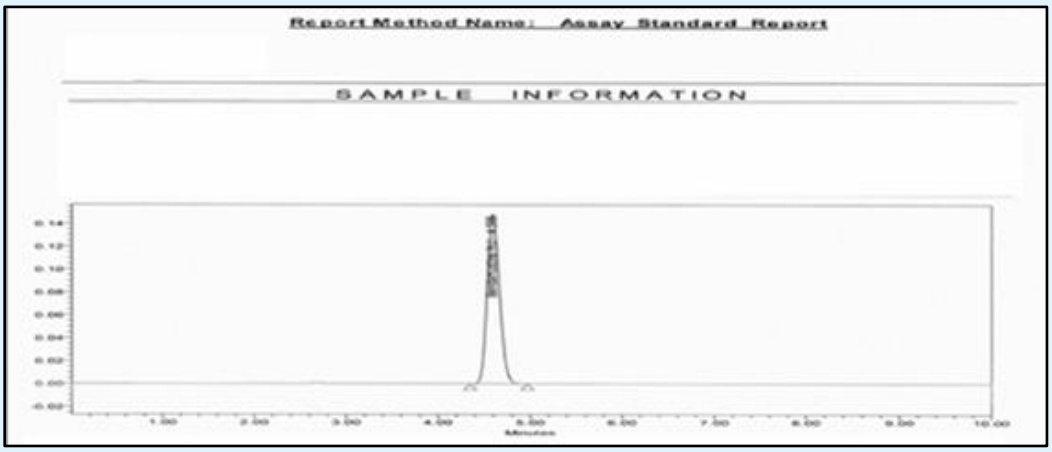

Figure 2: Typical chromatogram of linearity.

Precision

Sample #	Retention Time (Average)	% Assay
1	4.163	101.0
2	4.170	100.5
3	4.174	100.5
4	4.171	101.6
5	4.174	101.8
6	4.179	101.6
Mean		101.2
% RSD		0.6

Table 6: Results of Intermediate Precision.

Acceptance Criteria:

- The relative standard deviation of results obtained from six sample preparations should not be more than 2.0%

Conclusion: The result meets the acceptance criteria and found comparable, indicates that the method is precise.

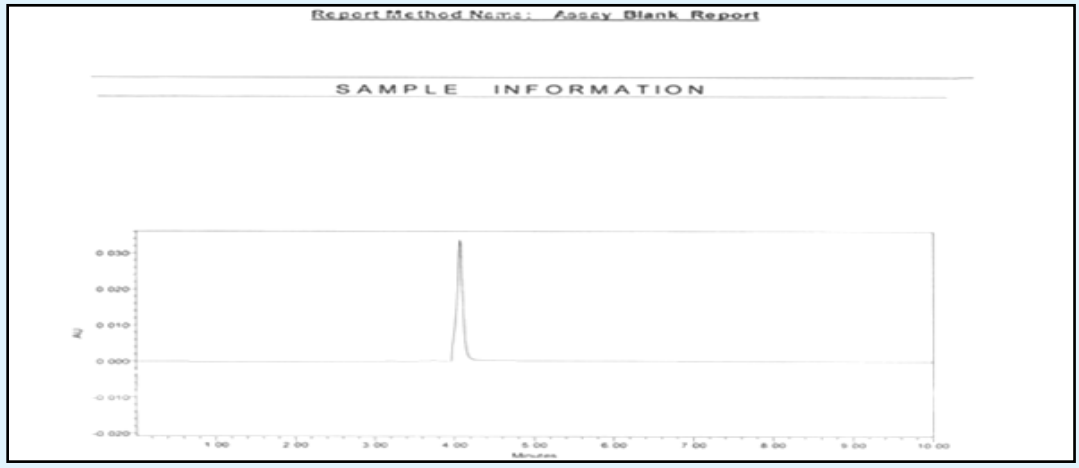

Figure 3: Chromatogram of Precision.

Accuracy Level	Sample #	Amount (mg added)	Amount (mg found)	% 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	100.0	99.4	0.9
	2	94.329	94.114	99.8		
	3	94.404	92.880	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		
% Recovery for 9 levels						99.7
% RSD for 9 levels						0.8

Table 7: Results for accuracy of bendamustine.

Accuracy (Recovery): To demonstrate the accuracy of assay test method, drug substance is spiked quantitatively in to placebo from 50% to 150% of working concentration of test concentration at each level with triplicate preparation and analyzed using the test method. The result for Bendamustine HCl is tabulated in Table 7**.** Typical chromatogram of Accuracy at 100 % level for is exhibited in figure 4.

Acceptance Criteria:

- % Recovery at each level and overall % recovery should be between 98.0 and 102.0 for Bendamustine HCl.
- The % RSD at each level and overall recovery should not be more than 2.0.

Conclusion: The results are well within the acceptance criteria; hence the method is accurate for its intended use.

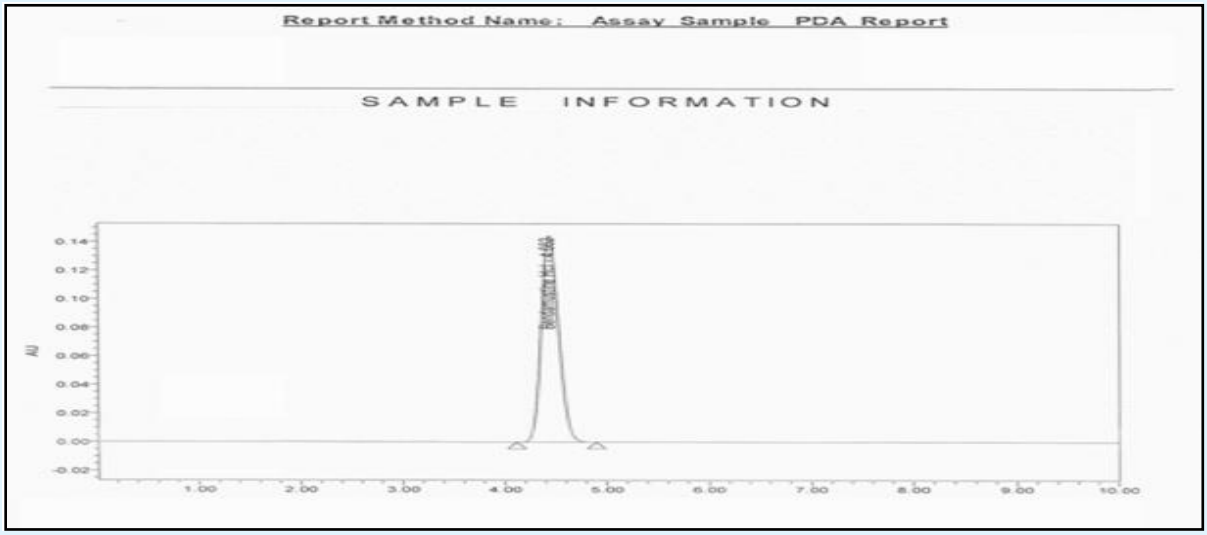

Figure 4: Typical Chromatogram for Accuracy.

Specificity

Specificity is the ability of the analytical procedure to assess unequivocally the analyte in the presence of components which may be expected to be present.

Name	Retention time (min.)	Results
Diluent	No Peak detected	No interference observed
Placebo 25mg per vial	No Peak detected	No interference observed
Placebo 100mg per vial	No Peak detected	No interference observed
Bendamustine Hydrochloride in Standard solution	4.595	N/A
Bendamustine Hydrochloride in Sample solution 25mg per vial	4.670	N/A
Bendamustine Hydrochloride in Sample solution 100mg per vial	4.664	N/A
Bendamustine Hydrochloride in Spiked sample solution	4.662	NA

Table 8: Specificity Results for bendamustine.

Acceptance Criteria:

- There should be no interference at the retention time of Bendamustine peak in the Chromatograms obtained from the diluent and the placebo solutions.

Conclusion:

No Interference is observed at the retention time of Bendamustine peak in the chromatograms obtained from the diluent, placebo. The results met the acceptance criteria; hence the method is specific for its intended use.

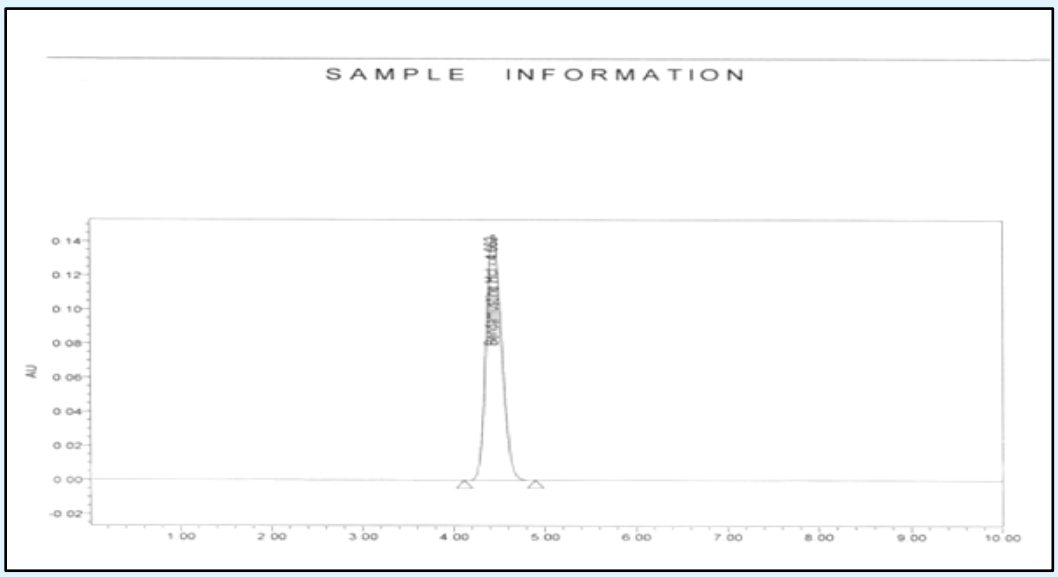

Figure 5: Typical chromatogram for specificity.

Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions. It was checked that the results were reproducible under differences in conditions, analysts and instruments.

Procedure: The standard solution and sample solution were injected by different analysts and the area for injections in HPLC was measured.

Injection	Analyst 1	Analyst 2
Rt	4.734	4.731
Peak Area	1439882	1426127

Table 9: Results for ruggedness of bendamustine.

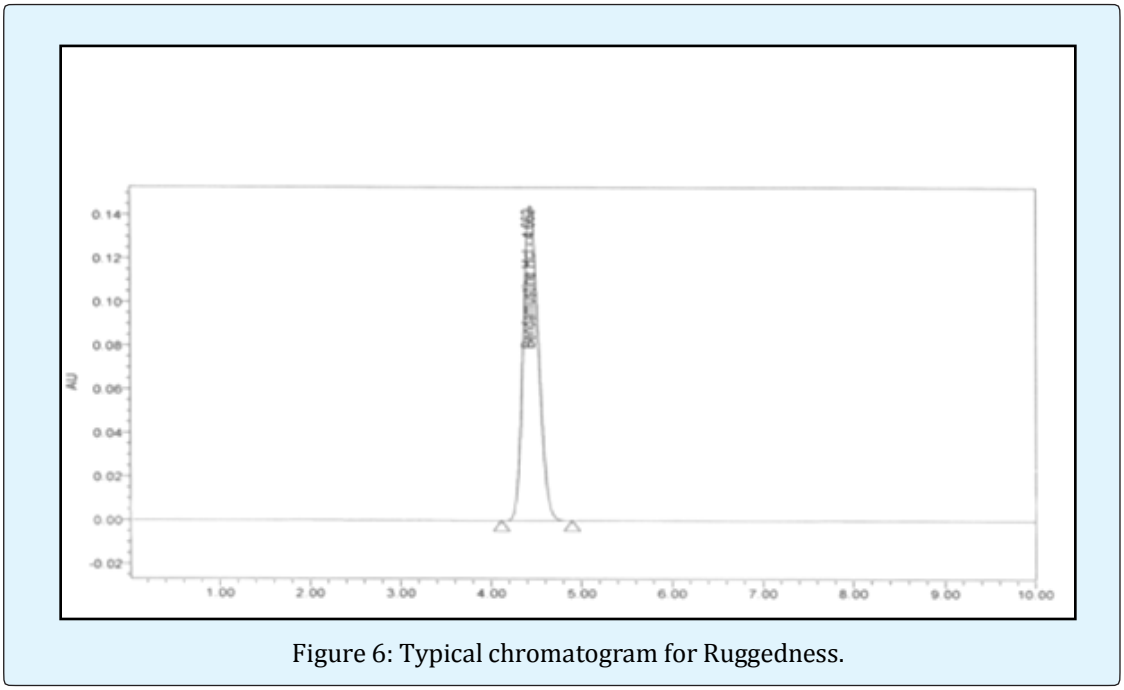

Figure 6: Typical chromatogram for Ruggedness.

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. 2 mL/min.): The standard was carried out by varying the flow rate of mobile phase to 1.3 mL/min. and 1.7 mL/min. in place of actual flow rate 1.5 mL/min. The results are summarized in Table 10.

Injection #	Flow Rate 1.3 mL/min.		Actual Flow Rate 1.5 mL/min.		Flow Rate 1.7 mL/min.	
	RT	Area	RT	Area	RT	Area
1	4.417	1604526	4.734	1439882	4.170	1245237
2	4.417	1608707	4.730	1420316	4.169	1243739
3	4.417	1610521	4.729	1426127	4.169	1245069
4	4.417	1601094	4.726	1418839	4.169	1249676
5	4.418	1608861	4.726	1427917	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	5598		5299		4842	

Table 10: Results of Robustness-Variation in Flow rate for Bendamustine.

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 column oven temperature 25°C. The results are summarized. The results are summarized in Table 11.

Injection #	Column Oven Temperature 23°C		Actual Column Oven Temperature 25°C		Column Oven Temperature 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	4.570	1422116
3	4.857	1392908	4.729	1426127	4.568	1423122
4	4.857	1403670	4.726	1418839	4.567	1421483
5	4.857	1406250	4.726	1427917	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 plate	4999		5299		5314	

Table 11: Results of Robustness-Variation in Column Oven Temperature for Bendamustine.

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 Mobile phase organic composition 70:30. The results are summarized. The results are summarized in Table 12.

Injection #	Mobile phase composition (68:32)		Actual Mobile phase composition (70:30)		Mobile phase composition (72:28)	
	RT	Area	RT	Area	RT	Area
1	4.731	1436831	4.734	1439882	4.454	1392300
2	4.731	1413689	4.730	1420316	4.454	1386460
3	4.731	1409539	4.729	1426127	4.455	1390738
4	4.731	1415929	4.726	1418839	4.456	1387169
5	4.732	1423610	4.726	1427917	4.456	1391037
Mean	NA	1420460	NA	1426616	NA	1389541
% RSD	NA	0.8	NA	0.6	NA	0.2
Tailing factor	1.2		1.2		1.2	
Theoretical plates	5603		5299		4568	

Table 12: Results of Robustness-Variation in organic composition for Bendamustine Hydrochloride.

Acceptance criteria:

- The Tailing factor for Bendamustine peak from first injection of standard solution should be not more than 2.0.
- Theoretical Plates for Bendamustine peak from first injection of standard solution should be not less than 2000.
- The relative standard deviation for Bendamustine peak from five replicate injections of standard solution should be not more than 2.0%.

Conclusion: The system suitability meets for each altered conditions. The results obtained with altered conditions are comparable with the results obtained with normal conditions. The robustness result indicates that the test method is robust enough as demonstrated by altering the Flow rate (±0.2 mL/min.), column temperature (±0.2°C) and organic composition (±2% of absolute).

Sensitivity: Limit of Detection (LOD), Limit of Quantification (LOD) was examined by injecting six consecutive injections of bendamustine solutions at lowest concentration (10 µg mL^{-1}). LOD can be calculated by using the formula, LOD = 3.3 × S.D/slope. LOQ can be calculated by using the formula, LOD = 10 × S.D/slope.
The obtained LOD and LOQ values were found to be 2.9 µg/ml and 8.75 µg/ml.

Summary and Conclusion

A simple, sensitive and reproducible HPLC method for determination of bendamustine has been developed in bulk and tablet dosage forms. The validation parameters

were found to be highly agreeable, indicating small sample volume, short retention time, system suitability, specificity, linearity, limits of detection and quantification, precision, accuracy and robustness. Hence, the proposed method can be easily applied for the quantification of bendamustine in routine quality control laboratories.

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