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A VALIDATED REPORT ON FORMULATION STABILITY STUDIES OF BENDAMUSTINE HYDROCHLORIDE

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ABSTRACT: The objective of this study was to adopt the analytical method. This adoption procedure was carried out by validating the method parameters and conducting the forced degradation studies to check the interference of the chromatographic response of the degradation products with the drug. In the present study an attempt was made to studies related to system suitability, linearity, accuracy, precision, and assay content, forced degradation, acid and base degradation, hydrolysis degradation and photo stability studies were conducted as per the standard procedures. System suitability conditions meet the recommended criterion. Linearity of the solution was demonstrated between 0.0002577 mg/ml and 0.51540 mg/ml concentration range. Recovery and % RSD are within the recommended limits. Injection reproducibility was demonstrated and the % relative standard deviation for retention time and area were within the limits. Limit of detection value is at 0.00004165mg/ml and limit of quantisation is at 0.00012621mg/ml. Solution was stable up to 38hrs at 5°C temperature. Precision was demonstrated and the percentage relative standard deviation for assay was within the limits. Specificity was also demonstrated and there was no interference with the diluent & mobile phase. Under forced degradation studies, the peak purity angle is always lower than purity threshold for Bendamustine hydrochloride and all degradant peaks were well resolved. Hence the method can be adopted for the formulation screening studies as a stability indicating method.

INTRODUCTION: Pharmaceutical analysis comprises those procedures necessary to determine the “*identity, strength, quality and purity*” of the drug. Again it may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. It also deals the analysis of raw materials and intermediates in the manufacture of drugs^{1, 2}. The pharmaceutical analyst must therefore, have a firm background in basic organic analysis and in addition he should have special skills in the quality evaluation of drug products.

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 kidneys³. The objective of this study was to adopt the analytical method. This adoption procedure was carried out by validating the method parameters and conducting the forced degradation studies to check the interference of the

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chromatographic response of the degradation products with the drug.

MATERIALS AND METHODS:

Standards and solvents

Bendamustine Hydrochloride drug substance was obtained as gift sample from Euticals Pharma Limited. All the other reagents were of analytical grade.

Chromatographic conditions:

Chromatographic system : Waters Alliance 2695 with PDA/UV 2995 detector with Empower software.
 Column : Zorbax SB C₁₈ 250mm x 4.6mm x 5µm
 Mobile Phase-A : Water: Acetonitrile: TFA:: 90:10:0.1
 Mobile Phase: B : Water: Acetonitrile: TFA:: 50:50:0.1
 Flow rate : 1.0 ml/min
 Detection wavelength : 230nm
 Column Temperature : 30°C
 Auto sampler temperature : 5°C
 Injection volume : 10µl
 Run time : 45 min.
 Diluent : Methanol
 Gradient programme :

Pump B. Conc.	Time (minutes)
0.01	0.00
3.00	0.00
16.0	50.0
33.0	70.0
35.0	90.0
40.0	90.0
41.0	0.00
45.0	0.00

System Suitability⁴:

Preparation of System suitability solution: Bendamustine hydrochloride drug substance (12.52mg) was accurately weighed into a 50ml volumetric flask, dissolved and made up to volume with diluents (concentration 0.2504mg/ml).

Procedure: Bendamustine hydrochloride system suitability solution (10µl) was injected into the chromatographic system connected to a Zorbax SB C₁₈ 250mm x 4.6mm x 5µm for 6 times and the relative standard deviation for area, theoretical plates and tailing factor for the replicate injections were calculated.

TABLE 1:

Injection Number	Peak Area	Theoretical plates	Tailing factor
Injection – 1	10688971	25834	1.74
Injection – 2	10613684	25241	1.71
Injection – 3	10622986	24865	1.71
Injection – 4	10592403	25024	1.71
Injection – 5	10618844	24740	1.68
Injection – 6	10593310	24853	1.69
Mean	10621700	25093	1.71
Acceptance	< 2.0%	≥ 2500	< 2.5
Result	Pass (RSD – 0.33)	Pass	Pass

Acceptance Criteria: Tailing factor for Bendamustine hydrochloride peak was < 2.5, Theoretical plates for Bendamustine hydrochloride was ≥ 2500 and %RSD for peak area was < 2.0%.

Linearity⁴:

Preparation of Bendamustine hydrochloride Stock and Level Solutions for Linearity:

Bendamustine hydrochloride Stock Solution for Linearity: Bendamustine hydrochloride drug substance (128.85mg) was accurately weighed into a 50ml volumetric flask, dissolved and made up to volume with diluent (concentration 2.5770mg/ml).

Level Solutions for Linearity: A series of solutions with concentrations of 0.1%, 5%, 10%, 20%, 50%, 80%, 100%, 120%, 150% and 200% were prepared. Level 200% was prepared with 4.0ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.51540mg/ml). This solution was prepared in duplicate. Level 150% was prepared with 3.0ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.38655mg/ml).

This solution was prepared in duplicate. Level 120% was prepared with 2.4ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.30924mg/ml). This solution was prepared in duplicate. Level 100% was prepared with 2.0ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.25770mg/ml).

This solution was prepared in duplicate. Level 80% was prepared with 1.6ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.20616mg/ml). This solution was prepared in duplicate.

Level 50% was prepared with 1.0ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration

0.12885mg/ml). This solution was prepared in duplicate. Level 20% was prepared with 0.4ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.05154mg/ml). This solution was prepared in duplicate. Level 10% was prepared with 0.2ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.02577mg/ml).

This solution was prepared in duplicate. Level 5% was prepared with 0.1ml of Bendamustine hydrochloride stock solution was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.012885mg/ml). This solution was prepared in duplicate. Level 0.1% was prepared with 0.2ml of level 10% solution for linearity was transferred into a 20ml volumetric flask and made up the volume with the diluent (concentration 0.0002577mg/ml). This solution was prepared in duplicate.

Demonstration of Linearity⁵:

The linearity of the method was demonstrated by chromatographic analysis of the above prepared solutions containing 0.1%, 5%, 10%, 20%, 50%, 80%, 100%, 120%, 150% and 200% of the target concentration of 0.2577mg/ml. 10 μ l of each of the above prepared solutions were injected into the chromatographic system connected to a Zorbax SB C₁₈ 250mm x 4.6mm, 5 μ m and the average peak area in each of the cases was calculated. Statistical evaluation was carried out by a graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation coefficient, slope, and y-intercept were calculated. Acceptance criteria were taken as R² value should not be less than 0.98.

Accuracy⁵:

Preparation of Bendamustine hydrochloride Stock and Level Solutions for Accuracy:

Bendamustine Hydrochloride Stock Solution for Accuracy:

Bendamustine hydrochloride drug substance (25.20mg) was accurately weighed into a 10ml volumetric flask, dissolved and made up to volume

with diluent (concentration 2.520 mg/ml). Level Solutions for Accuracy was prepared with a series of solutions with concentrations of 80%, 100% and 120% were prepared as described below. Level 80% was prepared with 0.8 ml of Bendamustine hydrochloride stock solution was transferred into a 10ml volumetric flask and made up to volume with the diluent (concentration 0.2016mg/ml).

This solution was prepared in triplicate. Level 100% was prepared with 1.0ml of Bendamustine hydrochloride stock solution was transferred into a 10ml volumetric flask and made up to volume with the diluent (concentration 0.2520mg/ml). This solution was prepared in triplicate. Level 120% was 1.2ml of Bendamustine hydrochloride stock solution was transferred into a 10ml volumetric flask and made up to volume with the diluent (concentration 0.3024mg/ml). This solution was prepared in triplicate.

Demonstration of Accuracy⁵:

Accuracy was calculated with respect to above prepared solutions at the levels of 80%, 100% and 120% of the normal or target concentration. The accuracy of the method was demonstrated by chromatographic analysis through recovery experiment on 3 samples of the above prepared solutions containing 80%, 100% and 120% of the target concentration of 0.25mg/ml of Bendamustine hydrochloride employed in the usual procedure. 10 μ l of each of the above prepared solutions were injected into the chromatographic system connected to a Zorbax SB C₁₈, 250 x 4.6mm, 5 μ m and the average peak area in each of the cases was recorded.

The amount of Bendamustine hydrochloride peak area found in each of these test solutions were calculated using the calibration curve. The table below summarizes the amount added vs. amount found and the percentage recoveries. The average percentage recovery should be between 98-102% and relative standard deviation of these recovery concentrations should be less than 2%.

Demonstration of Precision⁵:

The precision of the method was demonstrated through two parameters which are injection reproducibility and the method precision.

Bendamustine hydrochloride drug substance, 12.59mg was accurately weighed into a 50ml volumetric flask, dissolved and made up to volume with diluent (concentration 0.2518 mg/ml). The relative standard deviations of retention time and peak areas of the above system suitability solutions were calculated for injection reproducibility. Acceptance Criteria: The injection reproducibility requirements are met if the relative standard deviation for retention time is not more than 1% and area is not more than 2.0%.

Injection Reproducibility:

For injection reproducibility, considered six injections from the same system suitability solution and the relative standard deviation for the replicate injections was calculated. The relative standard deviations of retention time and peak areas of the above system suitability solutions were calculated for injection reproducibility. Acceptance Criteria: The injection reproducibility requirements are met if the relative standard deviation for retention time is not more than 1% and area is not more than 2.0%.

Method Precision⁶:

For method precision, six individual preparations were made from the same batch of Bendamustine hydrochloride drug substance with a target concentration of about 0.25mg/ml and individual peak areas were measured. The relative standard deviation was calculated for these six preparations.

Preparation-1 was made with Bendamustine hydrochloride drug substance (12.48mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-1 (concentration – 0.2496mg/ml). Preparation-2 was made with Bendamustine hydrochloride drug substance (12.50mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-2 (concentration – 0.2500mg/ml).

Preparation-3 was made with Bendamustine hydrochloride drug substance (12.52mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-3 (concentration –

0.2504mg/ml). Preparation-4 was made with Bendamustine hydrochloride drug substance (12.55mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-4 (concentration – 0.2510mg/ml). Preparation-5 was made with Bendamustine hydrochloride drug substance (12.45mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-5 (concentration – 0.2490mg/ml).

Preparation-6 was made with Bendamustine hydrochloride drug substance (12.47mg) was accurately weighed into a 50ml volumetric flask, dissolved, made up to volume with the diluent and labelled as Preparation-6 (concentration – 0.2494mg/ml) was made with 0µl of each of the above solutions were injected in duplicate into the chromatographic system connected to a Zorbax SB C₁₈ 250mm X 4.6mm X 5µm column and recorded the chromatographic response. The peak areas due to Bendamustine hydrochloride peaks were measured.

Acceptance Criteria was relative standard deviation for the assay preparations should not be more than 2.0%. The amount of Bendamustine hydrochloride in each preparation was calculated using the formula given.

Assay Content⁶:

Test Solution: Sample average area was taken from 6 sample solutions in the method precision study. The % of the Bendamustine hydrochloride individually in each sample solution was calculated using the following formula:

$$\text{Assay\%} = \frac{A_T}{A_S} \times \frac{W_S}{W_T} \times P$$

Where,

A_T = Average area of the Bendamustine hydrochloride in the test preparation

A_S = Average area of the Bendamustine hydrochloride in standard preparation

W_T = Concentration (mg/ml) of the test solution

W_S = Concentration (mg/ml) of the standard/system suitability solution

P = Potency of the Bendamustine hydrochloride working standard/drug substance

Specificity⁶:

The specificity of the method was demonstrated through establishing interference check by injecting the diluent blank and mobile phase to determine whether any peaks are co-eluting with the Bendamustine hydrochloride peak. 10 μ l each of diluent blank and mobile phase solutions were injected separately onto the chromatographic system connected to a Zorbax SB C₁₈ 250 x 4.6mm, 5 μ m and observed the chromatographic responses.

There should not be any interference of peaks, eluted in the diluent blank and mobile phase with the Bendamustine hydrochloride peak retention time. It was observed that peaks eluted in the chromatographic response of diluent blank and mobile phase does not show any interference with those eluted in the Bendamustine hydrochloride.

Limit of Detection and Limit of Quantification (LOD and LOQ) ⁷:

Preparation of Bendamustine hydrochloride Stock and Level Solutions for LOD and LOQ:

Preparation of Stock Solution for LOD and LOQ: Bendamustine hydrochloride drug substance, 12.78mg was accurately weighed into a 50ml volumetric flask, dissolved and made up to volume with diluent (concentration 0.2556 mg/ml). Transfer 1ml of this solution into a 100ml volumetric flask, dissolved and made up to volume with diluent (concentration 0.002556mg/ml).

Level solutions for LOD and LOQ: A series of the test solutions (level solutions) were prepared as described below at different concentrations using the above stock solution close to LOD and LOQ level.

Level 0.05% was made by transferring 2.5ml of the stock solution into a 50ml volumetric flask and made up to volume with the diluent (concentration 0.0001278mg/ml). Level 0.10% was made by transferring 5.0ml of the stock solution into a 50ml volumetric flask and made up to volume with the diluent (concentration 0.0002556mg/ml). Level 0.15% was made by transferring 7.5ml of the stock solution into a 50ml volumetric flask and made up to volume with the diluent (concentration

0.0003834mg/ml). Injected 10 μ l of each of the above prepared solutions on to the chromatographic system connected to a Zorbax SB C₁₈ 250 x 4.6 mm, 5 μ m for 6 times and calculated the average area and standard deviation in each of the cases.

Statistical evaluation:

Plot two different graphs, concentration vs. average area and concentration vs. drug substance deviation and calculate the slope, y-intercept and correlation coefficient.

Solution Stability⁸:

System Suitability Solution for Solution Stability was taken Initial, 4, 24 and 38hours. The stability of assay solutions was demonstrated for a period of 38 hours at 5°C temperature of sample cooler by chromatograph the same solution at periodic time intervals.

The system suitability solution injected initially was considered as solution stability initial and the same solution was injected at specified time intervals of 24 and 38hours.

Procedure: 10 μ l of the above prepared solution was injected into the chromatographic system connected to a Zorbax SB C₁₈ 250 x 4.6mm, 5 μ m. Vial was kept in auto sampler which is maintained at 5°C temperature for further study and was injected into the chromatographic system at periodic time intervals (initial, 24 and 38hrs).

Forced Degradation⁸:

Analytical method was tested by the forced degradation studies to determine adequate separation of degradant from Bendamustine hydrochloride peak.

Acid degradation⁸:**Preparation of the stock solution for acid degradation:**

Weighed accurately 10.25mg of Bendamustine hydrochloride drug substance into a 20ml volumetric flask and added 0.01N HCl, dissolved and made up to volume with 0.01N HCl. This solution was analyzed initially and stock solution was stored at 60°C for further study. Test solutions were prepared as per the following procedure prior to analysis.

Preparation of the test solutions for analysis:

At appropriate time interval, 2.5ml of acid degradation stock solution was transferred into a 5ml volumetric flask and made up to volume with diluent and analyzed by HPLC.

Preparation of Acid Blank solution for acid degradation:

10ml of 0.01 N HCl was marked as blank solution. This blank solution was analyzed initially and stored along with the test solution. At appropriate time interval, blank solution was also prepared similarly as sample solution and injected into chromatograph.

Base Degradation⁸:

Preparation of the stock solution for base degradation:

Weighed accurately 10.14mg of Bendamustine hydrochloride drug substance into a 20ml volumetric flask and added 0.1N NaOH, dissolved and made up to volume with 0.1N NaOH. This solution was analyzed initially and stock solution was stored at 60°C for further study. Test solutions were prepared as per the following procedure prior to analysis.

Preparation of the test solutions for analysis:

At appropriate time interval, 2.5ml base degradation stock solution was transferred into a 5ml volumetric flask and made up to volume with diluent and analyzed by HPLC.

Preparation of Base Blank solution for base degradation:

10ml of 0.1 N NaOH was marked as blank solution. This blank solution was analyzed initially and stored along with the test solution. At appropriate time interval, blank solution was also prepared similarly as sample solution and injected into chromatograph.

Oxidative degradation⁹:

Preparation of the stock solution for oxidative degradation:

Weighed accurately 10.22mg of Bendamustine hydrochloride drug substance into a 20ml volumetric flask and added 5% hydrogen peroxide, dissolved and made up to volume with 5%

hydrogen peroxide. This solution was analyzed initially and stock solution was stored at 60°C for

Preparation of the test solutions for analysis:

At appropriate time interval, 2.5ml oxidative degradation stock solution was transferred into a 5ml volumetric flask and made up to volume with diluent and analyzed by HPLC.

Preparation of oxidative Blank solution for oxidative degradation:

10ml of 5% hydrogen peroxide was marked as blank solution. This blank solution was analyzed initially and stored along with the test solution. At appropriate time interval, blank solution was also prepared similarly as sample solution and injected into chromatograph.

Hydrolysis Degradation⁹:

Preparation of the stock solution for Hydrolysis:

Weighed accurately 10.22mg of Bendamustine hydrochloride drug substance into a 20ml volumetric flask and added water, dissolved and made up to volume with water. This solution was analyzed initially and stock solution was stored at 60°C for further study. Test solutions were prepared as per the following procedure prior to analysis.

Preparation of the test solutions for analysis:

At appropriate time interval, 2.5ml hydrolysis degradation stock solution was transferred into a 5ml volumetric flask and made up to volume with diluent and analyzed by HPLC.

Preparation of Hydrolysis Blank solution for hydrolysis:

10ml of water was marked as blank solution. This blank solution was analyzed initially and stored along with the test solution. At appropriate time interval, blank solution was also prepared similarly as sample solution and injected into chromatograph.

Photo Stability Studies¹⁰:

Preparation of Bendamustine hydrochloride for Photo Stability Studies:

Bendamustine hydrochloride drug substance (50mg) was weighed into 4vials of 12.5mg each. The vials were, labelled as Uncovered (1 vial),

Covered with Aluminium foil (1 vial) & Covered with Aluminium foil and placed in Carton (1 vial), were exposed to UV (200 uw/cm²) & fluorescence light (1.2 million Lux hours) in the Photo stability chamber. The remaining 1 vial was kept in dark as control.

Preparation of Test Solutions for Analysis:

The content from the vial was transferred into a 50ml volumetric flask. The vial was rinsed with the diluent, adding the rinsing to the volumetric flask and made up to volume with the diluent. Directly transferred an aliquot of the solution into an HPLC vial and injected into chromatographic system. All the vials were prepared and injected individually.

RESULTS AND DISCUSSIONS:

Linearity of Bendamustine hydrochloride

TABLE 2. LINEARITY OF BENDAMUSTINE HYDROCHLORIDE

Level Solutions for Linearity	Concentration (mg/ml)	Area Response	Average Area Response
0.1%	0.0002577	7446 7516	7481
5%	0.0128850	564415 569060	566738
10%	0.0257700	1129591 1137451	1133521
20%	0.0515400	2297303 2288574	2292939
50%	0.1288500	5463240 5446366	5454803
80%	0.2061600	8860187 8845044	8852616
100%	0.2577000	11197387 11146172	11171780
120%	0.3092400	13722462 13652652	13687557
150%	0.3865500	16882903 16884768	16883836
200%	0.5154000	22150335	22107914

FIGURE 1. LINEARITY OF BENDAMUSTINE HYDROCHLORIDE

Accuracy of Bendamustine hydrochloride

TABLE 3. ACCURACY OF BENDAMUSTINE HYDROCHLORIDE

Level solution for Accuracy	Amount added (conc. mg/ml)	Mean Area Response	Amount found (conc. mg/ml)	% of Recovery
80%	0.2016	8704999	0.20158	100.0
	0.2016	8707994	0.20165	100.0
	0.2016	8673314	0.20080	99.6
100%	0.2520	10808272	0.25276	100.3
	0.2520	10787913	0.25227	100.1
	0.2520	10798086	0.25251	100.2
120%	0.3024	12854829	0.30257	100.1
	0.3024	12891915	0.30347	100.4
	0.3024	12765480	0.30039	99.3
			Mean	100.0
			SD	0.35
			% of RSD	0.35

Demonstration of Precision:

TABLE 4. DEMONSTRATION OF PRECISION

Injection Number	Peak Area	Theoretical plates	Tailing factor
Injection – 1	10626548	26353	1.69
Injection – 2	10563880	26759	1.70
Injection – 3	10693966	25999	1.67
Injection – 4	10729380	28071	1.77
Injection – 5	10803995	27795	1.79
Injection – 6	10689694	27771	1.74
Mean	10684577	27125	1.73
Acceptance	< 2.0%	≥ 2500	< 2.5
Result	Pass (RSD – 0.78)	Pass	Pass

Injection Reproducibility for Bendamustine hydrochloride

TABLE 5. INJECTION REPRODUCIBILITY FOR BENDAMUSTINE HYDROCHLORIDE

S. No.	Injection Number	Retention Time	Peak Area
1	Injection – 1	29.64	10626548
2	Injection – 2	29.63	10563880
3	Injection – 3	29.64	10693966
4	Injection – 4	29.63	10729380
5	Injection – 5	29.63	10803995
6	Injection – 6	29.63	10689694
Mean		29.63	10684577
Acceptance		< 1.0%	< 2.5%
Result		Pass (RSD 0.02)	Pass (RSD 0.78)

Method Precision for Bendamustine hydrochloride**TABLE 6. METHOD PRECISION FOR BENDAMUSTINE HYDROCHLORIDE**

S. No.	Solution ID	Conc. (mg/ml)	Bendamustine hydrochloride Area	Peak	Bendamustine hydrochloride Area	Mean	% of Assay
01.	Standard	0.2518	10626548 10563880 10693966 10729380 10803995 10689694		10684577		*96.1
02.	Preparation 1	0.2496	10557376 10544400		10550888		95.7
03.	Preparation 2	0.2500	10613664 10553470		10583567		95.9
04.	Preparation 3	0.2504	10565237 10613981		10589609		95.8
05.	Preparation 4	0.2510	10572735 10701201		10636968		96.0
06.	Preparation 5	0.2490	10574537 10560173		10567355		96.1
07.	Preparation 6	0.2494	10580373 10574176		10577275		96.1
Mean							95.9
SD							0.16
% of RSD							0.17

Note: *Potency of Bendamustine hydrochloride drug substance/ working standard.

Statistical evaluation:**TABLE 7. STATISTICAL EVALUATION**

S. No	% of Level Solution	Concentration (mg/ml)	Mean Area	Standard Deviation
1	0.05	0.0001278	5423	56.6
2	0.10	0.0002556	10600	322.7
3	0.15	0.0003834	13505	119.9

FIGURE: 2 STATISTICAL EVALUATION **FIGURE 3. STATISTICAL EVALUATION**

Limit of detection value is at 0.00004165mg/ml and limit of quantisation is at 0.00012621mg/ml.

Solution Stability of Bendamustine hydrochloride**TABLE 8. SOLUTION STABILITY OF BENDAMUSTINE HYDROCHLORIDE**

Time Period at 5°C	Average Peak Area	Impurity Area%	RRT	Impurity ID	% of Initial
Initial	10626548	0.10	0.58	HP1	100.0
24 hrs	10586758	0.11	0.57	HP1	99.6
38 hrs	10678544	0.12	0.57	HP1	100.5

Acid Degradation of Bendamustine hydrochloride at 60°C**TABLE 9. SUMMARY OF ACID DEGRADATION OF BENDAMUSTINE HYDROCHLORIDE AT 60°C**

Time period	BDM Area %	Degradant RRT	Degradant area %	Degradant ID	BDM Peak Purity Angle	BDM Peak Purity Threshold
Initial	98.3	0.57	1.29	HP1	0.06	0.29
		0.79	0.21	Unknown		
		1.26	0.16	Unknown		
		1.30	0.05	Unknown		
60°C 1hr	71.7	0.22	2.15	HP2	0.07	0.29
		0.57	25.8	HP1		
		0.68	0.05	Unknown		
		0.79	0.12	Unknown		
		1.26	0.13	Unknown		
		1.30	0.06	Unknown		

Base Degradation of Bendamustine hydrochloride at 60°C

TABLE 10. SUMMARY OF BASE DEGRADATION OF BENDAMUSTINE HYDROCHLORIDE AT 60°C

Time period	BDM Area %	Degradant RRT	Degradant area %	Degradant ID	BDM Peak Purity Angle	BDM Peak Purity Threshold
Initial	24.4	0.22	13.3	HP2	0.21	0.41
		0.38	18.1	Unknown		
		0.42	2.16	Unknown		
		0.55	7.43	Unknown		
		0.57	16.1	HP1		
		0.58	0.05	Unknown		
		0.78	18.3	Unknown		
		1.06	0.08	Unknown		

Oxidative Degradation of Bendamustine hydrochloride at 60°C

TABLE 11. SUMMARY OF OXIDATIVE DEGRADATION OF BENDAMUSTINE HYDROCHLORIDE AT 60°C

Time period	BDM Area %	Degradant RRT	Degradant area %	Degradant ID	BDM Peak Purity Angle	BDM Peak Purity Threshold
Initial	96.1	0.57	3.24	HP1	0.07	0.29
		0.66	0.07	Unknown		
		0.79	0.51	Unknown		
		1.30	0.05	Unknown		
		0.21	0.06	Unknown		
		0.22	5.79	HP2		
60°C 1hrs	58.2	0.33	0.31	Unknown	0.09	0.30
		0.39	0.08	Unknown		
		0.56	0.19	Unknown		
		0.57	34.3	HP1		
		0.65	0.74	Unknown		
		0.79	0.18	Unknown		
		1.30	0.14	Unknown		

Hydrolysis Degradation of Bendamustine hydrochloride at 60°C

TABLE 12. SUMMARY OF HYDROLYSIS DEGRADATION OF BENDAMUSTINE HYDROCHLORIDE AT 60°C

Time period	BDM Area %	Degradant RRT	Degradant area %	Degradant ID	BDM Peak Purity Angle	BDM Peak Purity Threshold
Initial	94.5	0.22	0.05	HP2	0.06	0.28
		0.57	4.76	HP1		
		0.79	0.67	Unknown		
		1.30	0.06	Unknown		
60°C 1hrs	56.4	0.22	6.95	HP2	0.10	0.31
		0.39	0.12	Unknown		
		0.57	36.1	HP1		
		0.79	0.16	Unknown		
		0.86	0.05	Unknown		
		0.95	0.06	Unknown		
		1.30	0.18	Unknown		

Photo Stability Studies of Bendamustine hydrochloride

TABLE 13. SUMMARY OF PHOTO STABILITY STUDIES OF BENDAMUSTINE HYDROCHLORIDE

Sample ID	BDM Area %	Impurity Area %	RRT	Name of the Impurity	BDM Peak Purity Angle	BDM Peak Purity Threshold
Control in dark	99.8	0.11	0.57	HP1	0.06	0.28
		0.06	1.30	Unknown		
		0.17	0.27	Unknown		
		0.47	0.29	Unknown		
Uncovered	97.6	0.27	0.30	Unknown	0.07	0.29
		1.13	0.56	Unknown		
		0.09	0.57	HP1		
		0.17	0.67	Unknown		
Covered with Aluminium Foil	99.8	0.11	0.57	HP1	0.07	0.29
		0.06	1.29	Unknown		
Covered with Aluminium Foil and placed in Carton	99.8	0.11	0.57	HP1	0.06	0.28
		0.05	1.30	Unknown		

Forced Degradation Chromatograms:

FIGURE 4: CHROMATOGRAM OF BENDAMUSTINE HYDROCHLORIDE STANDARD

FIGURE 5: ACID DEGRADATION CHROMATOGRAM OF INITIAL SAMPLE

FIGURE 6: ACID DEGRADATION CHROMATOGRAM OF 60°C - 1HR SAMPLE

FIGURE 10. HYDROLYSIS CHROMATOGRAM OF INITIAL

FIGURE 7: BASE DEGRADATION CHROMATOGRAM OF INITIAL SAMPLE

FIGURE 11: HYDROLYSIS CHROMATOGRAM OF 60°C 1hr

FIGURE 8. OXIDATIVE DEGRADATION CHROMATOGRAM OF INITIAL SAMPLE

FIGURE12: PHOTO STABILITY STUDIES -CONTROL IN DARK

FIGURE 9. OXIDATIVE DEGRADATION CHROMATOGRAM OF 60°C - 1HR SAMPLE

FIGURE13. PHOTO STABILITY STUDIES -COVERED WITH ALUMINUM FOIL

FIGURE14: PHOTO STABILITY STUDIES –COVERED WITH ALUMINUM FOIL AND PLACED IN CARTON

FIGURE 15. PHOTO STABILITY STUDIES –UNCOVERED

CONCLUSIONS: System suitability conditions meet the recommended criterion. Linearity of the solution was demonstrated between 0.0002577 mg/ml and 0.51540 mg/ml concentration range. Accuracy was demonstrated as reported. Recovery and % RSD are within the recommended limits. Injection reproducibility was demonstrated and the % relative standard deviation for retention time and area were within the limits. Limit of detection value is at 0.00004165mg/ml and limit of quantisation is at 0.00012621mg/ml. Solution was stable up to 38hrs at 5°C temperature. Precision was demonstrated and the % relative standard deviation for assay was within the limits.

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Specificity was also demonstrated and there was no interference with the diluent & mobile phase. Under forced degradation studies, the peak purity angle is always lower than purity threshold for Bendamustine hydrochloride and all degradant peaks were well resolved. Hence the method can be adopted for the formulation screening studies as a stability indicating method.

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