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A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR THE ESTIMATION OF BENDAMUSTINE HYDROCHLORIDE FOR INJECTION

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ABSTRACT

A simple, accurate and precise stability indicating RP-HPLC method has been developed and subsequently validated for quantitative estimation of Bendamustine Hydrochloride for injection. The proposed method is done by using column Zorbax SB-C18,5µ,250x4.6mm maintained at 8 ºC temperature. The optimum mobile phase consisted of ACN and triflouro acetic acid buffer in which mobile phase A is in the ratio of 90:10 v/v and mobile phase B in the ratio of 50:50 v/v, effluent flow rate monitored at 1.0 ml/min and UV detection was done at 230 nm by diode array detector. The method was validated according to ICH guidelines. It was found to be linear, precise, accurate and reproducible. Bendamustine Hydrochloride for injection was exposed to acid, alkali, hydrogen peroxide, thermal and UV conditions, then the stressed samples were analyzed by the proposed method. There were no interfering peaks from degradation products and impurities due to variable stress conditions and the proposed method is specific for the quantitative estimation of Bendamustine Hydrochloride for injection in the presence of their degradation products also. The method can be successfully applied in quality control of bulk manufacturing.

Keywords: Bendamustine Hydrochloride; Quantitative estimation; Forced degradation studies; RP-HPLC

1. INTRODUCTION

Bendamustine Hydrochloride (TREANDA) an alkylating drug, contains a mechlorethamine group and a benzimidazole heterocyclic ring with a butyric acid substituent[1]. It is chemically, 1H-benzimidazole-2-butanoic acid, 5-[bis(chloroethyl)amino]-1methyl-monohydrochloride with a molecular formula $C_{16}H_{21}Cl_2N_3O_2 \cdot HCl$. Its molecular weight is 394.7, white to off-white lyophilized powder. Its melting point is149-151ºC. It is freely soluble in water and methanol. It is used as for Chronic Lymphocytic Leukemia and Non-Hodgkin's Lymphoma.[2,3] 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 (Bendamustine hydrochloride) for Injection is intended for intravenous infusion only after reconstitution with Sterile Water for Injection, USP, and after further dilution with either 0.9% Sodium Chloride Injection, USP, or 2.5% Dextrose/0.45% Sodium Chloride Injection, USP. It is supplied as a sterile non-pyrogenic white to off-white lyophilized powder in a single-use vial. Each 25-mg vial contains 25 mg of Bendamustine hydrochloride and 42.5 mg of Mannitol, USP. Each 100-mg vial contains 100 mg of Bendamustine hydrochloride and 170 mg of mannitol, USP. The pH of the reconstituted solution is 2.5 - 3.5. Literature survey reveals that so far no method has been reported for the stability indicating Bendamustin HCl[4]. Hence, there was a need to develop an stability indicating RP-HPLC method for the routine estimation of Bendamustine Hydrochloride and the method is validated in accordance with USP and ICH guideline [5, 6] for the assay of active ingredients and their related substances[7] The chemical structure of Bendamustine HCl is given in **Figure 1**.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The working standard and sample of Bendamustine HCl, Bendamustine Hydrochloride for injection sample and Placebo were procured from strides Arcolab. HPLC grade acetonitrile, methanol were purchased from Merck India

Pvt Ltd. HCl, trifluoroacetic acid, Hydrogen peroxide, sodium hydroxide obtained from SD fine chemicals and water from MilliQ system.

2.2 Instruments and Glassware

The HPLC systems Agilent 1100 and 1200 series consisting of Quaternary Pump (G1311A), Degasser (G1379A/G1322A), Auto sampler (G1313A/G1329A), Thermo stated column compartment (G1316A), Diode Array Detector (G1314A/G1315B), Variable wavelength detector (G1317), Cooler (G1330B), software Chemstation (Ver. 3.02) and Chromalean were used to carry out RP-HPLC with gradient elution and UV detection. Balance with sensitivity of 0.01mg and microbalance (B-004), Digital pH meter (pHM-04).Borosilicate class-A grade general glass wares used throughout the analysis

2.3 Preparation of Solutions:

2.3.1 Standard solution:

5.0mg of Bendamustine HCl Standard was accurately weighed and transferred into a 10ml volumetric flask, dissolved and diluted to volume with methanol to obtain 0.5mg ml-1

2.3.2 Sample solution:

2 sample vials was taken and reconstituted each vial with 20ml of water and transferred whole reconstituted solution into a 200 ml volumetric flask, dissolved and diluted to volume with water. Diluted

5.0ml of this solution to 10ml with methanol to obtain 0.5mg ml-1

2.4 Procedure:

Standard and Sample solutions were separately injected (10 µL) into the chromatograph, the chromatograms were recorded and the area for the major peaks was measured.

Optimized chromatographic conditions

The analytical technique was developed by using a column Zorbax SB-C$_{18}$, 5µ, 250 x 4.6 mm using diode array detector at a wavelength of 230 nm with a flow rate of 1.0 ml/min. Mobile phase used is a binary gradient. The Injection volume was 10 µl and the retention time of Bendamustine HCl was found to be 25.00 minutes with a total Run time of 60 minutes. The standard and sample chromatogram was shown in **Figure 2 and 3**.

4.5 Mobile phase preparation

Preparation of Buffer: Transferred 1ml of trifluoroacetic acid in 1000ml volumetric flask containing 500ml of water, mixed well and diluted to volume with water.

Mobile phase A: Prepared a mixture of Buffer solution and acetonitrile in the ratio of 90:10, filtered and degassed.

Mobile phase B: Prepared a mixture of Buffer solution and acetonitrile in the ratio of 50:50, filtered and degassed.

3. RESULTS AND DISCUSSION

Validation of RP-HPLC method for determination of Bendamustine HCl [100mg/vial]. The method was validated in accordance with ICH guidelines for system suitability, specificity, system precision, method precision, intermediate precision, linearity, limit of detection, limit of quantitation, accuracy, range, robustness.

3.1 System suitability:

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. It is performed by injecting the blank solution, 5 injections of standard solution and checked the system suitability parameters and the result obtained was 0.6%.

3.2 Specificity:

Specificity is the ability of analytical method to assess unequivocally analyze in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. The specificity parameter of the developed method was observed by injecting blank (methanol), placebo, standard solution and sample solution into HPLC system. The diluent, placebo, peaks were not interfering with Bendamustine HCl peak. Recorded the retention time of blank, placebo, and Bendamustine HCl standard and sample peak and shown in **Table 1**.

3.3 Forced degradation:

The forced degradation of sample was carried out as per ICH guidelines in acid, alkali, hydrogen peroxide, neutral, sunlight, UV and thermal. The acid, alkali, hydrogen peroxide, neutral studies were carried out by heating at 80 ºC for 2 hours in water bath with 3ml of 0.1N HCl, 0.1N NaOH, 3% Hydrogen Peroxide and 3ml water respectively. The sample vials was exposed to sunlight for 8hrs, and UV light at 254nm and 365nm for 8hrs. The sample was kept at 80 C for 3hrs in oven to carry out thermal stress studies. All the stress results with purity factor were shown in Fig.4 to Fig.9 and in **Table 2 and Table 3**.

3.4 Precision:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurements.

System precision: The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and area of six determinations should be measured and percentage RSD should be calculated. Six replicate of standard

preparation were injected. The retention time, area responses were recorded and RSD was calculated and shown in **Table 4**.

3.5 Method precision: In method precision, a homogenous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results for a single batch. Six replicate of sample preparation were injected. The percentage recoveries were recorded and RSD was calculated and shown in **Table 5**.

3.6 Intermediate precision: The intermediate precision should be carried out to ensure that the analytical results will remain unaffected with change in instrument, analyst, column and day. The method was repeated by different analyst using different lot of column, different system on different day for intermediate precision studies, percentage recoveries and RSD were recorded. **Table 6**.

3.7 Linearity:

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The linearity studies were performed in the range of 50% to 150% of the working concentration. From the statistical treatment of the linearity data of Bendamustine HCl, it is clear that the

response of Bendamustine HCl is linear between 50% to 150% level of working concentration. The correlation coefficient and Regression coefficients were greater than 0.998. In addition, the analysis of residual shows that the values are randomly scattered around zero, the p-value was determined. The P value is >0.05 then the intercept is statistically equal to zero for Bendamustine. P value of Bendamustine is 0.16. Hence it is statistically equal to zero. In addition the origin is within the lower and the upper limit of the 95 % confidence interval that gives high degree of confidence to the value obtained for intercept. Moreover, the value of the % intercept is within ± 2 % of the area response at 100 % level and shown in **Figure 11 and Table 7**.

3.8 Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. The accuracy of the method was evaluated through the recovery test of known amount of Bendamustine HCl which is spiked into the placebo. These studies were performed in triplicate on three different levels at 50%, 100% and 150% to the placebo. Mean % recovery of Bendamustine HCl at each level should be

between 97.0 % and 103.0 %. The table was shown in **Table 8**.

3.9 Robustness: The robustness of an analytical method 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. The Robustness parameters are carried out by Change in column temperature $\pm5^{\circ}C$, flow rate ±0.2ml/min and organic phase ratio $\pm2\%$. From the results it was concluded that the method is robust.

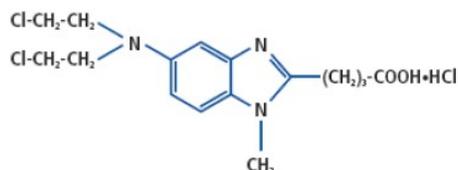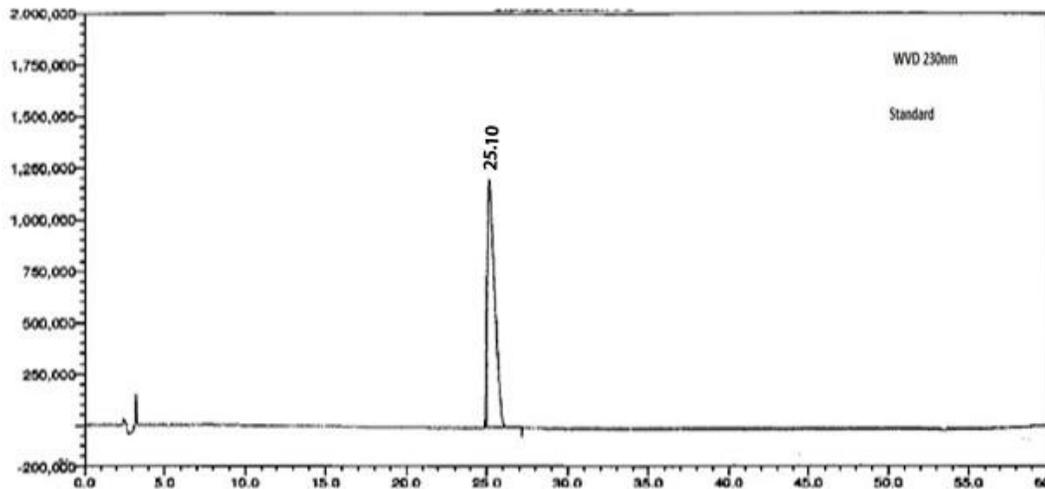**Figure 1: Chemical structure of Bendamustine HCl**
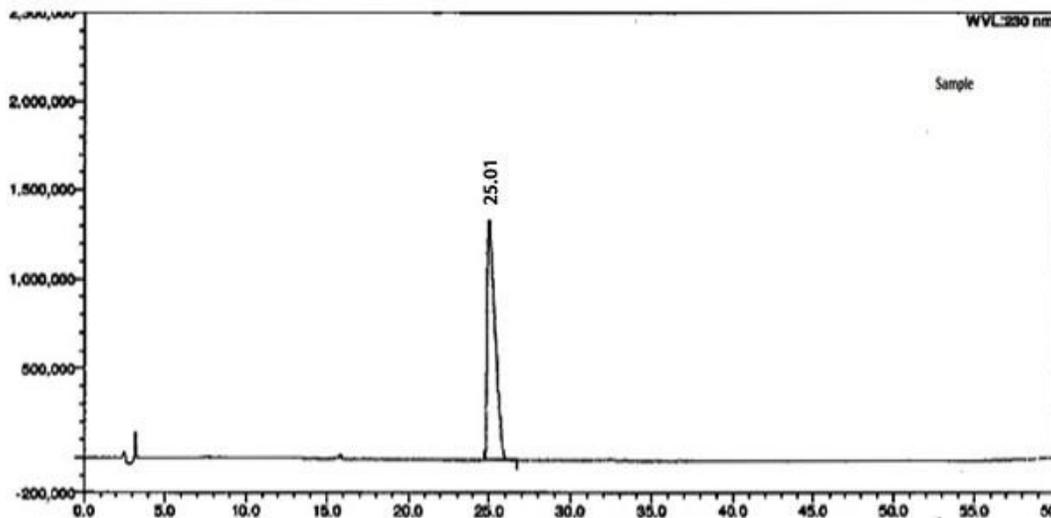

Figure 2: Chromatogram of Standard

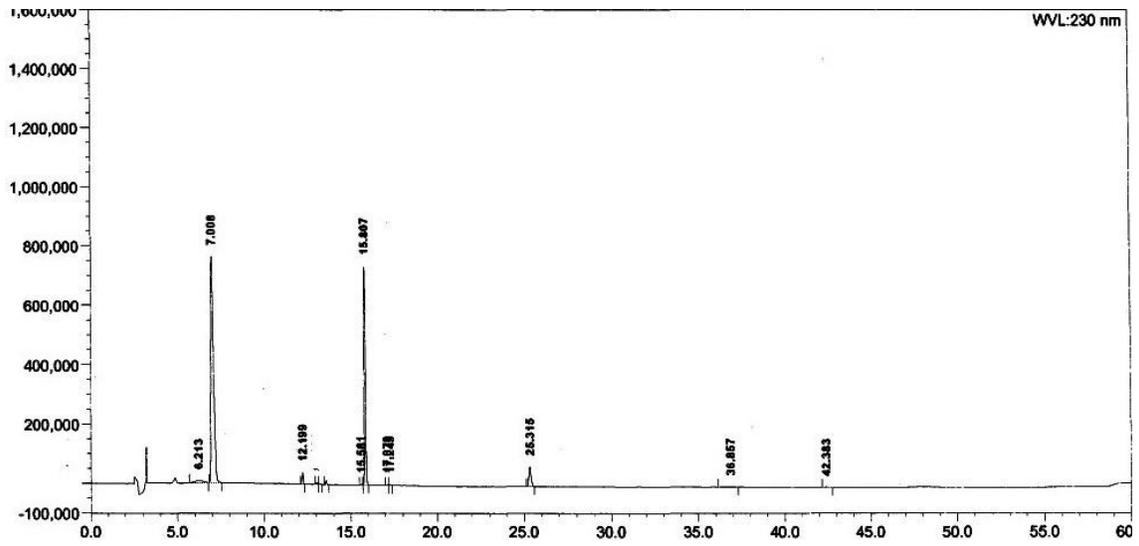
Figure 3: Chromatogram of Sample

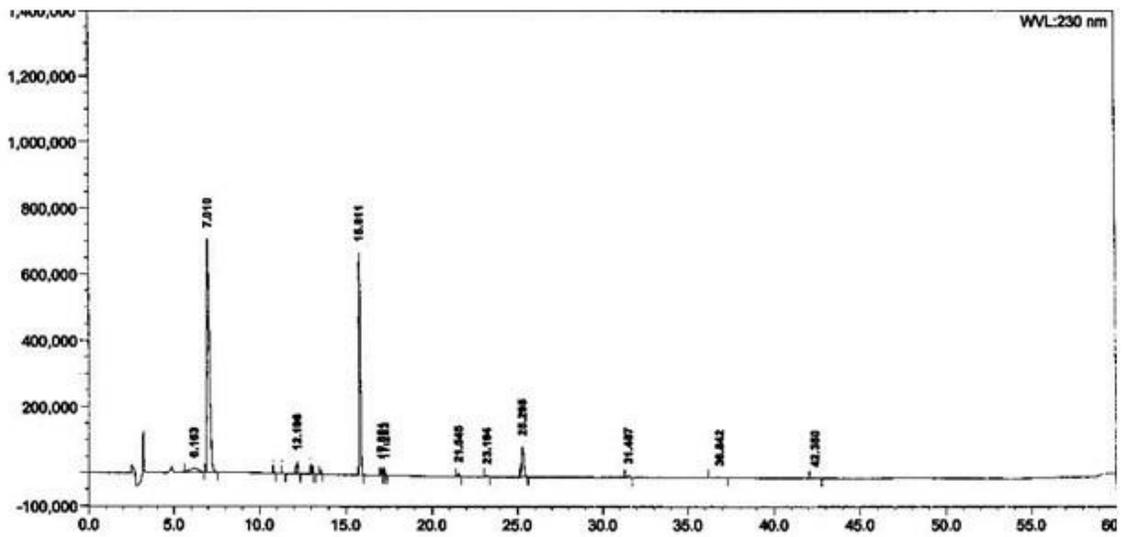**Figure 4: Chromatogram of Acid stressed sample**

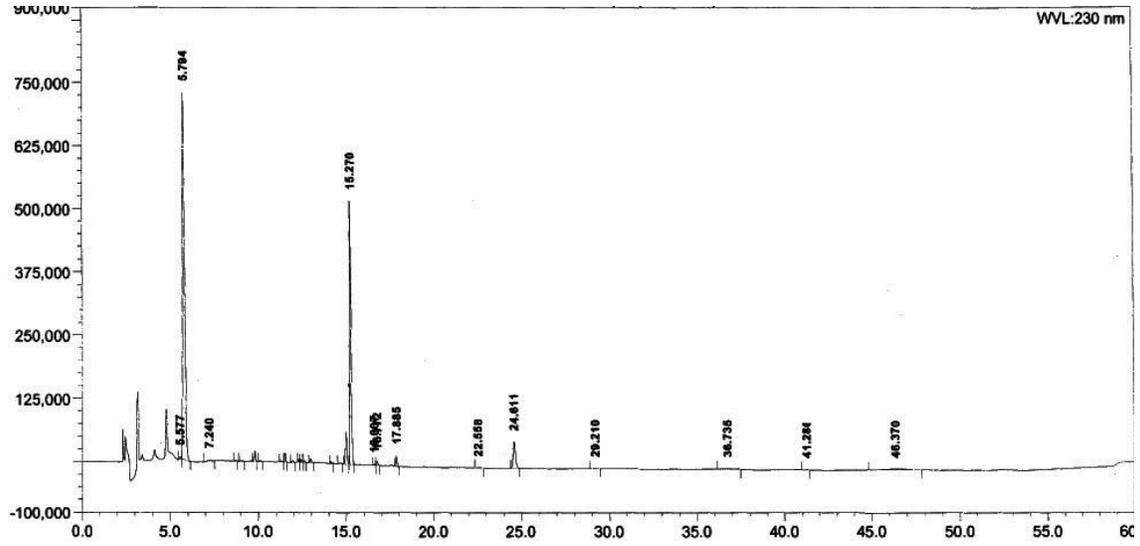**Figure 5: Chromatogram of Alkali stressed sample**

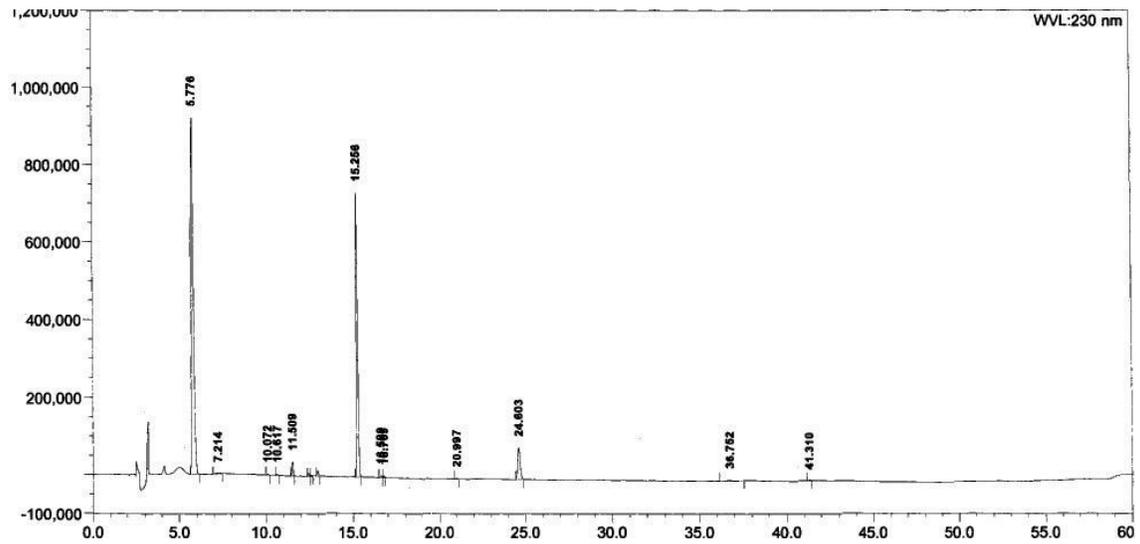**Figure 6: Chromatogram of Hydrogen peroxide stressed sample**

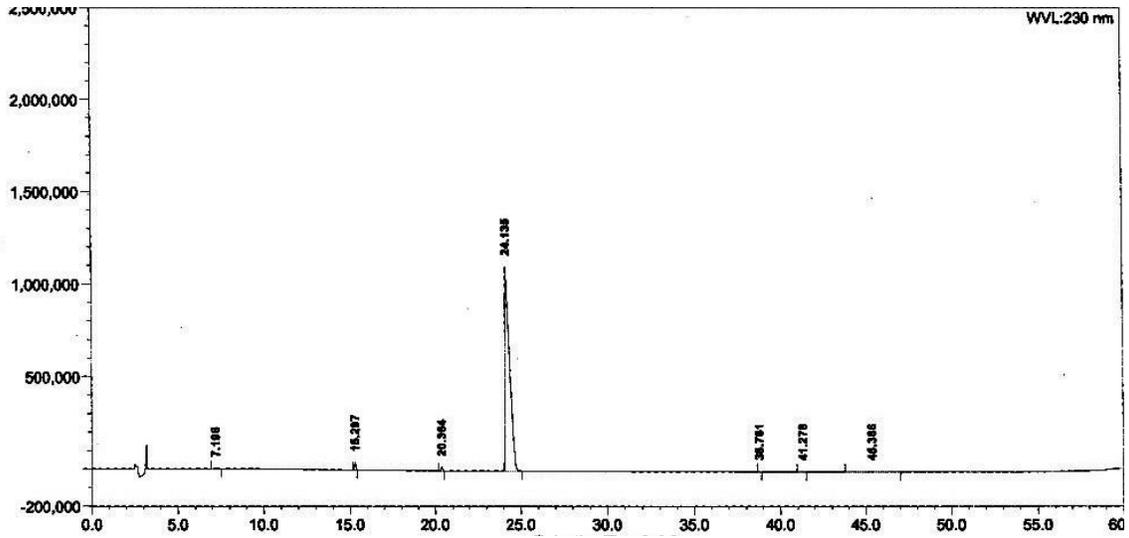**Figure 7: Chromatogram of Neutral stressed sample**

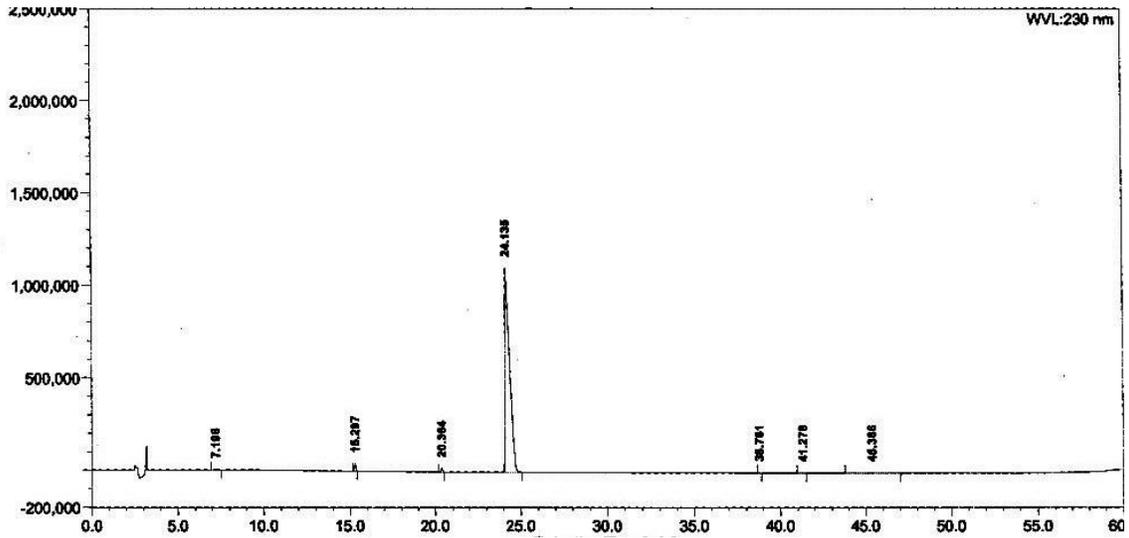**Figure 8: Chromatogram of sunlight stressed sample**
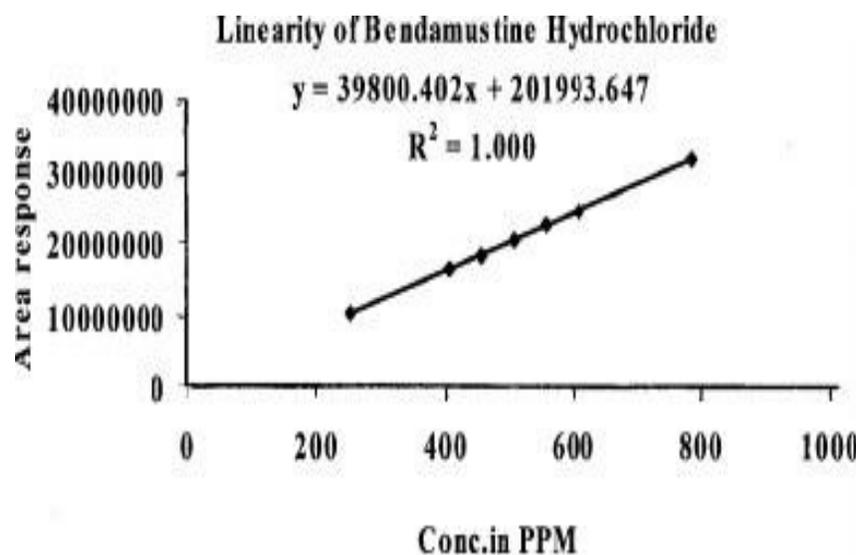

Figure 9: Chromatogram Thermal stressed sample

Figure 10: Linearity of Bendamustine Hydrochloride

Table 1: Retention time of blank, placebo, Bendamustine HCl standard and sample solution

SOLUTIONS	BLANK (Diluent)	PLACEBO	TREANDA Standard	TREANDA Sample
Retention Time(min)	2.3	2.4	25.10	25.01

Table 2: Results of sample with heating

Sl no	Samples Condition	**Peak purity	Peak factor	%Assay
1	Sample as such	P	999	102.7%
2	0.1N HCl	P	998	3.1%
3	1 N HCl	P	999	73.4%
4	0.1N NaOH	P	997	4.3%
5	1 N NaOH	P	999	4.2%
6	3%H2O2	P	999	2.3%
7	Neutral	P	998	3.7%
8	Sunlight	P	999	100.8%
10	Thermal	P	999	97.7%

Table 3: Results of sample without heating

SL. NO	Samples condition	**Peak purity	Purity factor	%Assay
1	0.1N HCl	P	999	97.3
2	1 N HCl	P	999	95.5
3	0.1 N NaOH	P	999	92.9
4	1 N NaOH	P	999	101.2
5	3% H2O2	P	999	96.9

Table 4: Results of system precision studies

RT (min)	25.007	25.025	25.021	25.016	25.022	25.052	25.024	0.1%
Area	19388280.3	19515875.6	19255099.5	19283217.4	19259116.2	19138569.6	19306693.1	0.7%

Table 5: Results of method precision studies

S. no.	1	2	3	4	5	6	Mean	%RSD
% Assay	103.1	103.0	102.3	102.5	102.0	102.2	102.5	0.4%

Table 6: Results of Intermediate precision studies

S. no.	1	2	3	4	5	6	Mean	%RSD
% Recovery	102.4	102.5	102.5	102.3	102.2	102.3	102.4	0.1%

Table 7: Results of Linearity studies

S. No.	Level	Standard stock solution added (in ml)	Bendamustine HCl Conc in PPM	AREA
1.	50%	1.00	250	10353458.448
2.	80%	1.60	400	16381763.542
3.	90%	1.80	450	18305068.728
4.	100%	2.0000	500	20200824.253
5.	110%	2.20	550	22396957.028
6.	120%	2.40000	600	24476711.958
7.	150%	3.10	750	31507346.113
Slope	**Intercept**	**%Intercept**	**Correlation coefficient**	**Regression coefficient**
39800.402	- 201993.647	1.0%	1.000	1.000

Table 8: Accuracy sample level at 50%, 100% and 150%

Sets	Levels (about)	mg added	mg recovered	Area Response	% Recovery	Mean % Recovery	% RSD
1	50%	47.49	47.13	9164267.865	100.4	100.3	0.2
2	50%	47.51	47.14	9151844.594	100.3		
3	50%	47.68	47.12	9138006.434	100.1		
1	100%	94.98	94.49	18146860.684	99.4	99.5	0.2
2	100%	94.96	94.46	18188508.978	99.7		
3	100%	94.97	94.47	18136993.916	99.4		
1	150%	142.47	140.87	27197934.788	99.4	99.0	0.4
2	150%	142.48	140.88	27004174.082	99.6		
3	150%	142.46	140.86	27067505.222	99.9		
				Across all levels		99.6	0.3

4. CONCLUSION

The proposed stability-indicating RP-HPLC method was validated as per ICH guidelines. The method was found to meet all the predetermined acceptance criteria. The validated HPLC method for Bendamustin HCl is specific, stable, linear, accurate, precise, rugged and robust. Based on the validation study results, it has been concluded that the HPLC method for Bendamustin HCl is suitable for intended purpose. The chromatogram of diluent (blank) indicates that there is no interference at the retention time Bendamustin HCl. The peak purity angle was less than the peak purity threshold and there was no tick mark in the purity flag column for Bendamustin HCl peak in all final stressed test solutions. Peak purity plot of stressed sample solutions indicates that Bendamustin peak is homogeneous and has no co-eluting peaks ensuring specificity of the method. Hence it can be successfully applied to perform the routine analysis of the drug in pharmaceutical formulations.

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