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Development and validation of RP-HPLC method for determination of related substances of bendamustine hydrochloride in bulk drug

E. Sasi Kiran Goud* and V. Krishna Reddy

Department of Chemistry, Sri Krishna Devaraya University, Anantapur, India

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ABSTRACT

*Three known impurities in Bendamustine Hydrochloride bulk drug were detected by a simple, sensitive and accurate gradient reverse phase high performance liquid chromatography (HPLC). These impurities were isolated from crude sample of Bendamustine Hydrochloride using reverse phase HPLC. The IUPAC names of impurities were Impurity-A is 4-6-[2-chloro ethyl)-2-hydroxy ethyl amino)3 methyl-benzimidazolyl-2)-butyric acid (HP-1),Impurity-B is 4-(-5-[(4-5-(bis(2-hydroxyethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoic acid and Impurity - C Isopropyl 4-(5-(bis(2-chloroethyl)amino-1-methyl-1-H-benzol[d]imidazol-2yl)butanoate. The effective separation was achieved on a Zorbax SB-$C_{18;}$ (4.6 mm x 25 cm) 5µm using a gradient mode by the Mobile phase A **:** 0.1% Trifluroacetic acid in Water**:** Acetonitrile (90:10) and mobile phase B : 0.1% Trifluroacetic acid in Water**:** Acetonitrile (50:50). The flow rate of the mobile phase was 1.0 mL/min and the total elution time, including the column equilibration was approximately 60.01minutes.The retention times of Bendamustine Hydrochloride and its impurities are 24.109,24.109,15.309,30.521 and 38.638. The UV detection wavelength was carried at 230 nm and experiments were conducted at 30°C. The developed method was validated in terms of system suitability, selectivity, linearity, range, precision, accuracy, limits of detection and quantification for the impurities following the ICH guidelines. Therefore, the proposed method was suitable for the simultaneous determination of Bendamustine Hydrochloride and its three related impurities.*

Key Words: Bendamustine hydrochloride, Impurities, Method Development, Zorbax SB-$C_{18,}$ and RP-HPLC.

INTRODUCTION

Novel water soluble polymer complexes of Bendamustine hydrochloride, a bifunctional alkylating agent with antimetabolic and cytotoxic activity[1]. The IUPAC name of Bendamustine hydrochloride was benzimidazole-2-butanoic aicd,5-[bis(2-chloroethyl)amino]-1- methyl- monohydrochloride ($C_{16}H_{21}Cl_2N_3O_2$).It is used to treat a type of cancer of the white blood cells called chronic lymphocytic leukemia (CLL)[2].It contains a mechlorethamine group and a benzimidazole heterocyclic ring with a butyric acid substituent. Mechlorethamine and its derivatives form electrophilic alkyl groups. These groups form covalent bonds with electron-rich nucleophilic moieties, resulting in interstrand DNA crosslink's. Bendamustine displays a distinct pattern of activity unrelated to other DNA-alkylating agents. Its mechanisms of action include activation of DNA-damage stress response and apoptosis, inhibition of mitotic checkpoints, and induction of mitotic catastrophe. In addition, unlike other alkylators, Bendamustine activates a base excision DNA repair pathway rather than an alkyltransferase DNA repair mechanism[3].The purpose of this method ,there was RP-HPLC methods and Spectroscopy methods were cited in the literature for the assay of Bendamustine Hydrochloride in bulk drug[4-7]. But there is no single method HPLC

method for the simultaneous determination of Bendamustine and three of its related impurities. The development and validation of RP- HPLC method for the simultaneous determination of Bendamustine and three of its related impurities as per ICH guidelines [8,9,10]. The unique feature and the novelty of the proposed method is that it is the first time that these thee impurity compounds were determined simultaneously with accurate, precise and sensitive. The chemical structure of Bendamustine Hydrochloride shown in the Fig:1

MATERIALS AND METHODS

Instrumentation and software:
Agilent 1200 series with high pressure liquid chromatographic instrument provided with Auto sampler and VWD UV detector, thermostatted column compartment connected with EZ Chrom software .

Chemicals and reagents:
All the reagents were of analytical reagent grade unless stated otherwise. Distilled and de-ionized HPLC-grade water,HPLC-grade Acetonitrile, Trifluroacetic acid and methanol were purchased from Merck, Mumbai . Samples of Bendamustine Hydrochloride and its impurities are gift sample of Bio-Leo labs Hyderabad.

Chromatographic Conditions:
The effective separation was achieved on a Zorbax SB-$C_{18;}$ (4.6 mm x 25 cm) 5µm using a gradient mode by the Mobile phase A : 0.1% Trifluroacetic acid in Water: Acetonitrile (90:10) and mobile phase B : 0.1% Trifluroacetic acid in Water: Acetonitrile (50:50). The flow rate of the mobile phase was 1.0 mL/min and the total elution time, including the column equilibration, was approximately 60.01minutes. The UV detection wavelength was carried at 230 nm and experiments were conducted at 30°C.The gradient program given in Table : 1.

Preparation of Standard Solutions:
Weigh and transfer 20.0 mg of Bendamustine Hydrochloride standard into a 20 mL volumetric flask, dissolve and dilute to volume with methanol. Further dilute 2.0 mL of this solution to 100.0 mL with diluent. Further, dilute 2.0 mL of this solution to 20.0 mL with diluent.

Preparation of sample solutions:
Weigh and transfer 25.0 mg of Bendamustine Hydrochloride standard into a 25 mL volumetric flask, dissolve and dilute to volume with methanol.

Method validation:
Validation of the developed method for the determination of Bendamustine Hydrochloride and the three impurities was performed according to the ICH guidelines with standards, bulk drug. Thus, system suitability along with method selectivity, specificity, linearity, range, precision (repeatability and intermediate precision),accuracy, limits of detection and quantification for the impurities.

System suitability:
The system suitability was conducted using diluted standard preparation and evaluated by injecting six replicate injections.

Specificity:
Specificity is the ability of analytical method to assess un equivocally the analyte in the presence of component that may be expected to be present, such as impurities, degradation products and matrix components. Performed the specificity parameter of the method by injecting Diluent, Standard preparation, , Sample preparation, Sample spiked with impurities, Impurity-A, Impurity-B and Impurity-C into the chromatographic system and evaluated by making three replicate injections.

Linearity and range:
The linearity of Bendamustine hydrochloride impurities was also studied by preparing standard solutions at 16 different levels. 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. Performed the linearity with Bendamustine Hydrochloride standard and impurities in the range of LOQ to 300% of specification limit. Recorded the area response for each level and calculated slope, intercept & correlation

coefficient. Tested the intercept for statistical equivalence to zero. Also performed precision at higher level by injecting six times into the chromatographic system.

Precision and Accuracy:
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. The system precision was conducted using all the impurities spiked to Bendamustine and evaluated by making six replicate injections. The Accuracy of the method by recoveries of all the impurities was determined by analyzing Bendamustine hydrochloride sample solutions spiked with each impurity at three different concentration levels ranging from 50% to 300%.

LOD and LOQ:
The LOD and LOQ were determined for Bendamustine hydrochloride and each of the impurities based on the standard deviation of (SD) of the response and slope (S) of the regression line as per ICH guidelines according to the formulae given below.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions:
Method development includes selection of appropriate chromatographic conditions/factors like detection wave length, selection and optimization of stationary and mobile phases. The longer wavelength of 230 nm was selected since it produces less noise, which minimizes problems that may exhibit around the active ingredient when attempting to quantify Bendamustine hydrochloride and its three process related impurities- A,B and C. Moreover, both the process related impurities are also detected satisfactorily at the same wavelength and hence it is selected as detection wavelength. Preliminary development trials were performed with various octadecyl columns (C18 columns) of different types and dimensions from different manufacturers were tested for the peak shape and the number of theoretical plates for Bendamustine hydrochloride raw material at 100 µg/mL concentration. Finally by switching to Zorbax SB-$C_{18;}$ (4.6 mm x 25 cm) 5µm column there was a substantial increase in the theoretical plates with a significant improvement in the peak shapes with 1.19 tailing factor. It also produced adequate resolution between Bendamustine hydrochloride and its three process related impurities.

Method validation:
System suitability:
The RSD from six replicate injections of diluted standard preparation was 0.8 %.The quantities of impurities and assay of Bendamustine Hydrochloride were calculated from their respective peak areas. System suitability data is given in Table-2

Selectivity:
For selectivity determination, all the known impurities were added to Bendamustine hydrochloride the response of each analyte in the mixture was compared with that of Bendamustine hydrochloride. During the selectivity studies, the retention times of individual standard solutions are coincide with the retention times of peak response obtained from the spiked sample solutions.The Selectivity of Bendamustine and its impurities Shown in the table : 3. The typical selectivity chromatograms are shown in Figure-2.

Linearity and range:
Peak areas of each compound were measured and used for quantification. Performed the linearity with Bendamustine Hydrochloride standard and impurities in the range of LOQ to 300% of specification limit. Plotted a graph of Bendamustine Hydrochloride standard and Impurities concentration (ppm) on X-axis and Area responses on Y-axis. The correlation & regression coefficients are more than 0.995. The P-value is >0.05. the origin was within the lower and upper limit of the 95% CL that gives high degree of confidence to the value obtained for intercept. Moreover, the value of intercept is within ± 5% of the area response at 100% level. Precision at higher level RSD was NMT 5.0%. .Linearity data of Bendamustine hydrochloride and its relative impurities given Figure 2.

Precision:
The repeatability and system precision were expressed as the percent relative standard deviation (% RSD) of each analyte concentration. The RSD of the Retention time for peaks obtained from six injections of Standard preparation was Zero. The RSD of the Area response for peaks obtained from six injections of Standard preparation was 0.8.The system precision, method precision data is given in Table-4 and 5.

Accuracy:
Accuracy of the method by recoveries of all the impurities was determined by analyzing Bendamustine hydrochloride sample solutions spiked with each impurity at three different concentration levels ranging from 50% to 300% in triplicate with respect to specified limit. The % recoveries of Bendamustine hydrochloride 98.1%.

Tables : Table 1:The gradient program

Time (Minutes)	Solution A (%)	Solution B (%)
0	100	0
3	100	0
16	50	50
33	30	70
35	10	90
50	10	90
55	100	0
60	100	0
60.01	STOP	

Table2: System suitability data of Bendamustine hydrochloride and related impurities.

Peak Name	LOQ (in %)	LOD (in %)	RRF	RRT
Bendamustine Hydrochloride	0.030	0.010	1.00	1.00
Impurity-A	0.024	0.008	1.17	0.64
Impurity-B	0.040	0.013	1.35	1.27
Impurity-C	0.024	0.008	0.97	1.57

Table 3: Selectivity of Bendamistine and Its related impurities

Solutions	Retention time (in min.)
Blank(Diluent)	-
Impurity-A	15.309
Impurity-B	30.521
Impurity-C	38.638
Bendamustine Hydrochloride	24.109

Table 4: system Precision study of Bendamustine hydrochloride and impurity-A,B and C

Injection No.	Bendamustine Hydrochloride	
	Retention Time	Area response
1	24.833	88860
2	24.840	88250
3	24.841	87923
4	24.828	86902
5	24.824	87794
6	24.825	87359
Mean	24.832	87848
% RSD	0.0	0.8

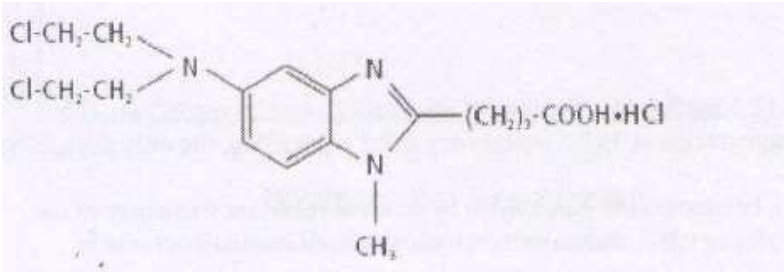
Table 5: Method Precision study of Bendamustine Hydrochloride and impurity-A,B and C

Sample set	Impurity A	Impurity B	Impurity C
1	0.181	0.176	0.166
2	0.180	0.178	0.168
3	0.180	0.178	0.164
4	0.180	0.176	0.164
5	0.180	0.177	0.162
6	0.180	0.176	0.164
Mean	0.180	0.177	0.165
% RSD	0.2	0.6	1.3

Table 6: LOQ and LOD of impurities with respect to Bendamustine Hydrochloride.

Parameter	Un Known	Impurity-A	Impurity-B	Impurity-C
LOD (ppm)	0.01	0.079	0.127	0.075
LOQ (ppm)	0.306	0.239	0.383	0.226
LOD(%) with respect to sample conc.	0.001	0.007	0.01	0.007
LOQ (%)with respect to sample conc.	0.03	0.02	0.03	0.02

Fig : 1 Bendamustine Hydrochloride (benzimidazole-2-butanoic aicd,5-[bis(2-chloroethyl)amino]-1- methyl-monohydrochloride($C_{16}H_{21}Cl_2N_3O_2$).

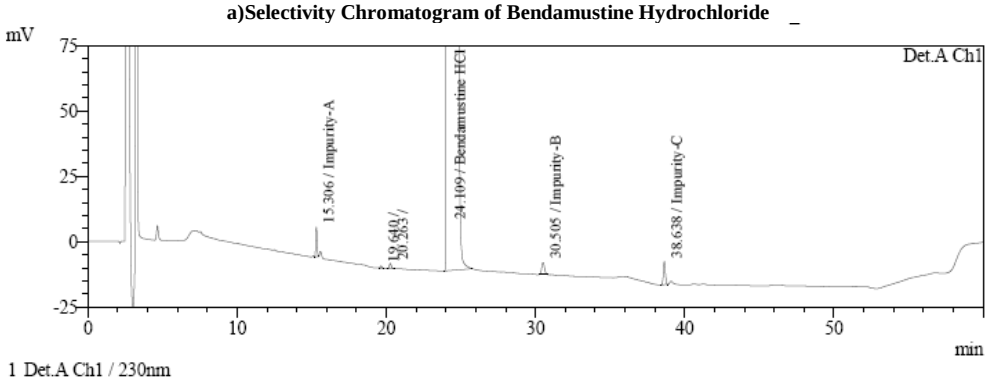


LOD and LOQ:

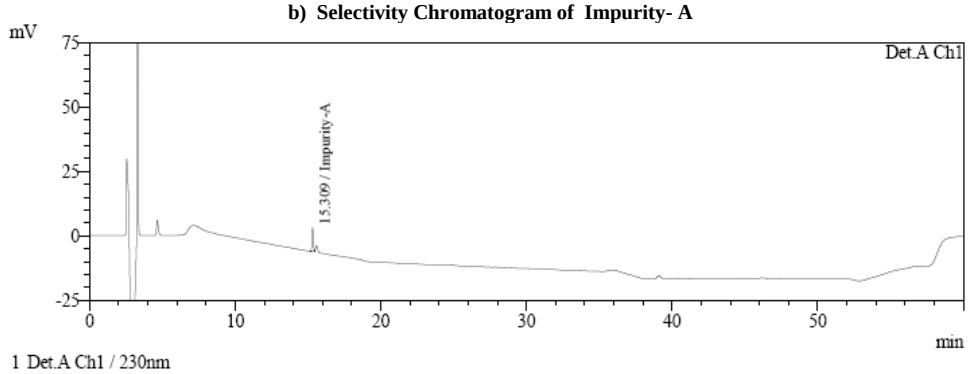
The LOD and LOQ for the impurity-A was found to be 0.007% and 0.02 % respectively. Similarly, the limit of detection (LOD) and limit of quantification (LOQ) for the impurity-B and impurity C were found to be 0.01% and 0.03%,0.007% and 0.02 % respectively. The concentrations of LODs and LOQs were verified for precision by the analysis of solutions having Bendamustine hydrochloride and its impurities at these levels in six replicates and found to be below 10.0% RSD. The LOD and LOQ data given table 6.

Figure 2: Selective Chromatograms of Bendamustine and Its related impurities.

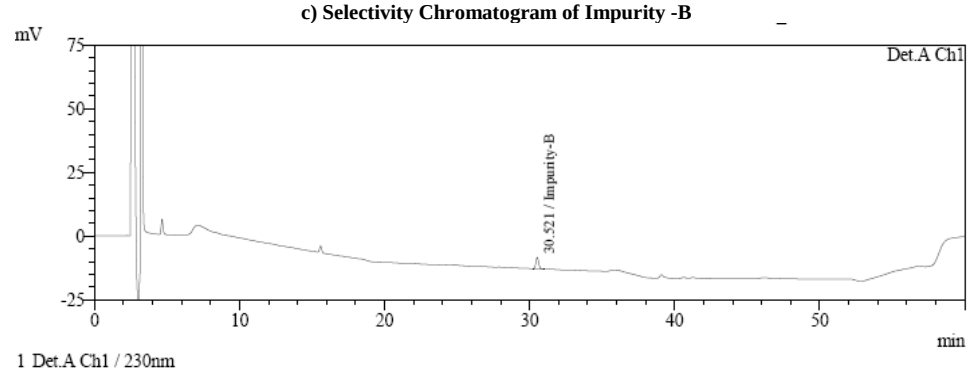
a)Selectivity Chromatogram of Bendamustine Hydrochloride



b) Selectivity Chromatogram of Impurity- A



c) Selectivity Chromatogram of Impurity -B



d) Selectivity Chromatogram of Impurity- C

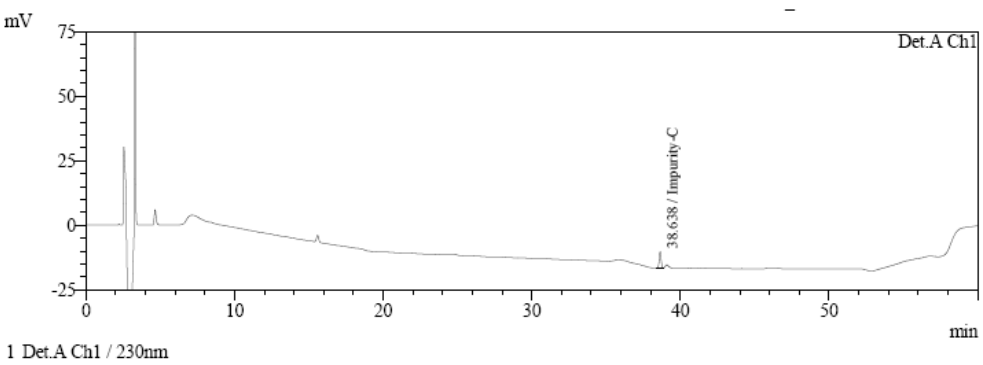
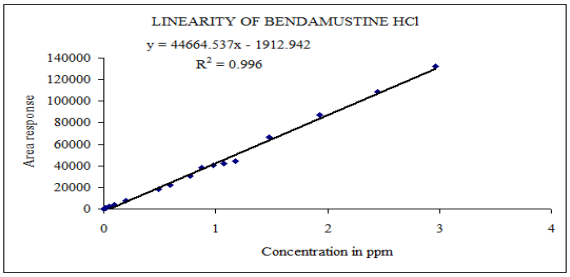
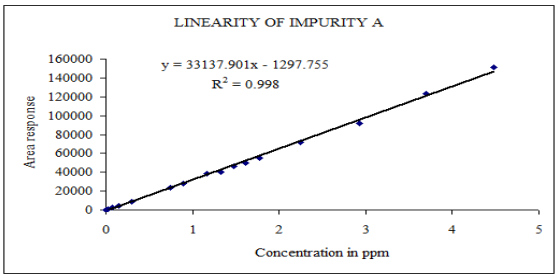


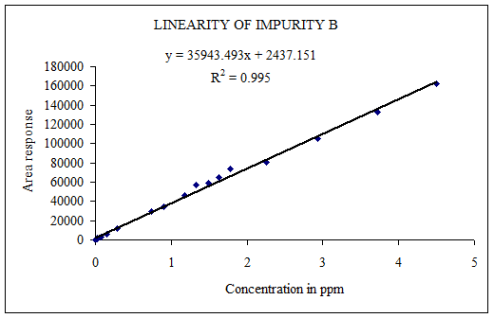
Figure 3: Linearity data of Bendamustine and its impurities



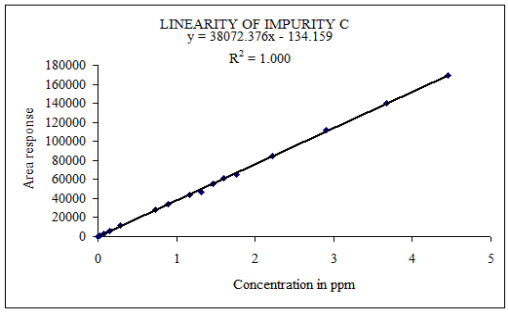
Regression Statistics	
Multiple R	0.998
R Square	0.996
Adjusted R Square	0.995
Standard Error	2581.18
Observations	17



Regression Statistics	
Multiple R	0.998
R Square	0.997
Adjusted R Square	0.997
Standard Error	2318
Observations	17



Regression Statistics	
Multiple R	0.997
R Square	0.995
Adjusted R Square	0.995
Standard Error	3575.85
Observations	17



Regression Statistics	
Multiple R	1.000
R Square	1.000
Adjusted R Square	1.000
Standard Error	781.20
Observations	17

CONCLUSION

The liquid chromatographic method with gradient elution developed for the simultaneous determination of Bendamustine hydrochloride and its three related impurities A,B and C in the bulk drug, was fully validated and proved to be reliable, sensitive, accurate, precise .The method has higher sensitivity towards the determination of impurities and it is the first time that such method appears in the literature and can be useful for routine analysis and quality control of Bendamustine hydrochloride in the relevant forms. The developed method was found to be accurate, precise, specific and linear. Thus, the method can be used for quality assurance of Bendamustine hydrochloride in bulk drugs and it can extend to validate the pharmaceutical formulations.

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