Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2016, 8 (12):183-192
(http://scholarsresearchlibrary.com/archive.html)

Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Development and validation of stability indicating HPLC method for estimation of related substances in bendamustine hydrochloride

Pavani Peddi[1]*, T. Raja Rajeswari[2] and Ramana Reddy Ganji[3]

[1]*Dept. of Chemistry, PVP Siddhartha Institute of Technology, Vijayawada-520010, India*
[2]*Reader in Chemistry, Dept. of Chemistry, Govt. College for Women, Guntur*
[3]*Dept. of Chemistry, Acharya Nagarjuna Univ, Guntur, India*

ABSTRACT

Estimation of related substances by using high-performance liquid chromatographic method was developed and validated for the determination of Bendamustine hydrochloride. Reversed-phase chromatography was performed on Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower[2] photodiode array detector using ACE C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 7.0 buffer: methanol as mobile phase at a flow rate of 1.0 mL/min. with UV detection at 235 nm. Linearity was observed in the concentration range of Monohydroxy impurity 0.05–1.16 µg/mL (R^2 = 0.998), the concentration range of BND-VI impurity 0.06–1.18 µg/mL (R^2 = 0.998), the concentration range of Bendamustine HCl 0.08-0.79 µg/mL (R^2 = 0.997) and the concentration range of Isopropyl ester 0.05-1.18 /mL (R^2 = 0.998). The limit of quantitation (LOQ) and limit of detection (LOD) were found to be Monohydroxy impurity 0.05&0.02 µg/mL, BND-VI impurity 0.06&0.02µg/mL, Bendamustine HCl 0.08&0.03µg/mL and Isopropyl ester 0.05&0.02µg/mL respectively. The method was validated as per ICH guidelines. The RSD for intra-day (0.14-0.32) and inter-day (0.47-0.66) precision were found to be less than 1 %. The percentage recovery was in good agreement with the labeled amount in the pharmaceutical formulations and the method is simple, specific, precise and accurate for the determination of Bendamustine hydrochloride in pharmaceutical formulations.

Keywords: Bendamustine HCl, Estimation of related substances, liquid chromatography.

INTRODUCTION

Bendamustine hydrochloride (BMH), **(Figure: 1.1)** chemically known as (4-{5-[bis-(2-chloroethyl) amino]-1-methyl- 1Hbenzimidazol-2-yl} butanoic acid) is an active nitrogen mustard [1]. It is used for the treatment of patients with chronic lymphocytic leukemia [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 crosslinks. The bifunctional covalent linkage can lead to cell death via several pathways [3]. Bendamustine is active against both quiescent and dividing cells. Besides biotransformation [4-7], Bendamustine, similar to other nitrogen mustards, undergoes degradation by hydrolysis. Two hydrolysis products of Bendamustine have been detected, namely monohydroxy and dihydroxy derivatives (4-{5-[(2-chloroethyl)-(2-hydroxyethyl) amino]-1-methyl-1H-benzimidazol-2-yl} butanoic acid and 4- {5-[bis-(2-hydroxyethyl) amino]-1-methyl-1Hbenzimidazol- 2- yl} butanoic acid) [8]. Because of the hydrolytic degradation in aqueous solutions, nitrogen mustards are often supplied for administration in a lyophilized form that requires reconstitution, usually in water. Literature review revealed that

there is only one HPLC method for the determination of stability of Bendamustine hydrochloride immobilized onto polyphosphoesters [9] and only one spectrophotometric method [10].

There is not even a single method estimation of impurities in BMH by using RP-liquid chromatographic method in pharmaceutical dosage forms. In the present work a simple estimation of impurities in BMH reverse phase liquid chromatographic method has been developed for the determination of BMH and validated as per ICH guidelines [11]. In the present work we developed simple, rapid and accurate reverse phase liquid chromatographic method for the determination of Bendamustine hydrochloride and its impurities.

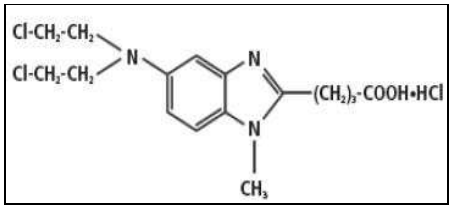

Figure: 1.1 Chemical Structure of Bendamustine hydrochloride (BMH)

1.1 Related Substance Structures:

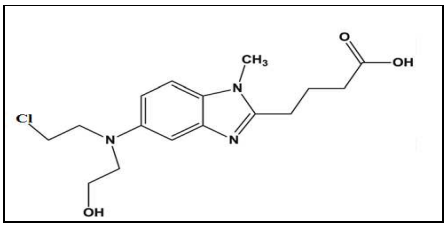

Figure: 1.2 Chemical Structure of Monohydroxy impurity

Molecular Formula:-$C_{16}H_{22}ClN_3O_3$.HCl, Molecular Weight:-339.82
4-[5-(2-chloroethyl (2-hydroxyethyl) amino)-1-methyl-benzimidazol-2-yl] butanoic acid

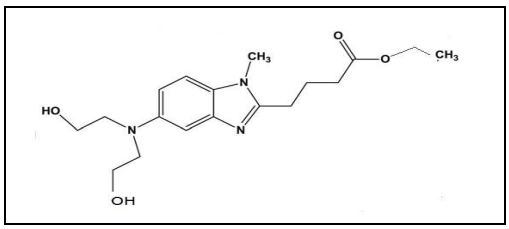

Figure: 1.3 Chemical Structure of Bendamustine VI

Molecular Formula: $C_{18}H_{27}N_3O_4$, Molecular Weight: 349.42
Ethyl 4-[5-(bis (2-hydroxyethyl) amino)-1-methyl-benzimidazol-2-yl] butanoate

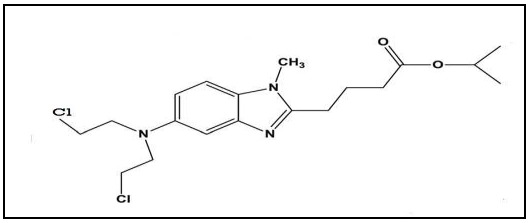

Figure: 1.4 Chemical Structure of Isopropyl ester

Molecular Formula: -$C_{19}H_{27}Cl_2N_2O_2$.HCl, Molecular Weight: 400.34

Isopropyl 4-[5-[bis (2-chloroethyl) amino]-1-methyl-benzimidazol-2-yl] butanoate

MATERIALS AND METHODS

2.1 Reagents& Chemicals:

Potassium dihydrogen Phosphate, Methanol (HPLC grade), Potassium hydroxide, were obtained from Merck (India). All chemicals were of an analytical grade and used as received.

2.2 Chromatographic conditions:

Chromatographic separation was achieved by using a Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower^2 photodiode array detector using ACE C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 7.0 buffer: methanol as mobile phase at a flow rate of 1.0 mL/min. with UV detection at 235 nm. Column maintained at temprature 35 ºC, sample temprature 5ºC. The overall run time was 60 min. and the flow rate was 1.0 mL/min. 10 µl of sample was injected into the HPLC system. Retention times of impurities were 5.03 for monohydroxy impurity, 7.43 for BND-VI, 17.02 for Bendamustine HCl, 35.04 for isopropyl.

3.0 Method Validation

3.1 System Suitability

Perform the system suitability by analyzing the reference solution six times and resolution solution once. Calculate the theoretical plates and tailing for main peak and resolution between BND-VI and Bendamustine HCl from resolution solution. Calculate %RSD for replicate injections of each component from reference solution.

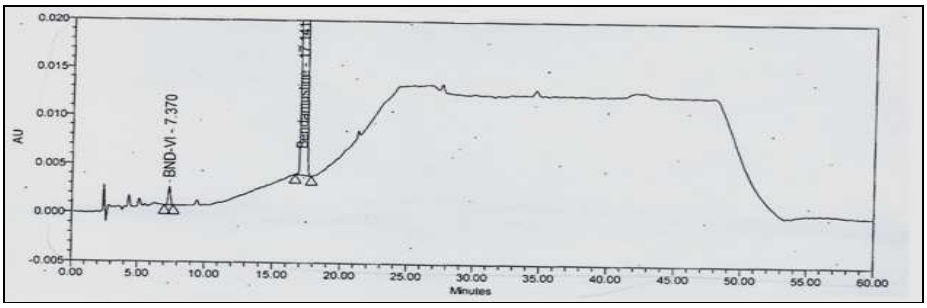

Figure: 1.5 System Suitability chromatogram of Bendamustine HCl and related substance

Table: 1.1 Summary of system suitability from resolution solution

Resolution between BND-VI and Bendamustine peak	Tailing factor for Bendamustine peak	Theoretical plates for Bendamustine peak
28.18	1.45	36512

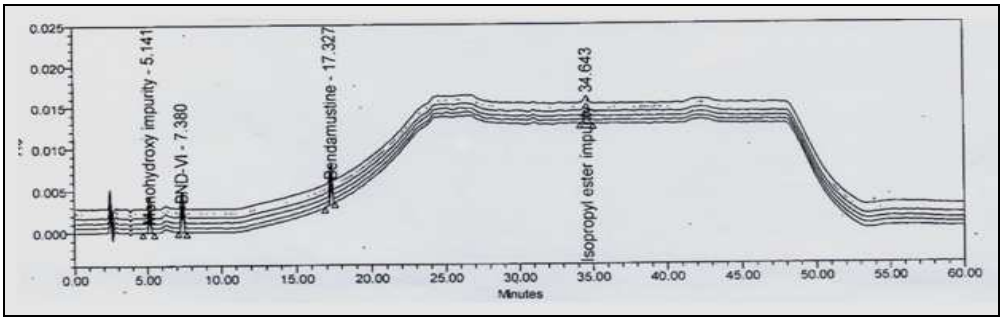

Figure: 1.6 Resolution Solution chromatogram of Bendamustine HCl and related substance

Table: 1.2 Summary of system suitability from Reference solution

Injection No	Monohydroxy impurity	BND-VI	Bendamustine	Isopropyl ester
1	17673	25561	20409	16030
2	17874	26154	21708	16984
3	17891	25673	21122	16974
4	17503	25898	21379	16002
5	17685	26799	22107	16836
6	17092	25919	21955	16613
Mean	17620	26001	21447	16573
%RSD	**1.68**	**1.70**	**2.91**	**2.73**

3.2 Specificity

Prepare and analyze the solutions of monohydroxy impurity, BND-VI, Isopropyl ester impurity and Bendamustine HCl each individually. Prepare a spiked solution of each potential impurity to the Bendamustine HCl drug substance and analyze. Perform the analysis using PDA detector and determine the peak purity.

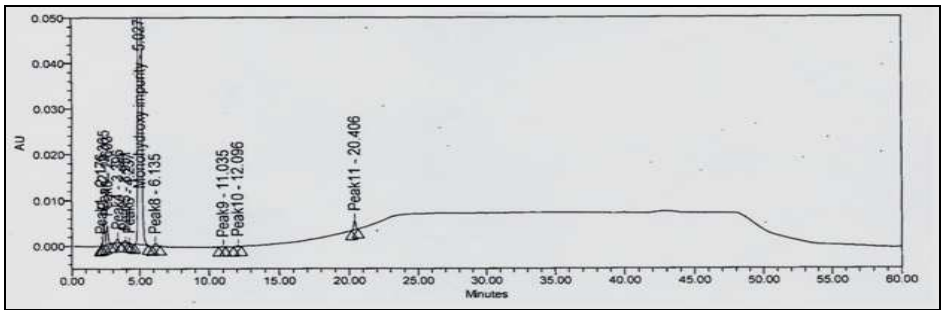

Figure: 1.7 Specificity chromatogram of Monohydroxy Impurity

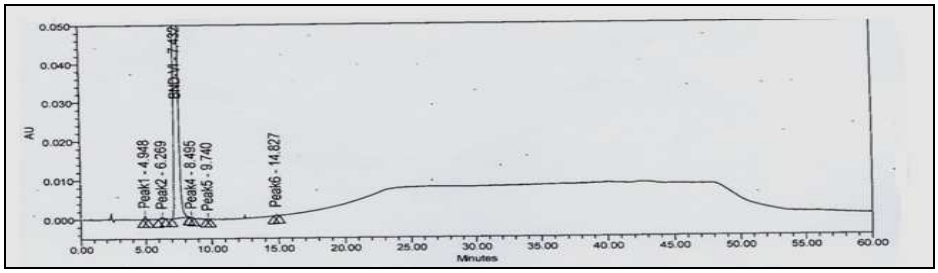

Figure: 1.8 Specificity chromatogram of BND VI

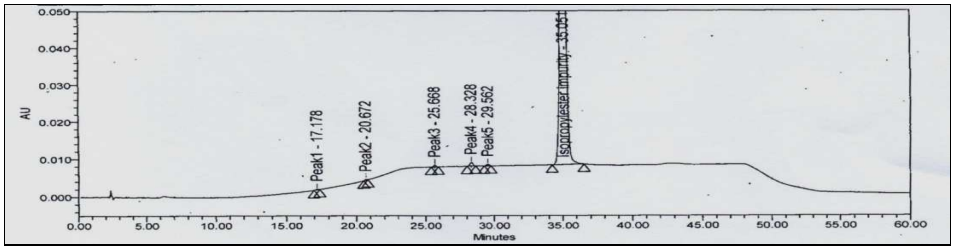

Figure: 1.9 Specificity chromatogram of Isopropyl Ester

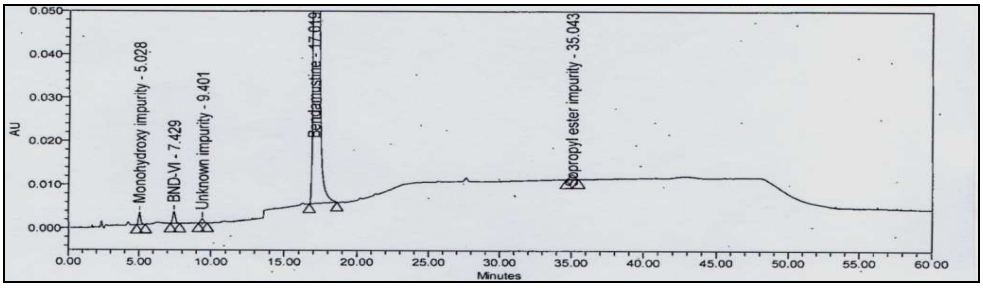

Figure: 2.0 Specificity chromatogram of Spiked Solution

Table: 1.3 summary of retention time, and relative retention time for known impurities

Peak Name	Retention Time	Relative retention time(RRT)
Monohydroxy impurity	5.03	0.30
BND-VI	7.43	0.44
Bendamustine HCl	17.02	1.00
Isopropyl ester	35.04	2.06

This study showed that all the known impurities of Bendamustine HCl are adequately resolved. Therefore the method is selective for the determination of related substances in Bendamustine HCl.

3.3 Limit of detection

Table: 1.4 Limit of detection (LOD) for Bendamustine HCl and impurities

Component	% of impurity	Concentration (mg/ml)	Signal to noise	LOD (%)
Monohydroxy impurity	0.0036	0.0000181	3.9:1	0.004
BND-VI	0.0037	0.0000184	3.9:1	0.004
Bendamustine HCl	0.0052	0.0000262	3.9:1	0.005
Isopropyl ester	0.0035	0.0000174	3.2:1	0.004

The limit of detection values obtained for each impurity and Bendamustine HCl are within the acceptance criteria.

3.4 Limit of Quantitation

Table: 1.5 Limit of Quantitation for Bendamustine HCl and impurities

Component	% of impurity	Concentration (mg/ml)	Signal to noise	LOQ (%)
Monohydroxy impurity	0.011	0.000054	11.2:1	0.011
BND-VI	0.011	0.000055	9.6:1	0.011
Bendamustine HCl	0.016	0.000079	12.6:1	0.016
Isopropyl ester	0.010	0.000052	10.6:1	0.010

Limit of quantitation values obtained for each impurity and Bendamustine HCl are within the acceptance criteria.

3.5 Precision at LOQ

The precision at LOQ is performed by analyzing six replicate injections of the standard solution containing all known impurities and Bendamustine HCl at LOQ level. Determine the percentage relative standard deviation of peak areas of each impurity and Bendamustine HCl. Results of peak area of impurities and Bendamustine HCl are summarized in table 9.

Table: 1.6 Summary of peak areas for precision at LOQ

Inj No	Monohydroxy impurity	BND-VI	Bendamustine	Isopropyl ester
1	2313	1920	2857	4326
2	2309	1794	2845	4276
3	2380	1870	2909	4283
4	2310	1733	2940	4430
5	2356	1874	2953	4255
6	2677	1882	2989	4382
Mean	2391	1846	2916	4325
%RSD	**5.99**	**3.72**	**1.93**	**1.58**

3.6 Linearty and Range

The linearty is determined by injecting the solutions in duplicate containing known impurities and Bendamustine HCl ranging from LOQ to 150% (LOQ, 20%, 40%, 80%, 100%, 120% and 150%) of the specified limit. Perform the regression analysis and determine the correlation coefficient and residual sum of squares. Determine the response factor for each impurity with respect to Bendamustine HCl. Report the linearty range as the range for determining the impurities.Results obtained are in the tables & figures show the line of best fit for peak area versus concentration for each impurity.

Table: 1.7 Linearity of Monohydroxy impurity

Level	% of Monohydroxy	Concentration (mg/ml)	Average Peak Area
LOQ	7.2	0.0000542	1876
20%	20.6	0.0001548	6474
40%	41.3	0.0003096	11166
80%	82.6	0.0006192	24184
100%	103.2	0.0007740	29813
120%	123.8	0.0009288	35363
150%	154.8	0.0011610	42814

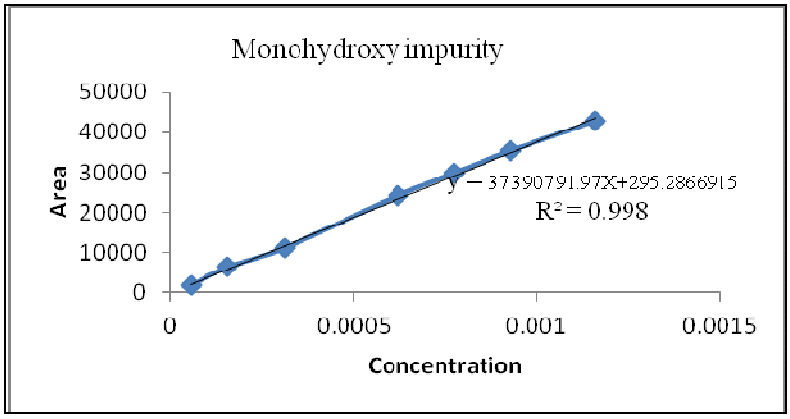

Figure: 2.1 Linearty graph of Monohydroxy impurity

Table: 1.7 Linearty of BND-VI

Level	% of BND-VI	Concentration (mg/ml)	Average Peak Area
LOQ	7.4	0.0000552	2029
20%	21.0	0.0001578	7416
40%	42.1	0.0003156	13457
80%	84.2	0.0006312	29723
100%	105.2	0.0007890	37139
120%	126.2	0.0009468	44214
150%	157.8	0.0011835	53844

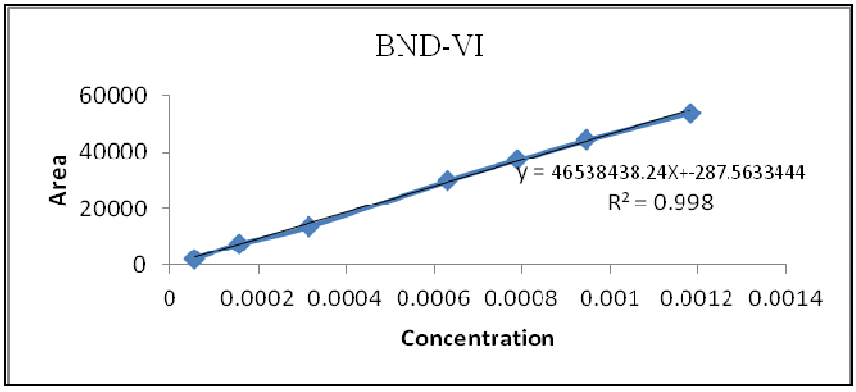

Figure: 2.2 Linearity graph of BND-VI

Table: 1.8 Linearity of Bendamustine HCl

Level	% of Bendamustine HCl	Concentration (mg/ml)	Average Peak Area
LOQ	15.7	0.0000785	3023
20%	20.9	0.0001047	4506
40%	41.9	0.0002094	8407
80%	83.8	0.0004188	17102
100%	104.7	0.0005235	21827
120%	125.6	0.0006282	25933
150%	157.1	0.0007853	30665

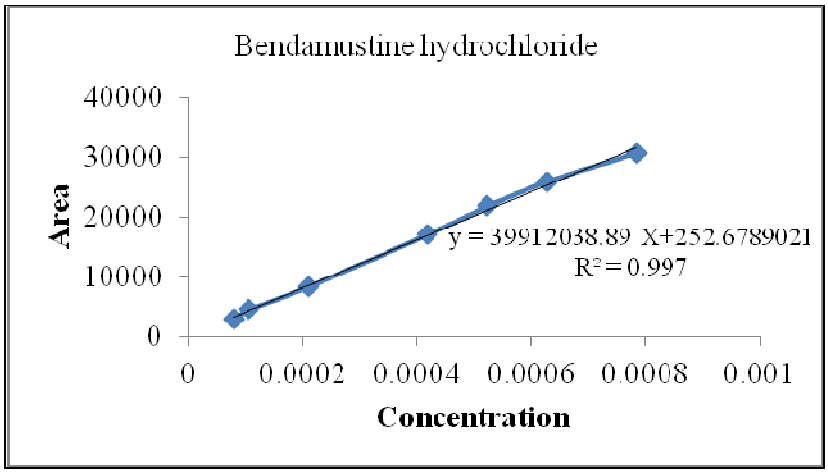

Figure: 2.3 Linearity graph of Bendamustine HCl

Table: 1.9 Linearity of Isopropyl ester

Level	% of Isopropyl ester	Concentration (mg/ml)	Average Peak Area
LOQ	6.9	0.0000521	3557
20%	21.1	0.0001580	5084
40%	42.1	0.0003159	9886
80%	84.2	0.0006318	20707
100%	105.3	0.0007898	25853
120%	126.4	0.0009477	29945
150%	157.9	0.0011846	37624

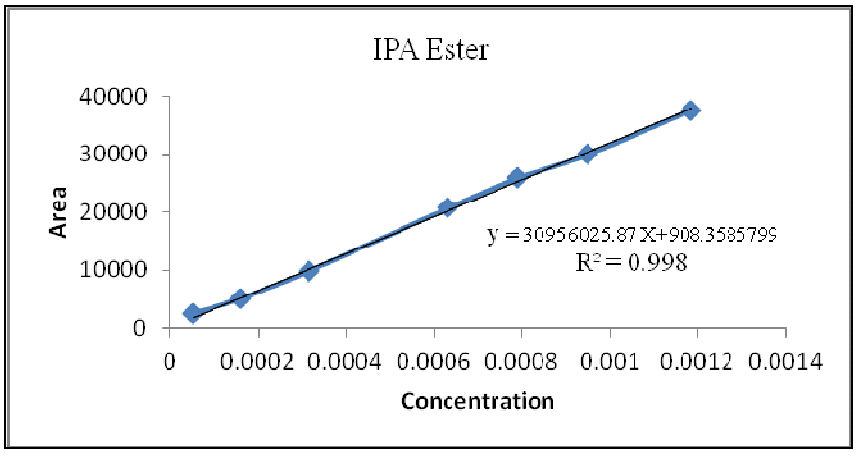

Figure: 2.4 Linearity graph of Isopropyl ester

The linearity results for Bendamustine HCl and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

3.7 Accuracy

Prepare Bendamustine HCl solution spiked with a known amount of each impurity at five levels each in triplicate (in total 15 determinations) and analyze as per the method. The impurities are to be spiked at LOQ, 25%, 50%, 100% and 150% of the specified limit.

Table:2.0 Summary of % recoveries for Monohydroxy impurity

Level	% of Monohydroxy	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	7.2	0.0000542	0.0000520	95.9	3.25
	7.2	0.0000542	0.0000550	101.5	
	7.2	0.0000542	0.0000550	101.5	
25%	25.8	0.0001935	0.0001780	92.0	0.54
	25.8	0.0001935	0.0001790	92.5	
	25.8	0.0001935	0.0001770	91.5	
50%	51.6	0.0003870	0.0004060	104.9	2.64
	51.6	0.0003870	0.0003850	99.5	
	51.6	0.0003870	0.0003960	102.3	
100%	103.2	0.0007740	0.0008170	105.6	0.44
	103.2	0.0007740	0.0008150	105.3	
	103.2	0.0007740	0.0008100	104.7	
150%	154.8	0.0011610	0.0012150	104.7	0.25
	154.8	0.0011610	0.0012200	105.1	
	154.8	0.0011610	0.0012140	104.6	

Table: 2.1 Summary of % recoveries for BND-VI

Level	% of BND-VI	Theoretical conc. (mg/ml.)	Measured conc.(mg/ml)	% Recovery	% RSD
LOQ	7.4	0.0000552	0.0000520	94.2	1.10
	7.4	0.0000552	0.0000530	96.0	
	7.4	0.0000552	0.0000520	94.2	
25%	26.3	0.0001973	0.0001810	91.7	0.32
	26.3	0.0001973	0.0001800	91.2	
	26.3	0.0001973	0.0001810	91.7	
50%	52.6	0.0003945	0.0003990	101.1	1.56
	52.6	0.0003945	0.0003990	98.9	
	52.6	0.0003945	0.0003870	98.1	
100%	105.2	0.0007890	0.0008480	107.5	0.47
	105.2	0.0007890	0.0008450	107.1	
	105.2	0.0007890	0.0008400	106.5	
150%	157.8	0.0011835	0.0012810	108.2	0.35
	157.8	0.0011835	0.0012840	108.5	
	157.8	0.0011835	0.0012760	107.8	

Table: 2.2 Summary of % recoveries for Isopropyl ester

Level	% of Isopropyl ester	Theoretical conc. (mg/ml.)	Measured conc. (mg/ml)	% Recovery	% RSD
LOQ	6.9	0.0000521	0.0000510	97.9	3.08
	6.9	0.0000521	0.0000540	103.6	
	6.9	0.0000521	0.0000510	97.9	
25%	26.3	0.0001974	0.0001840	93.2	0.81
	26.3	0.0001974	0.0001860	94.2	
	26.3	0.0001974	0.0001870	94.7	
50%	52.7	0.0003949	0.0003750	95.0	1.56
	52.7	0.0003949	0.0003820	96.7	
	52.7	0.0003949	0.0003870	98.0	
100%	105.3	0.0007898	0.0007920	100.3	1.25
	105.3	0.0007898	0.0007750	98.1	
	105.3	0.0007898	0.0007910	100.2	
150%	157.9	0.0011846	0.0011720	98.9	1.83
	157.9	0.0011846	0.0012020	101.5	
	157.9	0.0011846	0.0011610	98.0	

The percentage recovery values obtained for each impurity are in the range of about 91.2-108.5, which are within the specified criteria. The relative standard deviation values of recoveries obtained for all impurities are in the range of 0.25%-3.25%

3.8 Precision

3.8.1 System precision

Perform the analysis of reference solution six times and determine the percentage relative standard deviation of peak area of replicate injections of each impurity and Bendamustine HCl.

Table 2.2: Summary of peak areas of the bendamustine and its impurities

Injection No	Monohydroxy Impurity	BND-VI impurity	Bendamustine hydrochloride	Isopropyl ester
1	29788	36830	22028	26147
2	29896	36827	22448	24841
3	29841	36688	22010	25569
4	29529	36979	22781	26340
5	29655	36993	21815	24876
6	29668	36924	22293	25612
Mean area	29730	36874	22229	25564
%RSD	**0.46**	**0.31**	**1.58**	**2.44**

The relative standard deviation observed for Bendamustine HCl and impurities are less than 10%.The results comply with the acceptance criteria and indicate acceptable precision of the system.

3.8.2 Method precision

The precision of the method is determined by analyzing a sample of Bendamustine HCl solution spiked with impurities at 100% of the specification limit.

Table 2.3: Summary of results for precision of the method

Inj. No	% of Monohydroxy Impurity	% of BND-VI	% of Isopropyl ester	% of Any other individual impurity	% of total impurities
1	0.14	0.16	0.15	0.07	0.52
2	0.14	0.16	0.15	0.07	0.52
3	0.14	0.15	0.15	0.07	0.51
4	0.14	0.15	0.15	0.07	0.51
5	0.14	0.16	0.15	0.07	0.52
6	0.14	0.16	0.15	0.07	0.52
Mean (%)	0.14	0.16	0.15	0.07	0.52
% RSD	**0.00**	**3.30**	**0.00**	**0.00**	**1.00**

CONCLUSION

A simple, economic, accurate and precise HPLC method was successfully developed. In this method it was carried out by using ACE C18, (250× 4.6mm) with 5µm particle size. Injection volume of 10µl is injected and eluted with the mobile phase Methanol and buffer of KH_2PO_4 pH 7.0 with potassium hydroxide, which is pumped at a flow rate of 1.0 ml/min. Detection, was carried out at 235 nm. The peaks obtained were sharp with retention time of 5.20 for Monohydroxy impurity, 7.43 for BND-VI, 17.2 for Bendamustine hydrochloride, 34.06 for Isopropyl ester. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, robustness, and stability of solution.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Bendamustine and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore the method is selective for the determination of related substances in bendamustine hydrochloride.

The limit of detection (LOD) and limit of quantitation (LOQ) for monohydroxy was found to be 0.018µg/ml,0.05µg/ml, for BND-VI 0.018µg/ml,0.05µg/ml,bendamustine 0.02µg/ml,0.07µg/ml, for Isopropyl ester 0.017µg/ml,0.05µg/ml respectively.Using the optimized chromatographic conditions, the retention times were found to be of 5.20 for Monohydroxy impurity, 7.43 for BND-VI, 17.2 for Bendamustine hydrochloride, 34.06 for

Isopropyl ester respectively. The linearity results for bendamustine hydrochloride and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Bendamustine hydrochloride and its impurities found to be 0.9991, 0.9993, 0.9987, and 0.9987 respectively.

The accuracy studies were shown as % recovery for Bendamustine and its impurities at 25%, 50%, 100% and 150%.The limit of % recovered shown is in the range of 90 and 110% and the results obtained were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the Bendamustine and its related substances in the range 91.2-108.5 respectively.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Bendamustine and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits. For intermediate precision the bias is not more than ± 0.03, the bias observed for individual impurities are within the acceptance criteria.

Hence, the chromatographic method developed for Bendamustine and its related substances are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality during its formulation.

REFERENCES

[1] Neil M.J.O', The Merck Index, Merck Research Laboratories, Whitehouse Station, NJ (**2006**)
[2] Friedberg J. W., Cohen P., Chen L., et al., *J. Clinical Oncology*, 26(2), 204-210 (**2008**)
[3] Lissitchkov T., Arnaudov G., Peytchev D., Merkle Kh., *J. Cancer Research and Clinical Oncology*, 132(2), 99-104 (**2006**)
[4] Teichert J., Sohr R., Baumann F., Hennig L., et al., *Drug Metabolism and Disposition*, 33(7), 984-992 (**2005**)
[5] Matt Kalaycio, Clinical Experience with Bendamustine : A new treatment for patients with chronic lymphocytic leukemia, Clin Leukemia, 2(4), 223-229 (**2008**)
[6] Teichert J., Baumann F., Chao Q., Franklin C.,et al., *Cancer Chemother. Pharmacol*, 59(6), 759-770 (**2007**)
[7] Rasschaert M., Schrijvers D., Van den Brande J. et al., *British Journal of Cancer*, 96, 1692–1698 (**2007**)
[8] Preiss R., Sohr R., Matthias M., Brockmann B., Huller H., *Pharmazie*, 40, 782-784 (**1985**)
[9] Ivanka Pencheva, Anita Bogomilova, Neli Koseva, Danka Obreshkova, Kolio Troev, *J. Pharm. Biomed. Analysis*, 48(4), 1143–1150 (**2008**)
[10] Mathrusri Annapurna M., Pavani S., Anusha S., Harika Mahanti and Venkatesh B., *Journal of Chemical and Pharmaceutical Research*, 4(3), 1696-1701 (**2012**)
[11] ICH Validation of analytical procedures: Text and methodology Q2(R1), International Conference on Harmonization, (**2005**)
[12] ICH Stability Testing of New Drug Substances and Products Q1A (R2), International Conference on Harmonization, (**2003**)