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A VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF RELATED SUBSTANCES FOR BENDAMUSTINE HCl FOR INJECTION

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ABSTRACT

A novel, simple, sensitive and stability-indicating high-performance liquid chromatography method was developed and validated for the related substances of Bendamustine hydrochloride in Bendamustine hydrochloride for Injection 25 mg/vial. Reversed-phase chromatography was performed on Agilent 1100 series with Software Empower-2 and Waters 2998 PDA 2695 pump Software Empower-2 photodiode array detector using Inertsil ODS-3V (250 mm × 4.6 mm, 5 µm particle size) column with pH 3.0 (adjusted with Phosphoric acid) of 0.2% TEA buffer: methanol in the ratio of 9:1 as mobile phase-A and Methanol:Acetonitrile in the ratio of 6:4 at a flow rate of 1.0 mL/min. Gradient profile at Initial: 95-5, 11 minutes: 89-11, 20 minutes: 76-24, 44 minutes: 40-60, 51: 95-5, 60minutes: 95-5 and with UV detection at 235 nm. Linearity was observed in the concentration range of Dihydroxy impurity 0.25-744 µg/mL (R2 = 0.999), Monohydroxy impurity 0.30–14.93 µg/mL (R2 = 0.999), the concentration range of Bendamustine 0.29-14.34 µg/mL (R2 = 0.999), the concentration range of Dimer impurity 0.46-22.95 µg /mL (R2 = 0.999) and the concentration range of Impurity-A 0.10–3.05µg/mL (R2 = 0.999),). The limit of detection (LOD) and limit of Quantitation (LOQ) were found to be Dihydroxy impurity 0.01&0.03 µg/mL, Monohydroxy impurity 0.01&0.03 µg/mL, Bendamustine 0.01&0.03 µg/mL, Dimer impurity 0.01&0.04 µg/mL and Impurity-A 0.004&0.01 µg/mL, respectively. The method was validated as per ICH guidelines. The RSD for intra-day (0.19-1.94) and inter-day (0.41-2.53) precision were found to be less than 10.0 %. The percentage recovery was in good agreement with the labelled 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, HPLC, validation.

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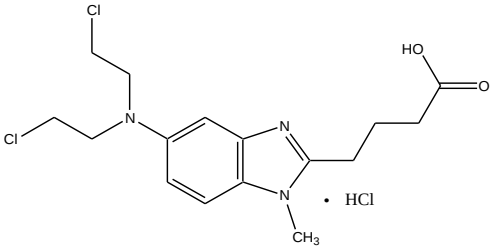
I. INTRODUCTION

Bendamustine hydrochloride (BMH), (Figure: 1.1) chemically (IUPAC name) 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-1Hbenzimidazol-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 there is only one HPLC method for the determination of stability of Bendamustine hydrochloride immobilized onto polyphosphoesters [9] and only one spectrophotometric method [10].

No liquid chromatography (LC) methods have been reported in major pharmacopeia's like United States Pharmacopeia (USP), European Pharmacopeia (EP), Japanese Pharmacopeia (JP) and British Pharmacopeia (BP). A literature review reveals that there is only one high-performance liquid chromatography (HPLC) method for the determination of the stability of Bendamustine immobilized onto polyphosphoesters [11].

An extensive literature survey reveals that there is no stability-indicating LC method for the determination of related substances or for the quantitative estimation of Bendamustine (BEN) in bulk drugs and pharmaceutical dosage form. All reported literature methods are not suitable for the quantification of all specified impurities. An exhaustive study on the stability of Bendamustine is necessary, because the current International Conference on Harmonization (ICH) guidelines require that stability analysis should be conducted by using stability-indicating methods, which must be developed and validated after stress testing on the drug under a variety of conditions, including hydrolysis (at various pH levels), oxidation, photolysis and thermal degradation (12–16]. Moreover, the structural characterization and synthesis of the degradation products both establish the degradation pathways and quantitative determination in drug substances and products. Hence, in the present work, the chemical degradation pathways of Bendamustine were established through a forced degradation study and a selective, precise and accurate LC method was also developed for the simultaneous estimation of BEN and its degradation products. The validation of the proposed method was also conducted and its applicability was evaluated in the analysis of its commercial form.

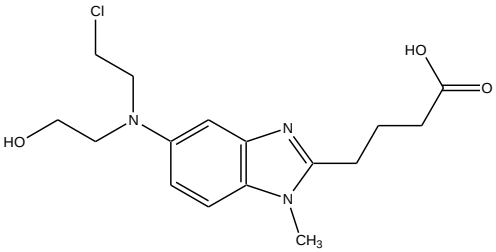
i. Bendamustine Hydrochloride Impurity



Molecular Formula: $C_{16}H_{22}Cl_3N_3O_2$

Molecular Wt.: 394.72

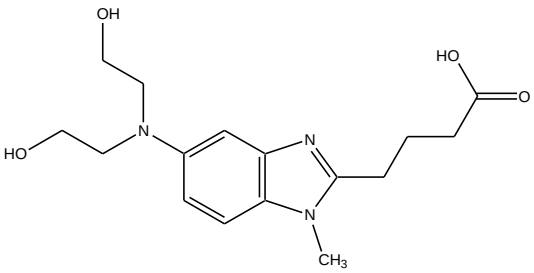
ii. Bendamustine Monohydroxy



Molecular Formula: $C_{16}H_{22}ClN_3O_3$

Molecular Wt.: 339.82

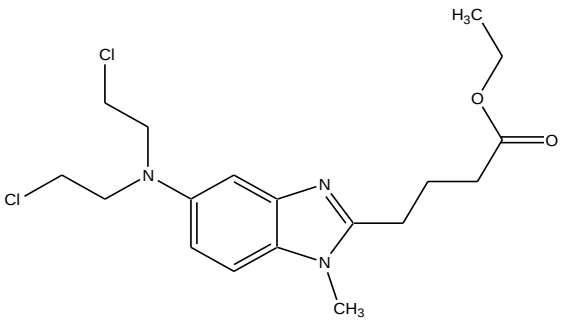
iii. Bendamustine Dihydroxy Impurity



Molecular Formula: C16H23N3O4

Molecular Wt.: 321.37

iv. Impurity-A



Molecular Formula: C18H25Cl2N3O2

Molecular Wt.: 386.32

v. Dimer Impurity

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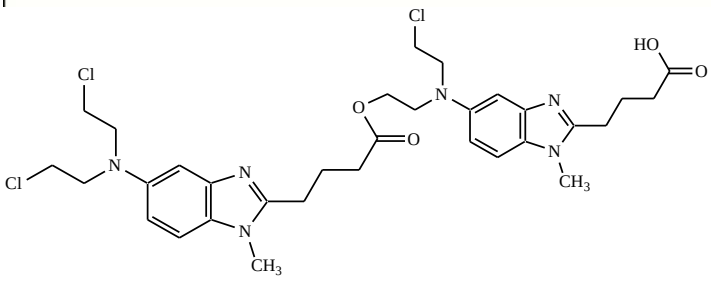

Molecular Formula: $C_{32}H_{41}Cl_3N_6O_4$
Molecular Wt.: 680.06

II. MATERIALS, EQUIPEMENTS DETAILS, CHEMICAL NAME FOR BENDAMUSTINE AND IT'S IMPURITIES, METHOD PROCEDURE

1. **Reagents and Solvents:**

Triethylamine, Phosphoric acid, Methanol, Acetonitrile (HPLC grade), Dimethysulfoxide (GC grade) were obtained from Merck (India) and Rankem. Milli-Q water purification system from Millipore.

2. Equipment Details:

Two LC systems were used for method development and validation. LC 1 was a Agilent (2695 separation module and a 1100 series of variable wavelength absorbance detector) with empower software. LC 2 was a Waters [2695 separation module and a 996-photodiode array (PDA) detector] with Empower-2 software.

3. Chemical Name for Bendamustine and it's Impurities:

The chemical names are as described in the following

Bendamustine: 4-[5-[bis(2-Chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid hydrochloride.

Bendamustine Monohydroxy Impurity: 4-{5-[(2-Chloroethyl)(2-hydroxyethyl)amino]-1-methyl-1H-benzimidazol-2-yl}butanoic acid.

Bendamustine Dihydroxy Impurity: 4-{5-[bis(2-Hydroxyethyl)amino]-1-methyl-1H-benzimidazol-2-yl}butanoic acid.

Impurity-A: [Ethyl 4-(5-(bis(2-chloroethyl)amino]-1-methyl-1H-benzo[d] imidazol-2-yl)butanoate].

Dimer Impurity: 4-{5-[{2-[(4-{5-[bis(2-chloroethyl)amino]-1-methyl-1H-benzimidazol-2-yl}butanoyl)oxy]ethyl}(2-chloroethyl)amino]-1-methyl-1H-benzimidazol-2-yl}butanoic acid.

4. Method Procedure:

A new gradient method was developed for separating process impurities of Bendamustine from its degradation peaks, thus proving the method to be stability indicating. The chromatographic method employed mobile phase A, consisting of a mixture of *pH 3.0 (adjusted with Phosphoric acid) of 0.2% TEA buffer: methanol in the ratio of 900:100* (v/v), and mobile phase B, consisting of a mixture of *Methanol:Acetonitrile in the ratio of 600:400*.

The method employed the gradient programs listed in Table I for the analysis of impurities. The method was developed by using an Inertsil ODS-3V column, 4.6 × 250 mm, 5 µm (GL Sciences, Inc., Torrance, CA). The flow rate of the mobile phase was 1.2 mL/min. The column temperature was maintained at 30°C, the sample cooling rack temperature was maintained at 25°C and the detection wavelength was monitored at 235 nm. The injection volume was 10 µL. Dimethylsulphoxide used as diluent.

Table I

HPLC Gradient Program for Analysis of Impurities

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0.00	95.0	5.0	1.2
11.0	89.0	11.0	1.2
20.0	76.0	24.0	1.2
44.0	40.0	60.0	1.2
51.0	95.0	5.0	1.2
60.0	95.0	5.0	1.2

III. ANLYTICAL METHOD VALIDATION

Solution stability

The stability of BEN and its impurities in solution for the related substance method was determined by injecting the spiked sample solution at 25°C temperature for 66 hours and measuring the amounts of the four impurities every about 9 hours and cumulative RSD of Bendamustine Monohydroxy Impurity was found 0.89%, Bendamustine Dihydroxy Impurity was found 0.24%, Impurity-A was found 0.91%, Dimer Impurity was found 0.23% found. Injecting the standard solution at 25°C temperature for about 70 hours and measuring the cumulative RSD of Bendamustine was found 8.58 % at about 46 hours (stability hours). Injecting the sample solution at 25°C temperature for about 65 hours and measuring the cumulative RSD of Bendamustine Monohydroxy Impurity was found 9.52% at about 36 hours (stability hours) and for Bendamustine Dihydroxy Impurity was found 2.82% at about 65 hours and remaining impurities were observed BQL.

Specificity

Specificity is the ability of the method to measure the response of the analyte in the presence of its potential impurities and degradation products. The specificity of the developed LC method for Bendamustine was tested in the presence of its impurities. The sample was subjected to acid hydrolysis, alkaline hydrolysis and oxidation conditions. The sample was also subjected to thermal and photo degradation in a dry state. Different stress conditions were used to achieve degradation. The degraded samples were diluted to produce 1.0 mg/mL of Bendamustine.

Linearity

Linearity solutions for the method of impurities were prepared by diluting impurity stock solutions to the required concentrations. Linearity was established over a specified range of the LOQ to the 150% of the specification limit of known impurities and Bendamustine

Limits of detection and quantification

The Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of known impurities and that of Bendamustine were determined based on calibration curve plotted between concentration of impurity and their respective responses. The respective LOD and LOQ of impurities were calculated from the residual standard deviation obtained from calibration curve. Precision at limit of Quantitation and verification of limit of detection value were performed. A precision study was also conducted at the LOQ level by injecting six individual preparations of all four impurities and Bendamustine and calculating the relative standard deviation (RSD) of the area.

Accuracy

The accuracy was performed by spiking respective impurity standards with Bendamustine sample at 50%, 100%, and 150% of specification level and by spiking respective impurity standards with Bendamustine HCl API + Placebo at LOQ level. The solution was prepared in triplicate at each level.

Precision

System precision was performed by injecting six replicate injections of standard solution of Bendamustine. Method precision was performed by analysing six sample preparations, as per method. Intermediate precision was performed by analysing six sample preparations, as per method by a different analyst, on a different day, on a different instrument, using a column of different serial no.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Bendamustine and its impurities and the tailing factor, plate count for Bendamustine and its impurities were recorded. The effect of flow rate was studied at 1.0 & 1.4 mL/min and compared with the flow rate of the method at 1.2 mL/min. The effect of Mobile phase Buffer pH was studied at 2.8 & 3.2 and compared with the Mobile phase Buffer pH of the method at 3.0. The effect of column temperature was studied at 25 & 30°C and compared with the column temperature of the method at 30°C. The effect of gradient programme was studied with ± 2% absolute.

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IV. RESULTS OF PARAMETERS

Selectivity

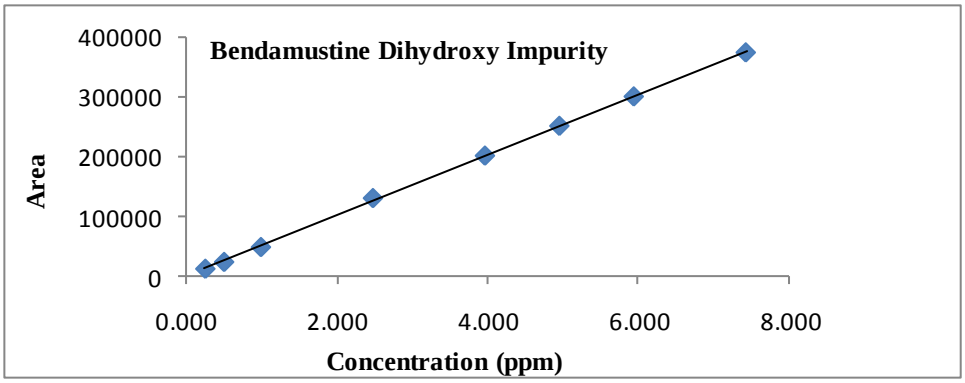
Name of the component	Retention time in Individual solutions	Retention time in Spiked sample solution
Dihydroxy Bendamustine	8.5	7.9
Monohydroxy Bendamustine	20.9	20.8
Bendamustine	31.2	31.2
Bendamustine Dimer	34.7	34.7
Impurity-A	37.4	37.3

Specificity

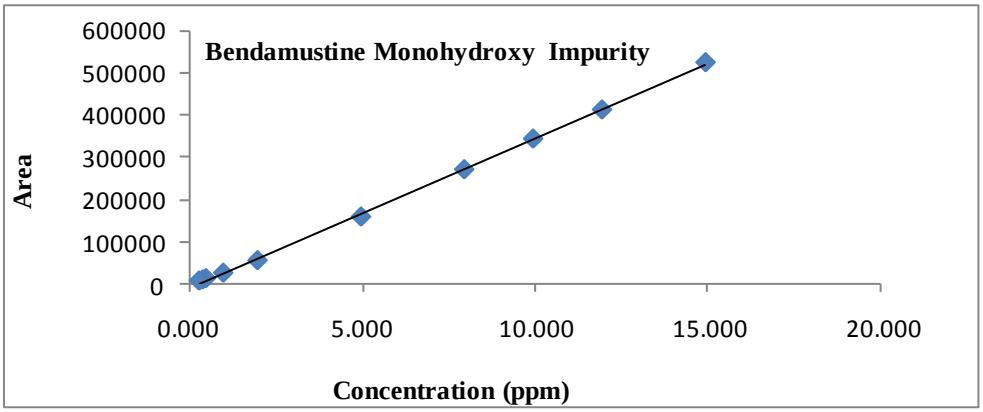
Stress condition	Degradation in Control Sample in %w/w
Control (Unstressed)	-
Thermal Sample(24 Hrs at 105°C)	1.4
Acid Sample 5mL of 1N HCl(5hours)	5.1
Peroxide Sample 5mL of 30%H2O2 (5hourrs)	1.2
Alkali Sample 5mL of 1N NaOH (20 minutes)	11.8
Photolytic stress (1.2 million lux hours)	8.1

Linearity and Range

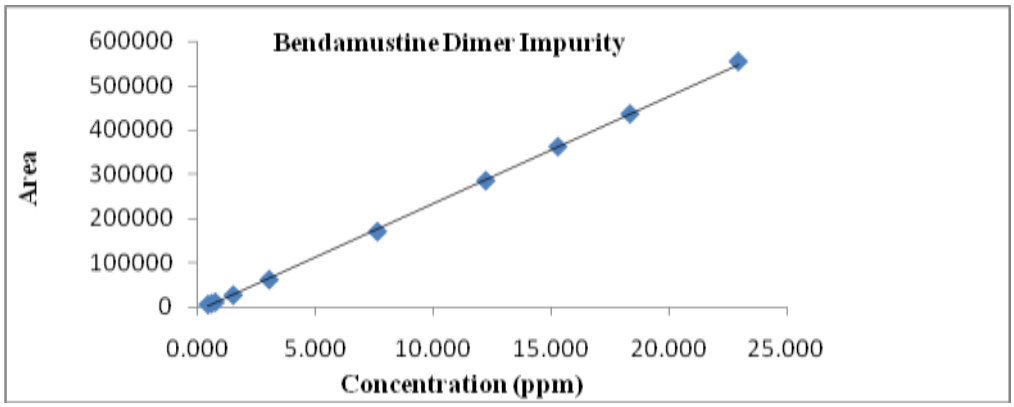
Bendamustine Dihydroxy Impurity		
Level (%)	**Concentration (µg/mL)**	**Area**
150%	7.44	374236
120%	5.95	300405
100%	4.96	251048
80%	3.97	201120
50%	2.48	129934
20%	0.99	47805
10%	0.5	22824
LOQ	0.25	11390
Slope		50635.3
Intercept		-637.4
Correlation coefficient		0.9999
Residual sum of squares		35964516



Bendamustine Monohydroxy Impurity		
Level (%)	**Concentration (µg/mL)**	**Area**
150%	14.93	524839
120%	11.94	412649
100%	9.95	343531
80%	7.96	270615
50%	4.98	158350
20%	1.99	54691
10%	1.00	24990
LOQ	0.50	12071
Slope		35452.8
Intercept		-9583.6
Correlation coefficient		0.9997
Residual sum of squares		206859696

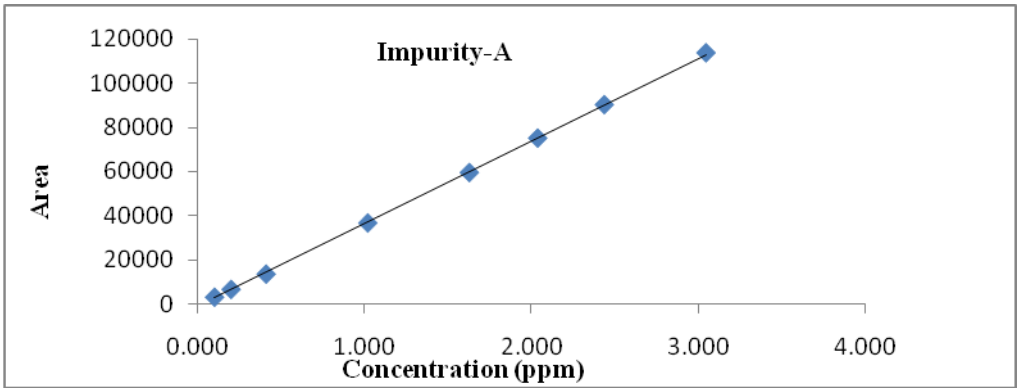


Bendamustine Dimer Impurity		
Level (%)	**Concentration (µg/mL)**	**Area**
150%	22.95	553097
120%	18.36	435046
100%	15.30	361416
80%	12.24	284860
50%	7.65	170203
20%	3.06	62270
10%	1.53	27373
5%	0.77	11374
4%	0.61	8407
LOQ	0.46	6276
Slope		24270.3
Intercept		-9241.2
Correlation coefficient		0.9998
Residual sum of squares		116789436



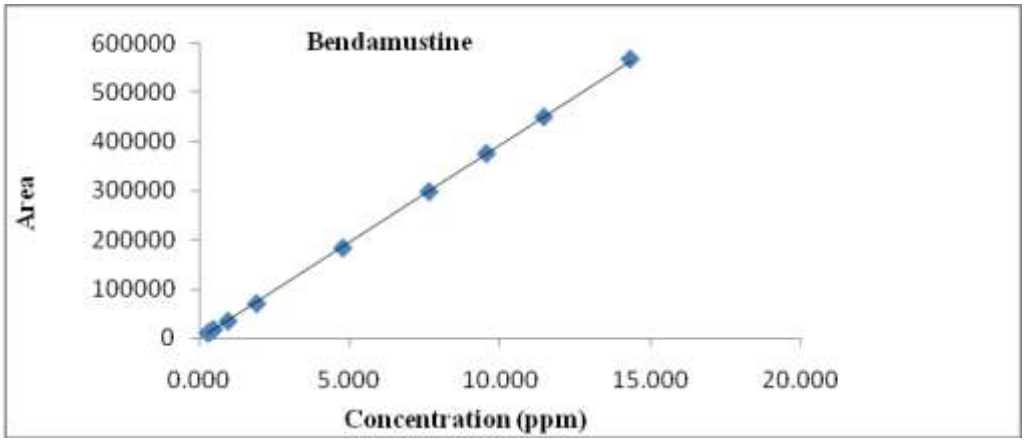
Impurity-A		
Level (%)	**Concentration (µg/mL)**	**Area**
150%	3.05	113615
120%	2.44	90216
100%	2.04	75136

80%	1.63	59612
50%	1.02	36860
20%	0.41	13825
10%	0.20	6915
LOQ	0.10	3394
Slope		37391.4
Intercept		-952.5
Correlation coefficient		0.9999
Residual sum of squares		1395129



Bendamustine		
Level (%)	**Concentration (µg/mL)**	**Area**
150%	14.34	566830
120%	11.47	449407
100%	9.56	375145
80%	7.65	297596
50%	4.78	183372
20%	1.91	70291
10%	0.96	34630
5%	0.48	17686
4%	0.38	13910
LOQ	0.29	10670
Slope		39526.7

Intercept	-2873.6
Correlation coefficient	0.9999
Residual sum of squares	36049799



Limits of detection and quantification

Component	Concentration LOD (%) w/w	Concentration LOQ (%) w/w
Bendamustine Monohydroxy	0.01	0.03
Bendamustine Dihydroxy	0.01	0.03
Bendamustine Dimer	0.01	0.04
Impurity-A	0.004	0.01
Bendamustine	0.01	0.03

Verification of Limit of detection

Injection	Area of Bendamustine Monohydroxy	Area of Bendamustine Dihydroxy	Area of Bendamustine Dimer	Area of Impurity-A	Area of Bendamustine
1	2622	2633	1666	1251	3230
2	2669	2681	1713	1166	3241
3	2441	2645	1704	1254	3192
4	2445	2782	1602	1320	3227
5	2384	2703	1745	1281	3155
6	2410	2680	1612	1182	3232
Mean	2495	2687	1674	1242	3213
%RSD	**4.8**	**2.0**	**3.4**	**4.7**	**1.0**

Precision at Limit of Quantitation

Injection	Area of Monohydroxy Bendamustine	Area of Dihydroxy Bendamustine	Area of Bendamustine Dimer	Area of Impurity-A	Area of Bendamustine
1	10109	11419	7312	4297	11218
2	9879	11066	7070	4258	10980
3	9715	11247	6963	4262	11217
4	9782	11228	6859	4234	11046
5	9378	11066	6694	4044	10848
6	9418	10992	6620	4232	10972
Mean	9714	11170	6920	4221	11047
%RSD	**2.9**	**1.4**	**3.7**	**2.1**	**1.3**

Accuracy

Bendamustine Dihydroxy

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average % Recovery at each level
LOQ	1	0.01520	0.01428	94.0	95.7
	2	0.01520	0.01484	97.6	
	3	0.01520	0.01451	95.5	
50%	1	0.12667	0.13250	104.5	104.9
	2	0.12667	0.13329	105.2	
	3	0.12667	0.13287	104.9	
100%	1	0.25334	0.26268	103.7	103.9
	2	0.25334	0.26319	103.9	
	3	0.25334	0.26388	104.2	
150%	1	0.38002	0.38892	102.3	102.2
	2	0.38002	0.38869	102.3	
	3	0.38002	0.38721	101.9	
			Overall mean		**101.7**
			Overall SD		**3.0**
			Overall % RSD		**2.9**

Bendamustine Monohydroxy

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average % Recovery at each level
LOQ	1	0.01614	0.01559	96.6	96.3
	2	0.01614	0.01602	99.3	
	3	0.01614	0.01503	93.1	
50%	1	0.24651	0.27989	113.5	113.9
	2	0.24651	0.27997	113.6	
	3	0.24651	0.28231	114.5	
100%	1	0.49302	0.55171	111.9	112.0
	2	0.49302	0.55116	111.8	
	3	0.49302	0.55396	112.4	
150%	1	0.73953	0.8112	109.7	109.8
	2	0.73953	0.81205	109.8	
	3	0.73953	0.81161	109.8	
			Overall mean		**108.0**
			Overall SD		**5.8**
			Overall % RSD		**5.4**

Bendamustine Dimer

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average % Recovery at each level
LOQ	1	0.02080	0.02041	98.1	101.2
	2	0.02080	0.02186	105.1	
	3	0.02080	0.0209	100.5	
50%	1	0.39042	0.42630	109.2	109.2
	2	0.39042	0.42475	108.8	
	3	0.39042	0.42806	109.6	
100%	1	0.78084	0.84637	108.4	108.4
	2	0.78084	0.84413	108.1	
	3	0.78084	0.84794	108.6	
150%	1	1.17126	1.24606	106.4	106.6
	2	1.17126	1.25036	106.8	
	3	1.17126	1.24863	106.6	
			Overall mean		**106.3**

	Overall SD	**2.555**
	Overall % RSD	**2.40**

Impurity-A

Level	**Sample**	**Amount added (mg)**	**Amount recovered (mg)**	**% Recovery**	**Average % Recovery at each level**
LOQ	1	0.00791	0.00730	92.3	98.9
	2	0.00791	0.00861	108.9	
	3	0.00791	0.00756	95.6	
50%	1	0.04970	0.05549	111.7	112.6
	2	0.04970	0.05601	112.7	
	3	0.04970	0.05643	113.5	
100%	1	0.09941	0.11171	112.4	111.8
	2	0.09941	0.11033	111.0	
	3	0.09941	0.11143	112.1	
150%	1	0.14911	0.1617	108.4	109.1
	2	0.14911	0.1636	109.7	
	3	0.14911	0.16252	109.0	
			Overall mean		**108.1**
			Overall SD		**4.722**
			Overall % RSD		**4.37**

Precision

System Precision

Injection	**Peak area of Bendamustine**
1	68718
2	68265
3	67073
4	66901
5	66494
6	66504
Mean	67326
%RSD	**1.4**

Method Precision (in % w/w)

Sample preparation	Bendamustine Dihydroxy	Bendamustine Monohydroxy	Bendamustine Dimer	Impurity-A	Total Impurity
1	0.514	0.090	0.095	0.227	1.016
2	0.515	0.089	0.097	0.229	1.025
3	0.513	0.089	0.099	0.230	1.025
4	0.512	0.089	0.096	0.228	1.020
5	0.514	0.088	0.100	0.231	1.026
6	0.514	0.088	0.098	0.229	1.036
Mean	**0.514**	**0.089**	**0.098**	**0.229**	**1.025**
%RSD	**0.2**	**0.9**	**1.9**	**0.6**	**0.7**

Intermediate Precision (in % w/w)

Sample preparation	Bendamustine Dihydroxy	Bendamustine Monohydroxy	Bendamustine Dimer	Impurity-A	Total Impurity
1	0.484	0.078	0.100	0.192	0.958
2	0.481	0.078	0.104	0.193	0.979
3	0.483	0.079	0.103	0.192	0.965
4	0.479	0.076	0.103	0.189	0.950
5	0.483	0.079	0.099	0.191	0.958
6	0.480	0.081	0.101	0.193	0.961
Mean	**0.482**	**0.079**	**0.102**	**0.192**	**0.960**
%RSD	**0.4**	**2.5**	**2.0**	**0.8**	**1.0**

Relative Retention Time and Response Factor

Name of the Peak	RRT About	RRF
Bendamustine Dihydroxy	0.3	1.0
Bendamustine Monohydroxy	0.7	0.9
Bendamustine	1.0	1.0
Bendamustine Dimer	1.1	0.7

Impurity-A (BD-6)_	1.2	1.0

Robustness

The system suitability parameters complied in every robustness condition ((flow rate, mobile phase buffer pH, column temperature and gradient programme). The method was found to be robust.

Chromatograms

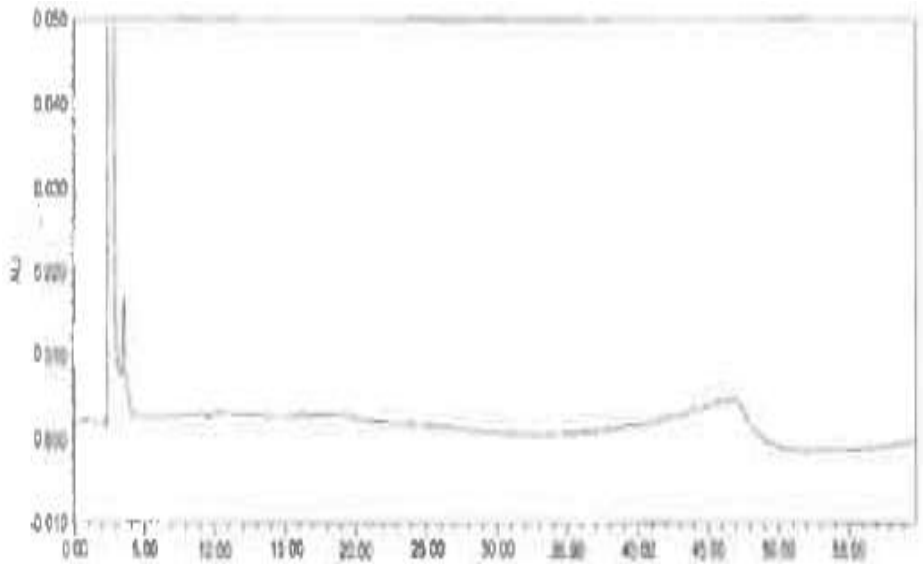


Fig.1: Blank

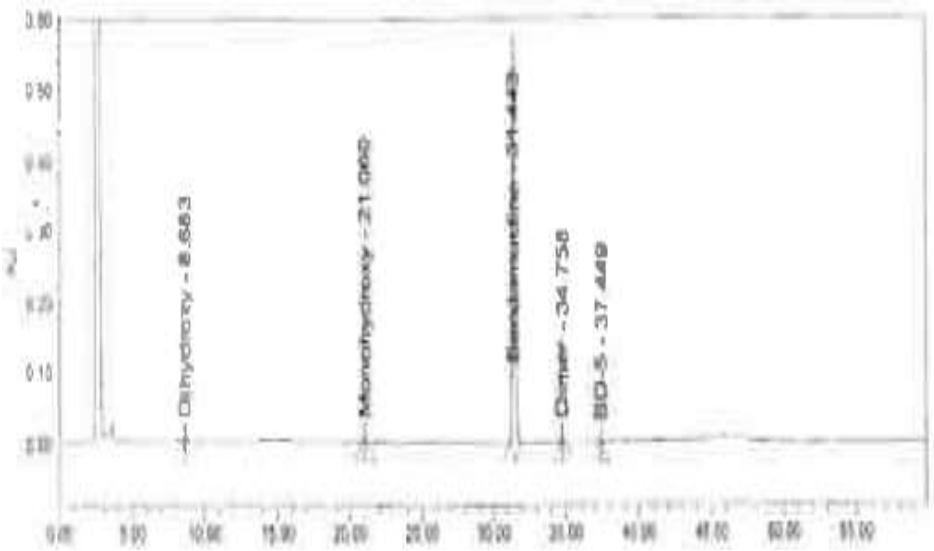


Fig.2: Diluted Spiked Sample solution

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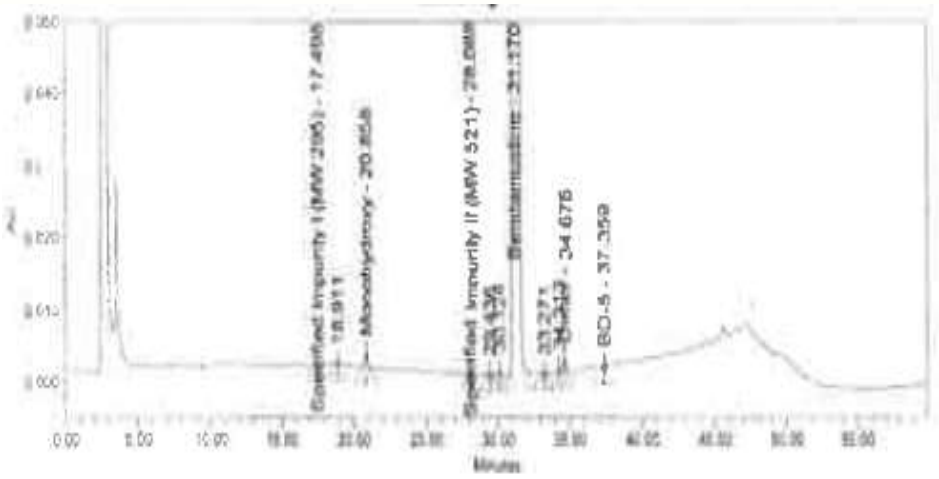

Fig.3: Sample solution

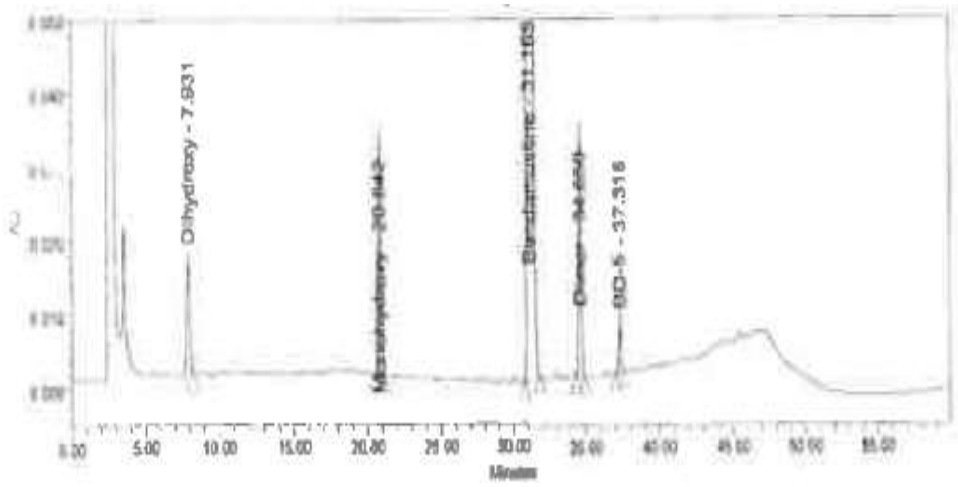

Fig.4: Spiked sample solution

V. CONCLUSION

The present study emerged with a suitable method for impurities for evaluation of the pharmaceutical quality of Bendamustine. The impurities method was designed by taking adequate care to separate process-related impurities and degradation products from each other and from Bendamustine. The method also identified the retention times of known impurities and accurately ensured their quantification by employing RRF's. A simple and accurate assay method for the determination of drug content was established. The developed method is stability indicating and can be used for the routine analysis of production samples and to check the stability of Bendamustine for injection samples.

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