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Method development and validation of bendamustine HCl injection by using RP-HPLC

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Abstract

Bendamustine hydrochloride in its pure and parenteral form has been validated using a precise, easy, affordable, repeatable, and accurate reverse phase high-performance liquid chromatographic approach. Chromatography was carried out on a zorbax SB C18 (250 x 4.6 mm) 3.5 µm column with a mobile phase of water: acetonitrile (with 0.01% of trifluoroacetic acid) mixed in the ratio of 65:35% v/v at a flow rate of 1.0 ml/min, detection was carried out at 230 nm, column temperature was set to 300 C and water: methanol (50:50 v/v) was used as diluent; conditions were finalized as optimized method. Bendamustine hydrochloride had a retention time of 7.768 min. The system's suitability parameters largely satisfied the requirements for acceptance. The approach generates linear answers with a correlation coefficient of 0.999 in the concentration range of 50 to 150%. For repeatability, the precision was 1.75% RSD and the test determination's intermediate precision was less than 2.0% RSD. The technique can therefore be used to check the quality of medication formulations.

1. Introduction

Bendamustine hydrochloride is a compound of nitrogen mustard with alkylating antineoplastic properties, is employed to treat non-Hodgkin B-cell aggressive lymphoma (Chowku *et al*., 2001) (NHL) and chronic lymphocytic leukaemia (CLL) that has developed during or six months after receiving treatment with rituximab (Friedberg *et al*., 2008) or a rituximab-containing regimen (Weide *et al*., 2002). Bendamustine belongs to class of benzimidazoles.

Bendamustine is a bifunctional mechlorethamine derivative that may covalently link with other molecules; it has the ability to create electrophilic alkyl groups (Martina *et al*., 2018). Through, its role as an alkylating agent, bendamustine produces intra and interstrand cross linkages between DNA bases, which leads to cell death (Shi, *et al*., 2019). This substance seems to cross-link and alkylate macromolecules, which inhibits the synthesis of proteins, DNA, RNA and finally triggers apoptosis (Dubbelman *et al*., 2013).

Bendamustine largely undergoes hydrolysis metabolism to form HP1 (monohydroxy) and HP2 (dihydroxy-bendamustine) metabolites, which have modest cytotoxic potential (Matt Kalaycio, 2008). M3 (gamma-hydroxy bendamustine) and M4 (N-desmethyl-bendamustine) are two potent minor metabolites; nonetheless, their plasma concentrations are 1/10th and 1/100th those of the parent molecule, respectively, indicating that bendamustine alone is responsible for the majority of the cytotoxic activity (Preiss *et al*., 1985; Atousa *et al*., 2022).

The major goal of this research was to provide an RP-HPLC (reverse phase-high performance liquid chromatographic) method was used to validate bendamustine HCl in its pure and parenteral dose that was easy to use, precise, accurate, affordable and reproducible. The created and verified approach has better sensitivity, is quick, reproducible and requires less chromatographic run time. The potential use of this approach in a human pharmacokinetic investigation under comparable chromatographic conditions can be investigated further (Mathrusri *et al*., 2012).

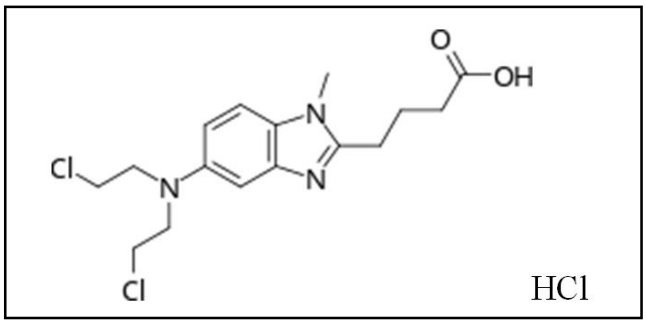

Figure 1: Structural formula of bendamustine HCl.

2. Materials and Methods

2.1 Chemicals

The source of the bendamustine was Hetero Laboratory in Hyderabad. Trifluoroacetic acid, acetonitrile and methanol of HPLC grade, milli-Q water purchased from Merck.

2.2 Instrumentation and chromatographic circumstances

A waters alliance 2998 separation module, empower 3 software and PDA detector made up the HPLC system. A zorbax SB C18 (250 x 4.6 mm) 3.5 µm column was used for the chromatography with mixture of water: acetonitrile (with 0.01% of trifluoroacetic acid) mixed 65:35% v/v ratio as the mobile phase at 1.0 ml/min flow rate, the detection was performed at 230 nm, column temperature was maintained at 30ºC and water: methanol (50:50 v/v) used as diluent. The injection had a 10 µl volume.

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2.3 Method development using HPLC

2.3.1 Solution A preparation

Mix trifluoroacetic acid (1 ml) with 1000 ml of water. Filter with a membrane of pore size 0.45 µm.

2.3.2 Solution B preparation

Mix 1 ml of trifluoroacetic acid with 1000 ml of acetonitrile. Filter with a membrane of pore size 0.45 µm.

2.3.3 Mobile phase preparation

Solution A and solution B should be combined in a degassed mixture at a 65:35 volume ratio.

2.3.4 Diluent preparation

Make a degassed combination of water and methanol in a 50/50 volumetric ratio.

2.3.5 Standard solution preparation

In a 50 ml volumetric flask 25 mg of bendamustine HCl working standard was added. Mix with diluent (10 ml) and then sonicate to dissolve it. Mix after diluting with diluent to volume. A 25 ml volumetric flask was filled with 5 ml of solution. Mix after diluting with diluent.

2.3.6 Sample solution preparation

5 vials should be reconstituted, each with roughly 20 ml of diluent. Then, combine the reconstituted solutions in a 250 ml volumetric flask, swirl it, add diluent to volume and mix. Remove the first few millilitres of the filtrate after passing the solution through membrane filter of 0.45 µm. A 100 ml volumetric flask was filled with 5 ml of solution. Mix after diluting with diluent (both these sample and standard solutions are stable at 5°C temperature up to 48 h).

2.4 Validation of the method

2.4.1 Suitability of the system

After injecting the standard solution five times, measured the area. Five replicate injections were made in the same region and the % RSD was calculated.

2.4.2 Method specificity

Specificity was assessed in order to quantify the analyte precisely and specifically in the presence of components that would be anticipated to be present in the sample. A solution containing all known contaminants was generated at a test concentration of between 0.1% and 1%, and their interference with analyte peak responses was examined (Sasi Kiran and Krishna Reddy, 2013).

2.4.3 Method linearity

The purpose of linearity is to produce test findings that are directly proportional to the analyte concentration in the sample. To measure the peak responses, standard solutions at various concentrations (50 ppm, 75 ppm, 100 ppm, 125 ppm and 150 ppm) were prepared and injected.

2.4.4 Accuracy

Drug substance is quantitatively spiked into a placebo from 50% to 150% of the working test concentration at each level with triplicate preparation and the test technique is then used to assess the results to determine the assay test method's accuracy.

2.4.5 Precision

The 10 µl of blank (diluent), standard solution and sample solutions (six preparations) should be injected into the chromatographic system, recorded and the peak responses should be measured on the same day in order to evaluate precision.

To evaluate intermediate precisions for assay method, six samples were prepared and analyzed through the use of various HPLC systems, columns, analysts and days.

2.4.6 Solution stability

By injecting the sample solution at regular intervals for up to 48 h, the stability of the sample solution was assessed (Mathrusri Annapurna *et al*., 2012).

2.4.7 Robustness

The investigation was done to determine the variability of test findings at various 0.8 ml/min, 1 ml/min and 1.2 ml/min flow rates, temperatures (35°C, 40°C, and 45°C), and mobile phase composition (5%).

3. Results

3.1 Optimization of chromatographic method

The technique was used with a variety of C18 columns, including the ODS, xterra and symmetry columns. Zorbax SB C18 (250 x 4.6 mm) 3.5 µm was discovered to be ideal because, at 1 ml/min flow, it produced good peak form and resolution. Initially, different ratios of trifluoroacetic acid to water and trifluoroacetic acid to acetonitrile were used as the mobile phase. Finally, acetonitrile and water in a ratio of 65:35 v/v each, along with 0.01% trifluoroacetic acid, were chosen for the mobile phase. With good peak shape, retention time (7.76 min), tailing factor 1.3 (not more than 2) and theoretical plate count 10770 (not less than 2000) bendamustine HCl was eluted. The optimized chromatogram was depicted Figure 2.

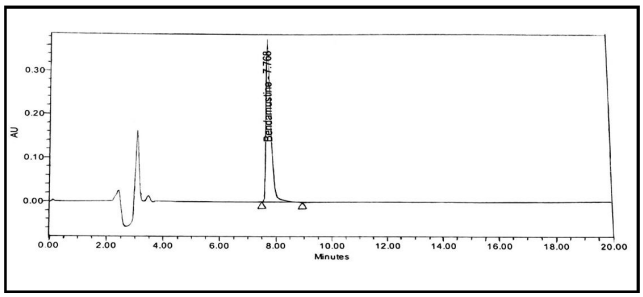

Figure 2: Optimized chromatogram.

3.2 Validation of the method

3.2.1 Suitability system

The results were shown in Table 1 and revealed that the % RSD for the peak region of five replicate injections was within the predetermined limitations, *i.e*., should not be greater than 2.

Table 1: Peak outcomes of bendamustine HCl system suitability

S.No	Name of the peak	RT	Peak area	Peak height	USP plate count	USP tailing
1	Bendamustine HCl injection-I	7.789	4121478	355392	10780	1.32
2	Bendamustine HCl injection-II	7.783	4121754	355148	10743	1.32
3	Bendamustine HCl injection-III	7.780	4110197	353922	10812	1.32
4	Bendamustine HCl injection-IV	7.771	4116198	355430	10756	1.32
5	Bendamustine HCl injection-V	7.779	4117782	355921	10787	1.31
Mean		7.779	4117782	-	-	1.3
SD		0.007	3890.20	-	-	-
% RSD		0.09	0.12	-	-	-

3.2.2 Specificity

The chromatograms made from the diluent, placebo and contaminants in the spiked sample show no interference at the retention period of the bendamustine peak. The chromatogram of the unspiked and spiked samples is given in Figures 3, 4 and the results met the acceptance requirements. Bendamustine HCl in parenteral dose form was determined to be 99.1% pure, indicating that the technique is suitable for the goal for which it is used.

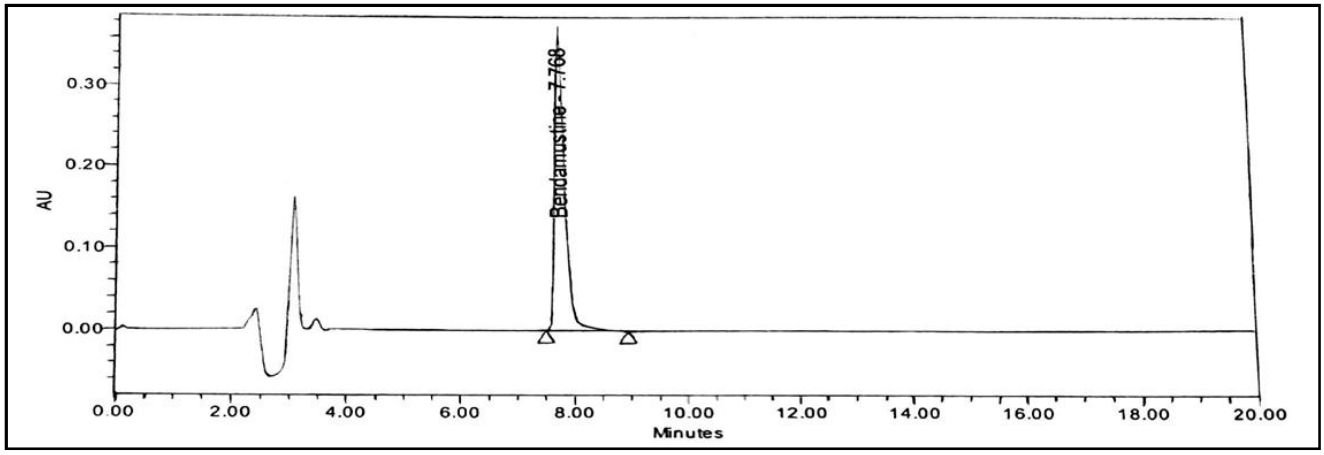

Figure 3: Chromatogram showing unspiked sample.

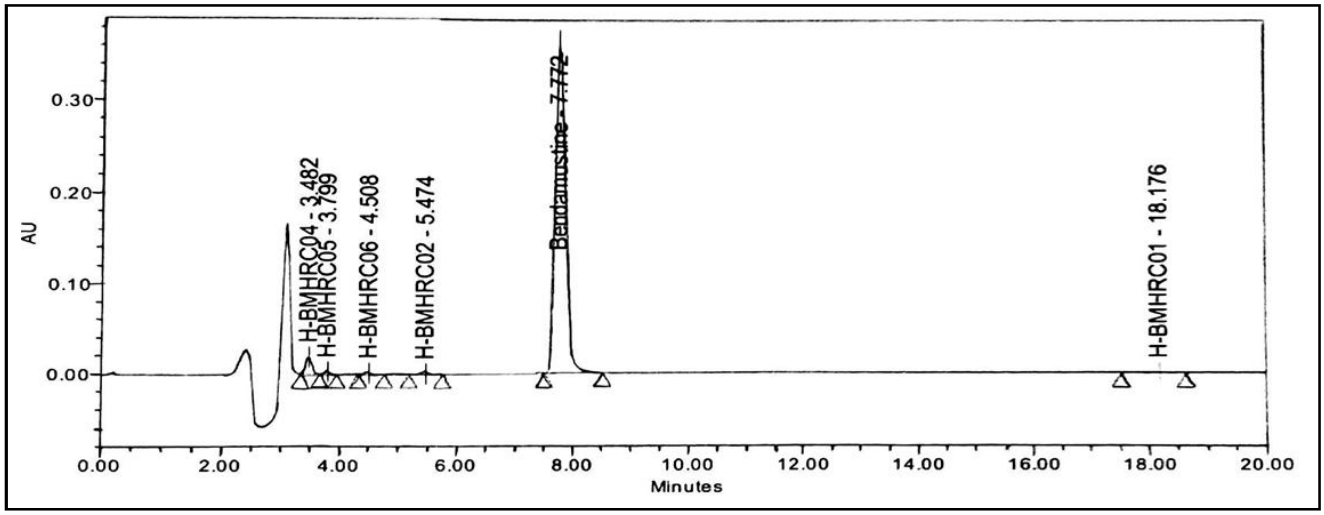

Figure 4: Chromatogram showing spiked sample.

3.2.3 Linearity of the method

The calibration curve's typical equation is as follows: Y = 37780x + 42011.74, r = 0.999. The findings revealed a significant connection between peak area ratio at each concentration of bendamustine HCl within the concentration range. Calibration curve of linear graph was depicted in Figure 5.

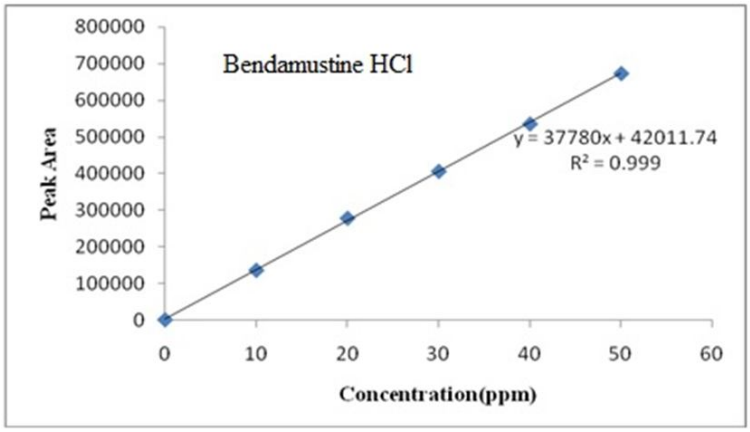

Figure 5: Calibration curve of linearity.

3.2.4 Accuracy of the method

Accuracy was prepared at various concentrations (50%, 100% and 150%), and the recovery percentage was computed. Table 2 lists the results of accuracy. Bendamustine HCl was shown to have a 99% recovery rate overall and a % recovery at each stage. The total mean % RSD and the % RSD at each level were found to be 0.39. The approach is accurate for the use intended since the results meet the acceptance criteria.

Table 2: Accuracy data

Level of accuracy	The sample number	Amount (added in mg)	Amount (found in mg)	Recovery %	Average% recovery	% RSD
50%	1	46.36	46.12	99.48	99.4	0.28
	2	46.42	45.97	99.03		
	3	46.40	46.19	99.37		
100%	1	91.60	90.64	98.75	98.8	0.19
	2	91.67	90.51	98.73		
	3	91.62	90.31	98.57		
150%	1	137.70	135.86	98.66	98.9	0.46
	2	137.78	135.78	98.54		
	3	137.74	136.89	99.38		
Mean % recovery					99.0	
Mean % RSD						0.39

3.2.5 Precision

The results of interday and intraday repeatability for bendamustine HCl was depicted in Table 3, 4. The approach is precise because the percentage RSD for both outcomes was attained within the allowed range.

Table 3: Repeatability findings for bendamustine HCl

S.No.	Name of the peak	Peak area	RT	USP plate count	USP tailing	% assay
1.	Bendamustine HCl injection-I	4157160	7.787	10768	1.17	99.7
2.	Bendamustine HCl injection-II	4133698	7.788	10867	1.18	99.2
3.	Bendamustine HCl injection-III	4149830	7.790	11312	1.22	99.6
4.	Bendamustine HCl injection-IV	4154958	7.791	11349	1.22	99.7
5.	Bendamustine HCl injection-V	4149315	7.792	11334	1.22	99.6
6.	Bendamustine HCl injection-VI	4149684	7.793	11333	1.22	99.6
Mean		4149107	7.790	-	-	99.6
SD		70934.02	0.002	-	-	0.19
% RSD		1.75	0.02	-	-	0.19

Table 4: Results for bendamustine HCl of intermediate precision

S.no	Name of the peak	RT	Peak area	USP plate count	USPtailing	% assay
1.	Bendamustine HCl injection-I	8.216	4374737	12247	1.32	100.9
2.	Bendamustine HCl injection-II	8.220	4295803	12263	1.32	99.1
3.	Bendamustine HCl injection-III	8.218	4278623	12269	1.32	98.7
4.	Bendamustine HCl injection-IV	8.217	4284143	12260	1.32	98.8
5.	Bendamustine HCl injection-V	8.217	4288025	12260	1.32	98.9
6.	Bendamustine HCl injection-VI	8.221	4275805	12252	1.33	98.6
Mean		8.217	4299522	-	-	99.2
SD		0.001	15827.281	-	-	0.87
% RSD		0.01	0.38	-	-	0.88

3.2.6. Stability of the solution

The quantity of bendamustine HCl recovered after 48 h remained constant with the original amounts, which are displayed inTable 5.

Table 5: Results of solution stability for bendamustine HCl

S.no	Peak name	Time intervals (h)	Peak area	RT	% assay	% assay difference
1.	Bendamustine HCl injection-I	Initial	4157160	7.787	99.74	-
2.	Bendamustine HCl injection-II	6	4143937	7.796	99.52	0.22
3.	Bendamustine HCl injection-III	12	4168580	7.795	99.43	0.31
4.	Bendamustine HCl injection-IV	24	4201324	7.807	100.0	0.28
5.	Bendamustine HCl injection-V	48	4020235	8.221	100.8	1.06

3.2.7. Robustness

For bendamustine HCl, the robustness was tested for flow rate fluctuations from 0.8 ml/min to 1.2 ml/min and mobile phase ratio adjustments from more organic phase to less organic phase ratio. The results are provided in Table 6 and the approach is robust at low flow conditions. It is also robust when the mobile phase changes by up to 5% and when the temperature changes.

Table 6: Bendamustine HCl robustness results

Sample parameter	Peak area	RT	Theoretical plates	Tailing factor	% assay	% RSD
Less 0.8 ml/min flow rate	5278576	9.654	11931	1.27	101.7	1.19
More 1.2 ml/min flow rate	3500348	6.533	9948	1.16	99.6	0.19
Low temperature – 35°C	4230545	7.364	11306	1.25	100.2	0.22
High temperature – 45°C	4223167	8.253	10740	1.18	99.7	0.37
Less organic phase	4209086	9.330	11607	1.25	98.8	0.38
More organic phase	4216386	6.821	10754	1.19	100.2	0.33

4. Discussion

Different ratios of trifluoroacetic acid to water and trifluoroacetic acid to acetonitrile were initially utilised as the mobile phase for the optimization of the chromatographic technique. Finally, acetonitrile, water and 0.01% trifluoroacetic acid in a 65:35 v/v ratio were selected as the mobile phase. Bendamustine HCl was successfully eluted with a theoretical plate count of 10770 (not less than 2000), a tailing factor of 1.3 (not more than 2), a nice peak shape and a retention period of 7.76 min.

Bendamustine HCl in parenteral dose form was found to be 99.1% pure, proving that the method is effective for the intended purpose. Both the overall mean % RSD and the % RSD at each level were found to be 0.39. Since the outcomes satisfy the acceptance criteria, the methodology is appropriate for the use intended.

The method is accurate because the percentage RSD for both results was achieved within the permitted range. The fact that the amount of bendamustine HCl recovered after 48 h remained constant with the original levels shows that the medicine is stable. Additionally, it is resilient to temperature variations and changes of up to 5% in the mobile phase.

The peak shape, retention period and tailing of bendamustine HCl were all good. The findings showed that every system suitability parameter for the devised approach was within the acceptable range. The procedure is accurate because the % RSD was obtained within the accepted range.

5. Conclusion

The quantitative determination of bendamustine HCl in parenteral dose form was developed and validated using an accurate, sensitive and precise RP-HPLC approach. The established analytical method is affordable, simple and takes less time to complete than methods that use pre-column derivatization. It is especially useful when tandem mass spectrometric detection is not feasible. As a result, the suggested approach can be quickly used to quantify bendamustine HCl in standard quality control laboratories.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

References

Atousa, H.; Masoud, M.; Mohammad, J.N. and Tahereh, GS. (2022). Bendamustine and pneumocystis pneumonia:A systematic review. Health Sci. Rep., **5**:1-7.

Chow, K.U; Bocher, S.; Geduldig, K.; Krapohl, A.; Hoelzer, D.; Mitrou, P.S. and Weidmann, E. (2001). *In vitro* studies of apoptosis of neoplastic cells in low-grade non-hodgkin´s lymphomas using combinations of established cytotoxic drugs with Bendamustine. Haematologica, **86**(5):485-493.

Dubbelman, A.C.; Rosing, H.; Darwish, M.; Andrea, D. and Bond, M. (2013). Pharmacokinetics and excretion of bendamustine in patients with relapsed or refractory malignancy. Drugs R.D, **13**(1):17-28.

Friedberg, J.W. ;Cohen, P.; Chen, L.; Sue Robinson, K. and Forero-Torres, A. (2008). Bendamustine in patients with rituximab-refractory indolent and transformed non-hodgkin's lymphoma: Results from a phase II multicenter, single-agent study. J. Clinical Oncology, **26**(2):204-210.

Mathrusri Annapurna, M.; Pavani, S.; Anusha, S.; Harika, M. and Venkatesh, B. (2012). New analytical methods for the determination of bendamustine hydrochloride: An anti-neoplastic drug. J. Chem. Pharm. Res., **4**(3):1696-1701.

Mathrusri Annapurna, M.; Venkatesh, B.; Anusha, S. and Neelima, B. (2012). Stability indicating liquid chromatographic method for the determination of bendamustine hydrochloride in parenterals. Research Journal of Chemical Sciences, **2**(9):72-78.

Matt Kalaycio (2008). Clinical experience with bendamustine: A new treatment for patients with chronic lymphocytic leukemia. Clinical Leukemia, **2**(4):223-229.

Martina, B.P.; Vibor, M.; Karla, M.J.; Drazen, P.; Inga, M.R.; Marin, K.; Ana, P.P. and Slobodanka, O.K. (2018). Bendamustine: an old drug in the new era for patients with non-hodgkin lymphomas and chronic lymphocytic leukemia. Acta Clin. Croat., **57**(3):542-553.

Preiss, R.; Sohr, R.; Matthias, M.; Brockmann, B. and Huller, H. (1985). The pharmacokinetics of bendamustine (Cytostasane) in humans. Die Pharmazie, **40**(11):782-784.

Sasi Kiran, E. and Krishna Reddy, V. (2013). Development and validation of RP-HPLC method for determination of related substances of bendamustine hydrochloride in bulk drug. Der Pharmacia Sin., **4**(1): 29-36.

Shi, Y.; Hong, X.; Huang, H.; Xu, W.; Han, X.; Yang, J.; Hellriegel, E. and James, L. (2019). Pb2008 An open-label study of bendamustine hydrochloride in chinese patients with indolent non-hodgkin lymphoma (Nhl) refractory to rituximab treatment. Hema Sphere, **3**(S1):908.

Weide, R.; Heymanns, J.; Gores, A. and Hubert, K. (2002). Bendamustine, mitoxantrone, rituximb (BMR): A new effective regimen for refractory (or) relapsed indolent lymphomas. Leuk Lymphoma, **43**(2):327-331.

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