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RESEARCH ARTICLE

A Validated RP-HPLC Method for the Determination of Bendamustine hydrochloride in Tablet Dosage Form using Gemcitabine hydrochloride as Internal Standard

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A Validated RP-HPLC Method for the Determination of Bendamustine hydrochloride in Tablet Dosage Form using Gemcitabine hydrochloride as Internal Standard

Summary

Using gemcitabine hydrochloride as an internal standard, an accurate, sensitive, precise and robust reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the estimation of bendamustine in bulk and dosage forms. The proposed method was validated as per the ICH guidelines (2005) and can be applied for the quantitative analysis of bendamustine in bulk and pharmaceutical dosage forms. The chromatographic separation was performed on ODS C-18 RP column (4.6 mm i.d x 250 mm) at ambient temperature using a mixture of methanol : water (50:50 v/v) as a mobile phase and at a flow rate of 1.0 ml/ min, while UV detection was performed at 232 nm. The retention times of gemcitabine and bendamustine were in the ranges of 6.647–6.797 and 11.66–12.49 minutes, respectively. The method was found to be linear in the range of 1 – 10 µg/ml. The LOD and LOQ for bendamustine were found to be 0.0422 and 0.1279 µg/ml respectively. Analytical recovery varied from 98.9% to 99.13%. The percentage RSD for precision and accuracy of the method was found to be less than 2%.

Key Words: *Internal Standard, Bendamustine, Gemcitabine, HPLC, Validation.*

İnternal Standart Olarak Gemsitabin Hidroklorür Kullanılarak Tablet Dozaj Formlarında Bendamustin Hidroklorür Tayinini Sağlayan Valide Edilmiş Bir RP-HPLC Yöntemi

Özet

Kütle (bulk) ve dozaj formlarında, bendamustin tayini için gemsitabin hidroklorürün internal standart olarak kullanıldığı, kesin, hassas, doğru ve sağlam bir ters faz yüksek performanslı sıvı kromatografisi (RP-HPLC) yöntemi geliştirilmiştir. Önerilen yöntem, ICH (2005) kurallarına uygun olarak valide edilmiştir; kütle ve farmasötik dozaj formlarında bendamustinin kantitatif analizi için uygulanabilir. Kromatografik ayırım ODS C-18 ters faz kolonda (4.6 mm iç çapx 250 mm), oda ısısında, mobil faz olarak 1.0 ml'lik akış hızında methanol:su (50:50 h/h) kullanılarak gerçekleştirilmiş, ayrım 232 nm'de UV'de incelenmiştir. Gemsitabin ve bendamustinin retansiyon zamanları sırasıyla 6.647-6.797 ve 11.66-12.49 aralığında bulunmuştur. Yöntem, 1-10 µg/ml aralığında doğrusaldır. Bendamustin'in LOD ve LOQ değerleri sırasıyla 0.0422 ve 0.1279 µg/ml'dir. Analitik geri kazanım %98.9'dan %99.13'e değişmiştir. Yöntemin kesinlik ve doğruluğunun yüzde bağıl standart sapmasının %2'den daha az olduğu tespit edilmiştir.

Anahtar kelimeler: *İnternal Standart, Bendamustin, Gemsitabin, HPLC, Validasyon*

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INTRODUCTION

Bendamustine hydrochloride is an antineoplastic agent with minimal side effects. It is not an official drug in any pharmacopeia. Chemically it is 4-[5-[Bis (2-chloroethyl) amino]-1-methyl benzimidazol-2-yl] butanoic acid (Fig.1). Bendamustine is a bi-functional alkylating agent consisting of a purine and amino acid antagonist (a benzimidazole ring) and an alkylating nitrogen mustard moiety (1). It is used in the treatment of chronic lymphocytic leukemiae and sarcoma, and its route of

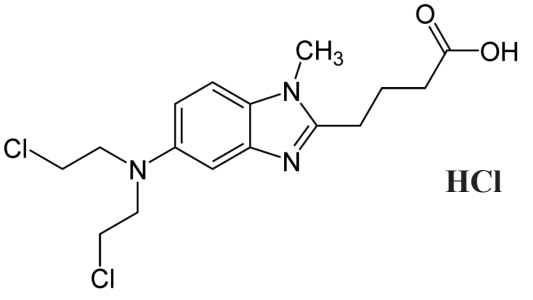

Figure 1. Structure of bendamustine hydrochloride

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administration is intravenous (2-3). It exhibits cytotoxic activity against human ovarian and breast cancers *in vitro.* Substituting bendamustine for cyclophosphamide in the CMF protocol (cyclophosphamide, methotrexate and fluorouracil) ended up with a prolonged remission from 6.2 to 15.2 months in patients with metastatic breast cancer (4). It exhibits a synergistic effect with fludarabine (5). A combination of bendamustine and gemcitabine (Fig.2) was used to study the efficacy and tolerability in patients suffering with metastatic breast cancer and relapsed ovarian cancer. In patients with breast cancer, partial remission and stable disease was observed in 22% and 44% respectively. But, this study was preterm discontinued due to severe myelotoxicity and fatigue (6). Combination of DNA repair inhibition with bendamustine or gemcitabine in the treatment of cancer was studied by Alfonso Bellacosa et al (7) wherein they reported enhancement of tumor cell sensitivity to the agent that induces DNA damage.

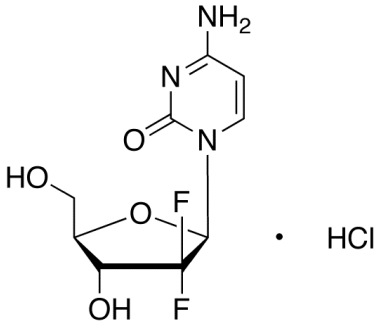

Figure 2. Structure of gemcitabine hydrochloride

Literature survey revealed that spectrophotometric (8–9), LC–MS (10-11) and capillary GC method (12) have been developed for the estimation of Bendamustine hydrochloride in blood plasma / urine samples. Earlier proposed HPLC (13–16) methods utilize acetonitrile as a solvent in mobile phase which is toxic in nature. To the knowledge of authors, there are no reports on the usage of internal standard for the determination of bendamustine. Hence, a new RP-HPLC method has been reported in the present investigation for the estimation of bendamustine hydrochloride in bulk and tablet dosage form using gemcitabine hydrochloride as internal standard (I.S.) and replacement of acetonitrile with a cheap and non-toxic solvent like methanol.

MATERIAL AND METHODS

Materials: The pharmaceutical grade pure sample of bendmustine (99.98%) and gemcitabine hydrochloride (99.73%) were procured from GSN Pharmaceutical Laboratories Limited, Hyderabad, India. HPLC grade (E. Merk) methanol and triple distilled water (TD water) were used.

Apparatus: Agilent 1120 Compact LC HPLC system consisting of a gradient liquid pump, LC UV-Visible spectrophotometric detector, ODS C-18 RP column (4.6mm x 250mm), 20 µl Hamilton injecting syringe and a window based single channel software were used. An Essae electronic balance was used for weighing. A nylon membrane filter having 0.45 µm pore size (Sigma–Adrich make) was used for filtering the mobile phase.

Preparation of standard stock solution: Standard stock solutions of bendamustine hydrochloride and gemcitabine hydrochloride were prepared by dissolving 25 mg of the drug in 25 ml of the mixture of methanol and water (50:50) to get 1.0 mg/ml solution. This standard stock solution was suitably diluted with mobile phase (methanol & water in the ratio of 50:50 v/v) to get the working solution of 100 µg/ml concentration.

Chromatographic conditions: Prior to use, mobile phase was filtered through nylon membrane filter having 0.45 µm pore size and then degassed by ultrasonification. A number of alterations in mobile phase characteristics (both solvent and composition) and flow rates were performed in order to optimize the operating conditions for isocratic RP-HPLC detection of bendamustine by facilitating separation as well as improvement of chromatographic system performance. Ease of separation, retention time, peak parameters (resolution, tailing and symmetry) were considered for the selection of mobile phase. Mixtures of various ratios of methanol: water (50:50, 40:60, 60:40 v/v) and of methanol: acetonitrile (50:50, 40:60, 60:40 v/v) were used for these studies to ascertain system suitability. The effect of flow rate on the separation of peaks of the studied compounds was studied in the range 0.8–1.2 ml/min. The appropriate wavelength in UV region (232 nm) was selected for the measurement of active ingredients in the proposed method.

RESULTS AND DISCUSSION

Internal Standard:

Gemcitabine hydrochloride was used as internal standard (IS) for the experiments of proposed method because (i) it gives a well-defined and symmetrical peak (retention time 6.647–6.797 minutes) which is well resolved from bendamustine peak (retention time 11.66-

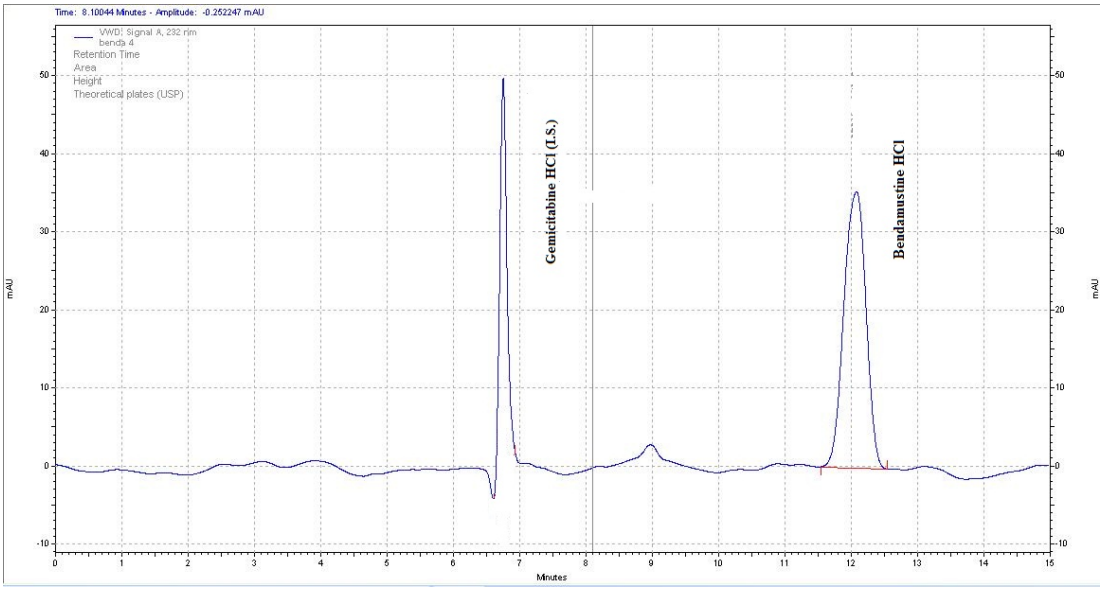

Figure 3. A typical chromatogram of bendamustine HCl (6.0 µg/ml) and gemcitabine HCl (I.S.) (10.0 µg/ml)

12.49 minutes), (ii) it can be detected comfortably at the measured wavelength (232 nm), (iii) could be eluted using a mixture of methanol and water without using any buffer, (iv) it is a chemical that is found easily in most of the quality control laboratories (17). In the present study, it was used in concentration of 10 µg/ ml.

Method development:

Well resolved and sharp peaks for bendamustine and gemcitabine were obtained by using a mobile phase of methanol: water (50:50 v/v) mixture (Fig.3), whereas, peak splitting was observed in 60:40 and 80:20 of methanol: water. The splitting of peak at higher volumes of methanol can be attributed to the anomalous weak peak of derivative developed by incubating either drug or internal standard with the traces of formaldehyde associated with methanol (18). Acetonitrile was the solvent in the methods suggested by others (13–16), but in the present method methanol replaced acetonitrile due to its toxic nature (19). Methanol was selected as a solvent in eluting mobile phase in view of added advantages viz., (i) it is inexpensive, (ii) it is non-toxic / environmental friendly / less damaging to health (20), (iii) it is suitable for repetitive separations and can be recycled by distillation (21), (iv) it provides improved efficiency by reducing peak tailing and forming hydrogen bonding with silanols (22), (v) it decreases the menace of salt precipitation in narrow capillaries as methanol is an organic polar solvent, which in turn was accountable for moderate back pressure for prolonged periods and hence an improved operation efficiency can be observed in HPLC (23). Moreover, the overlap between the peaks was observed when methanol was replaced by acetonitrile whereas; broadening of the peaks was observed when buffer was used instead of water. Hence, a mixture of methanol and water (50:50 v/v) was the most suitable composition. After performing the chromatographic separation of the drug at different flow rates, it was found that flow rate of 1.0 ml / min showed a well-defined and symmetrical peak for bendamustine peak (retention time 11.66-12.49 minutes), which is well resolved from internal standard (retention time 6.647–6.797 minutes).

In the present study, ODS C18 analytical column was chosen as the stationary phase due to excellent selectivity and low cost (24). Optimized chromatographic conditions are shown in Table 1.

Method validation:

After developing the method, the described method for the assay of bendamustine using gemcitabine as internal standard was validated by ICH guidelines (2005) (25) by evaluation of main validation parameters such as linearity and range, accuracy and precision, recovery, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity: Working standard solutions of bendamustine (concentration ranges from 1 to 10 µg/ml) containing gemcitabine (10 µg/ml) as an IS were prepared using mobile phase as a solvent. Twenty microliters of each solution were injected into the HPLC system to obtain the chromatogram. For each prepared standard solution,

Table 1. Optimized chromatographic conditions

Chromatograph	Agilent 1120 Compact LC HPLC system
Elution	Isocratic
Mobile phase	Methanol: water (50:50 v/v)
Column	ODS C-18 RP (4.6 mm i.d x 250 mm)
Flow rate	1 ml / min
Detection	UV at 232 nm
Injection volume	20 micro liters
Temperature	Ambient
Retention time of Gemcitabine HCl (I.S.) of Bendamustine HCl	 6.647-6.797 minutes 11.66-12.49 minutes
Run time	15 minutes
Resolution : Between the peaks of I.S. and Bendamustine HCl	5.427 min

Table 2. Calibration curve for the estimation of Bendamustine hydrochloride by proposed RP HPLC method

Concentration (µg/ml)		Ratio of AUC of Bendamustine HCl to I.S.
Bendamustine HCl	Gemcitabine HCl (I.S.)	
1	10	0.4875
2	10	0.6988
4	10	1.3471
6	10	1.7986
8	10	2.2192
10	10	2.5690
Regression equation Slope (a) Intercept (b) Correlation coefficient		Y = a X + b 0.23651 0.298067 0.994984

ratio of the peak height for drug (bendamustine) to the peak height for the internal standard (Gemcitabine) was calculated (Table 2). A standard curve was constructed by plotting peak area ratio [drug/I.S.] against the concentration in µg/ml. The graph was found to be rectilinear over the concentration range of 1.0 to 10 µg/ml. The linear regression equation for the taken concentration range was Y = 0.23651 X + 0.298067 with correlation coefficient (r^2) of 0.995. Furthermore, the regression equation was used to estimate the amount of bendamustine hydrochloride in tablet dosage form.

Precision: A standard solution containing 4 µg/ml of bendamustine hydrochloride and 10 µg/ml of gemcitabine hydrochloride was prepared to calculate the precision using the developed method. The prepared solution was injected into the HPLC system in six replicates under the same chromatographic conditions. The chromatograms were recorded for the intra and inter day variations. The values were given in Table 3. Percentage R.S.D. for the calculated values was 0.196. As RSD% < 2, the results of the precision study indicate the reliability of the method (26).

Recovery studies: The solutions of bendamustine hydrochloride containing 2 µg/ml were subjected to the proposed HPLC method of analysis for finding out the recovery. The recovery studies were carried out by adding known amount of bendamustine hydrochloride to the pre-analyzed sample and subjected them to the proposed HPLC method of analysis. Results of recovery studies were found in between 98.9% to 99.13% and are shown in Table 4.

Limit of Quantification (LOQ) and Limit of Detection (LOD): The LOQ and LOD were established at a signal to noise ratio 1:10. The LOD and LOQ of

Table 3. Precision data

Day	Mean Ratio of AUC of drug to I.S*	% R.S.D.*	Mean % R.S.D.
Day- 1	1.34465	0.247	0.196
Day-2	1.34020	0.151	
Day-3	1.34242	0.190	
* Average of six determinations			

Table 4. Recovery studies of the proposed HPLC method

Concentration of Bendamustine HCl in sample solution (µg/ml)	Amount added (µg/ml)	Total amount (µg/ml)	Amount found (µg/ml)	% Recovery*	Mean
2	1	3	2.974	99.13%	99.01%
2	2	4	3.956	98.90%	
2	3	5	4.950	99.00%	
* Average of three determinations					

Table 5. Results of analysis of tablet containing Bendamustine HCl and recovery studies

Pharmaceutical formulation	Amount of Bendamustine HCl (mg) *		% of recovery
	Labeled	Found	
Bendit	100	99.88	99.88%
* Average of three determinations			

bendamustine hydrochloride were calculated using σ (standard deviation of the response) and *S* (slope of the calibration curve), and found to be 0.0422 µg/ml and 0.1279 µg/ml respectively.

LOD = 3.3 × σ /S = 0.0422 µg /ml and

LOQ = 10 × σ /S = 0.1279 µg /ml

Estimation of Bendamustine hydrochloride in tablet dosage form: Ten tablets were procured and the tablet powder was used for estimation. To an accurately weighed portion of powder equivalent to 25 mg of bendamustine hydrochloride, an equal quantity of internal standard was added. The drug contents were dissolved in 25 ml of the mixture of methanol and water (50:50 v/v) with ultrasonification and filtered through 0.45 µm membrane filter. From the filtrate, 0.1 ml was pipetted in to 10 ml graduated test tube and made up to volume with the mobile phase. 20 µl of the sample was injected in to the column. The drug content in the tablet was quantified using the regression equation and the results are given in Table 5. Good recovery values of drug (99.88%) shows that the proposed method can be successfully applied to the determination of bendamustine in pharmaceutical formulations without any interference from common excipients.

CONCLUSION

A simple, selective, rapid, precise and economical reverse phase HPLC method has been developed for the determination of bendamustine hydrochloride in tablet using gemcitabine hydrochloride as an internal standard, where a well resolved peak was observed for internal standard from that of drug. As the run time is only 15 min, the method is rapid. In the present case, methanol replaced the acetonitrile, a costly and toxic solvent used by earlier workers. Linearity was found in range of 1–10 µg/ml for the standard curve constructed by plotting peak area ratio [drug/I.S.] against the concentration and linear regression equation was Y = 0.23651 X + 0.298067 with correlation coefficient (r^2) of 0.995. As RSD% < 2, the results of the precision study indicate the reliability of the method. Results of recovery studies were found in between 98.9% to 99.13%. The LOD and LOQ of bendamustine hydrochloride were found to be 0.0422 µg/ml and 0.1279 µg/ml respectively. The extracts of the formulations containing bendamustine hydrochloride showed no significant peaks except that of bendamustine hydrochloride which indicates that the excipients in the solid dosage form are not interfering in the estimation by this method and therefore this method is found to be specific.

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