WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 5, 720-729. Research Article ISSN 2277– 7105

LIQUID CHROMATOGRAPHY MASS SPECTROMETRIC METHOD FOR QUANTIFICATION OF POTENTIAL GENOTOXIC IMPURITY IN BENDAMUSTINE HYDROCHLORIDE

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Article Received on
28 Feb. 2017,

Revised on 19 March 2017,
Accepted on 09 April 2017

DOI: 10.20959/wjpr20175-8320

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ABSTRACT

A new, simple, accurate and sensitive method was developed for the quantification of potential genotoxic impurity at low level (5.4 ppm) in Bendamustine Hydrochloride using Liquid Chromatographic Mass Spectrometry (LC-MS/MS). The chromatographic separation was achieved on Reliant C18, 5µ (150 x 4.6) mm column with gradient programme and elution was monitored by mass spectrometer in Multiple Reaction Monitoring mode using electrospray ionization. The LOD and LOQ values found to be 0.15 ppm and 0.5 ppm for the impurity with respect to the test concentration 0.5mg/ml. The method was linear (r2>0.99), precise (RSD<2%), accurate and well within acceptable ICH limits.

KEYWORDS: Potential genotoxic impurities, Bendamustine Hydrochloride, LC-MS/MS, Multiple Reaction Monitoring (MRM), Threshold of Toxicological Concern (TTC).

INTRODUCTION

Bendamustine hydrochloride (figure 1) chemically known as (4-{5-[bis-(2-chloroethyl) amino]-1-methyl-1Hbenzimidazol-2-yl} butanoic acid) is an active nitrogen mustard. It is used for the treatment of patients with chronic lymphocytic leukaemia. Bendamustine was developed to have a dual action combining the alkylating activity of the nitrogen mustards with the antimetabolite properties of purine analogues. Even so, bendamustine appears to act

primarily as an alkylating agent which causes the formation of intra-strand and inter-strand cross-links between DNA bases. In vitro, bendamustine inhibited the growth of several leukaemia cell lines, including acute lymphocytic leukaemia (ALL), acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML) and non-Hodgkin's lymphoma cells.[1-5]

In order to synthesize APIs effectively, it is necessary to build up the molecular structure through the combination of simple structural moieties. This typically involves the formation of carbon-carbon, carbon-nitrogen and carbon-oxygen bonds. The current status of synthetic methodology is such that this is impractical to achieve without the use of electrophilic species that fall into the broad class of alkylating agents and are hence potentially genotoxic. Thus many reactive starting materials, intermediates and reagents used in the synthesis of APIs are potentially genotoxic and furthermore may present as residual impurities within the API. Hence it is important to identify the presence of genotoxic impurities in drug substances followed by monitoring and controlling in very low levels to ensure patient safety.[6-8] First, materials present in the synthesis are screened for potential genotoxicity, typically through the application of an appropriate structure activity relationship (SAR) process, using commercial systems such as SARAH and DEREK.[9]

The allowable levels of PGI's are determined by a staged toxicological threshold of concern (TTC) based on both the dose and duration of the intended clinical study. This allowable amount can be in low ppm range, which is much lower than the allowable levels of non-PGI impurities controlled under ICH Q3A guideline. This TTC value was estimated to be 1.5 µg/person/day.[10-15] From the evaluation study of genotoxic and carcinogenic impurities in the synthetic scheme of Bendamustine Hydrochloride, one intermediate (Fig.2) has been considered as one of the potential genotoxic impurities as per structural alert. The maximum daily dose of Bendamustine is 150mg/m^2 body surface. Assuming a standard body surface of 1.874 m^2 for a height of 1.70m and weight of 76 kg the maximum daily dose will be 281 mg (For calculation of Body Surface Area (BSA) the DuBois formula was used). So the TTC value for Bendamustine is 5.4 ppm (1.5/280*1000) justified. From the literature survey there is no method reported for the low level quantification of this potential genotoxic impurity in Bendamustine Hydrochloride.

Development of a method for the determination of potential genotoxic impurity at very low level using conventional analytical instruments (HPLC, UV and FID) is a great challenge in Pharmaceutical industry as the sensitivity of these instruments is low. Hence an instrument

with greater sensitivity is required for precise and accurate quantitation at low levels. This can be achieved by combining HPLC with Mass Spectroscopy (MS). In the present work high sensitive and reliable, LC/MS/MS method was developed and validated for the quantification of this impurity-I in Bendamustine Hydrochloride. The proposed method is a direct, sensitive and robust which involves MRM mode with electrospray ionization to achieve very low LOD and LOQ for quantification of potential genotoxic impurity in Bendamustine Hydrochloride.

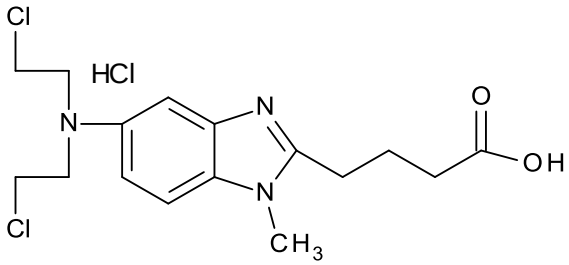

Fig.1: Bendamustine Hydrochloride

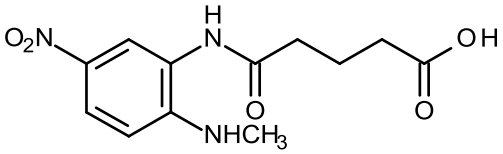

Fig.2: 5-(2-(Methylamino)-5-nitrophenylamino)-5-oxopentanoic acid (Impurity-I)

MATERIALS AND METHODS

LC-MS grade ammonium formate was purchased from Sigma-Aldrich. HPLC grade acetonitrile and methanol were purchased from JT Baker (Mumbai, India). Purified water collected through Milli-Q Plus water purification system (Millipore, USA). Bendamustine Hydrochloride and its potential genotoxic impurity (Impurity-I) were obtained from Natco Pharma Ltd, Natco Research Centre, and Hyderabad, India.

Instrumentation

The LC-MS/MS method development and validation was done using Waters Xevo TQ-S micro system including Acquity UPLC H-Class system connected to Mass detector equipped with electrospray ionization in positive mode.

LC-MS/MS chromatographic conditions

The analysis was carried out using Reliant C18, 5µ (150 x 4.6) mm with a flow rate of 0.6 ml/min. The mobile phase used was a mixture of 10mM ammonium formate as mobile phase-A and acetonitrile and methanol in the ratio of 50:50 as mobile phase-B using a gradient programme of Time(mins)/%A: 0/70, 10/70, 15/20, 20/20, 25/70, 30/70. The column temperature was maintained at 40°C and the injection volume was 10µl. Mass spectrometer was operated in electrospray ionization with positive ion mode with a capillary voltage of 3000V. The collision energy was set at 20V, cone voltage was 30V, the dissolvation gas flow was 1000 L/hr with a temperature of 525°C and source temperature was 150ºC. Under these conditions the potential genotoxic impurity in Bendamustine Hydrochloride was quantified by MRM mode with the transition 282.13 > 168.13(protonated).

Preparation of standards and test sample solutions

The standard stock solutions of impurity-I was prepared approximately at 10 ng/ml in diluent. The Bendamustine Hydrochloride test samples were typically prepared at 0.5mg/ml in diluent.

RESULTS AND DISCUSSION

Method development

The aim of the present work was to develop a method that could successfully separate and quantify the potential genotoxic impurity (Impurity-I) in Bendamustine Hydrochloride, different stationary phases and mobile phases were used and finally the desired chromatographic separation was achieved on a Reliant C18, 5µ (150 x 4.6) mm with a flow rate of 0.6 ml/min. The mobile phase used was a mixture of 10mM ammonium formate as mobile phase-A and acetonitrile and methanol in the ratio of 50:50 as mobile phase-B using a gradient programme of Time(mins)/%A: 0/70, 10/70, 15/20, 20/20, 25/70, 30/70.

Method validation

The method has been validated for the quantification of impurity-I in Bendamustine Hydrochloride to ensure that the performance characteristics of the method meet the requirements for its intended analytical applications. During the method validation the assessed parameters were specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, precision and accuracy.

Limit Of Detection (LOD) and Limit Of Quantification (LOQ)

The LOD and LOQ were calculated with signal to noise ratios of 3:1 & 10:1 respectively and by injecting a dilute solution having known concentrations of impurity-I and established the minimum level at which the impurity-I can be reliably detected. The LOD is 0.15 ppm and LOQ is 0.5 ppm obtained for the impurity.

System precision and system suitability

The precision and system suitability was performed by injecting six replicates of the working standard solution (5.4ppm of the impurity-I with respect to the test sample concentration). The %RSD for the peak areas obtained was calculated. The data presented in the table 1 establishes system precision. The blank chromatogram and Reference solution chromatogram for the impurity-I are represented in Figure-3 and figure-4.

Table 1: **System suitability and system precision**

S.No	Peak area for Impurity-I
1	20483.43
2	21420.46
3	21256.26
4	22014.80
5	22792.91
6	21296.22
Mean	21544.01
% RSD	3.63

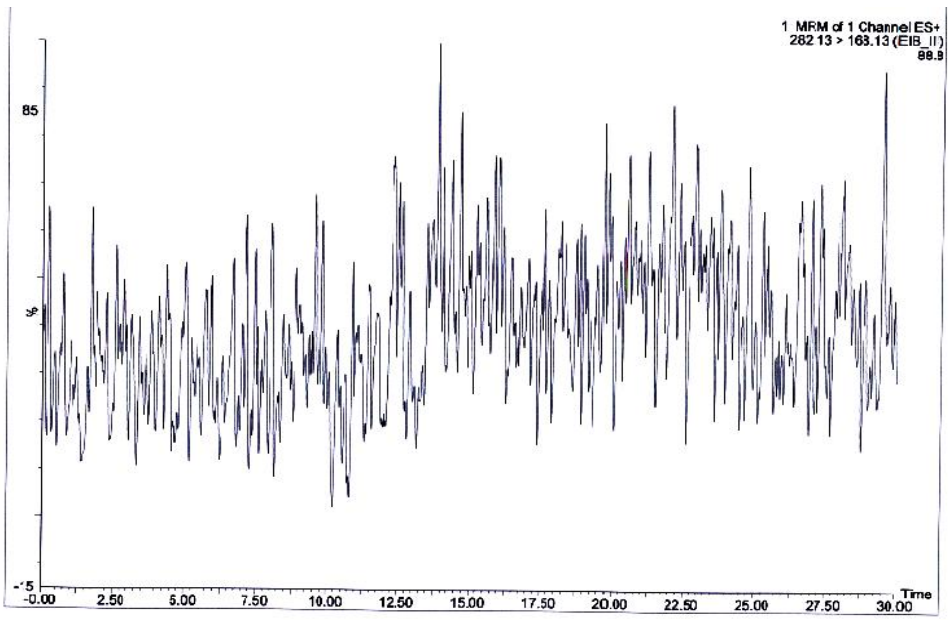

Fig.3: **Blank chromatogram**

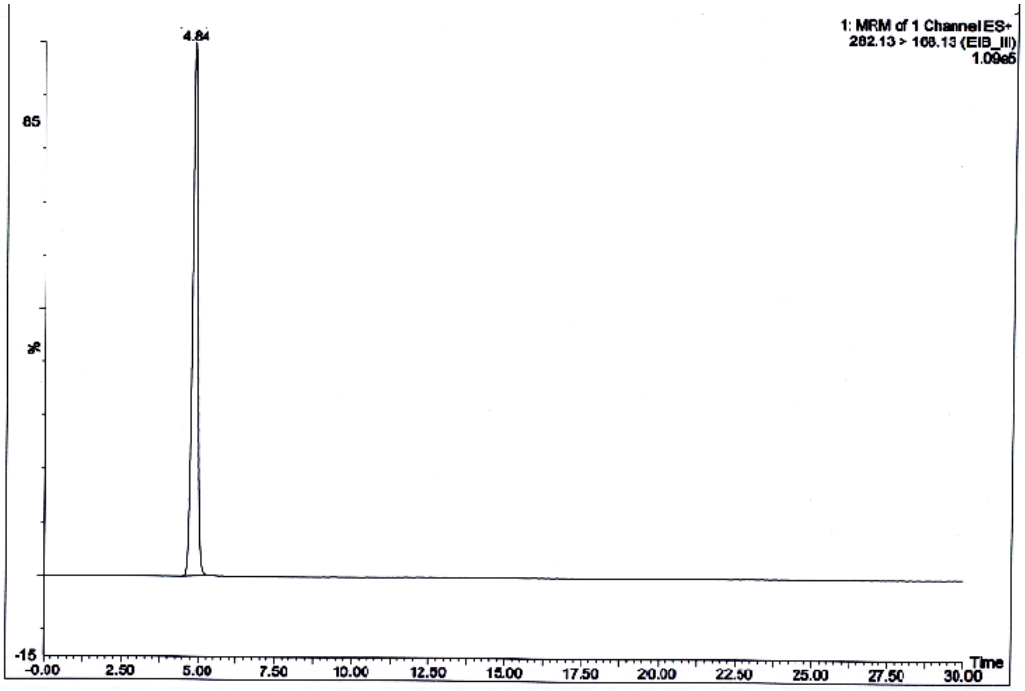

Fig.4: **Reference solution (5.4 ppm) chromatogram**

Specificity

The specificity of the optimized method was performed by injecting stock solution of impurity-I to check resolution between the impurity-I and Bendamustine Hydrochloride under the same conditions mentioned in LC-MS chromatographic conditions. The specificity is represented in the Figure.5. Summary of retention time and m/z value for Bendamustine Hydrochloride and impurity-I are mentioned in the given table 2.

Table 2: Summary of retention time and m/z values

Compound	**Retention Time(Min)**	**Mass(m/z) value** (+ve)
Impurity-I	4.9	282.1
Bendamustine Hydrochloride	16.4	358.1
Bendamustine thio impurity	15.3	354.1
Bendamustine di hydroxy impurity	2.6	322.1
Bendamustine IPA ester impurity	20.2	400.1
Bendamustine dimer impurity	18.5	679.4

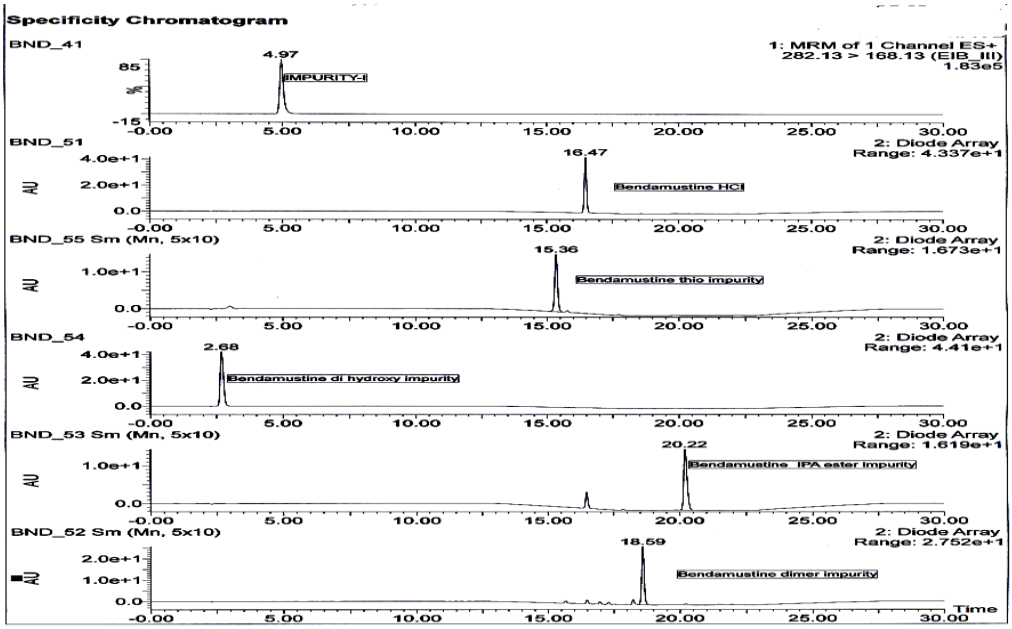

Fig.5: **Specificity chromatogram**

Linearity

Linearity of the method was checked by preparing the solutions at 7 concentration levels from LOQ to 150% of specification limit (0.5, 1.0, 2.7, 4.05, 5.4, 6.48 and 8.1 ppm). The mean responses recorded for impurity-I were plotted against concentration. The correlation coefficient of linear regression was found to be greater than 0.99 for impurity, indicating good linearity. Corresponding linearity graph is shown in Figure 6 and data is represented in the Table 3.

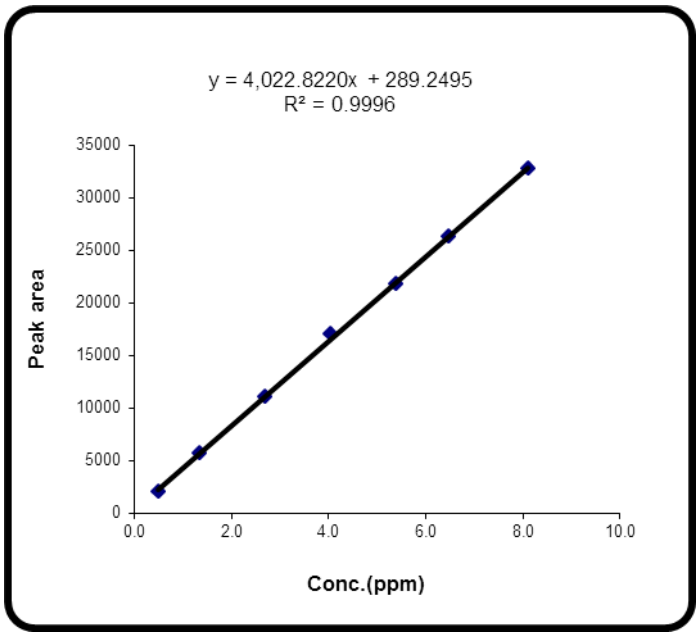

Fig.6: Linearity graph for Impurity-I

Table 3: Linearity data

LEVEL	Impurity-I peak area
LOQ (0.5 ppm)	2120
1.35 ppm	*5754*
2.7 ppm	*11086*
4.05 ppm	*17043*
5.4 ppm	21874
6.48 ppm	*26357*
8.1 ppm	*32763*
Slope	*4022.82*
R^2	***0.9996***

Accuracy

Accuracy of the method was evaluated by using two solutions containing Bendamustine Hydrochloride spiked with the impurity-I at LOQ, 50%, 100% and 150% of the specification limit (5.4 ppm). Each concentration level was prepared in triplicates. The percentage recovery results obtained for the impurity-I are listed in Table.4. A representative spiked chromatogram at LOQ level is shown in Figure 7.

Table 4: Summary of Recovery study for impurity-I

TEST+SPIKED (n=3)	% Recoveries for Impurity-I
Test+LOQ spiked (0.5 ppm)	98.7
Test+50% spiked (2.7 ppm)	99.5
Test+100% spiked (5.4 ppm)	96.4
Test+150% spiked (8.1 ppm)	93.2

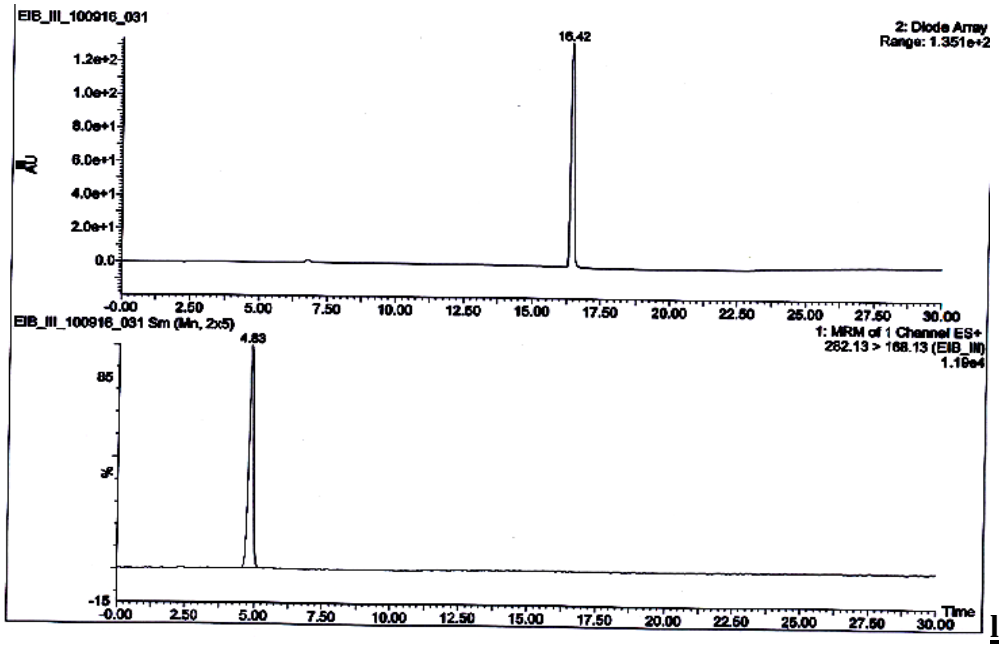

Fig.7: LOQ spiked chromatogram

CONCLUSION

The proposed LCMS method is simple, sensitive and accurate to quantify potential genotoxic impurity (Impurity-I) at ppm level present in Bendamustine Hydrochloride. The validated parameters are well within the limits and this method is found suitable for routine quality control test of Bendamustine Hydrochloride.

ACKNOWLEDGEMENT

The authors are grateful to the Management of Natco Pharma limited for the facility and support during the work.

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