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LIQUID CHROMATOGRAPHY MASS SPECTROMETRIC METHOD FOR QUANTIFICATION OF POTENTIAL GENOTOXIC IMPURITY 2, 6-DIAMINOPYRIDINE IN MINODRONIC ACID

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ABSTRACT

A new, simple, accurate and sensitive method was developed for the quantification of potential genotoxic impurity 2, 6-Diaminopyridine in Minodronic acid using Liquid Chromatographic Mass Spectrometry (LCMS). The chromatographic separation was achieved on ZIC HILIC, 5u (150 x 4.6) mm column with isocratic programme and elution was monitored by mass spectrometer in Single Ion Monitoring mode using electrospray ionization. The LOD and LOQ values were found to be 1.0 ppm and 3.0 ppm respectively for the impurity at the test concentration of 0.5mg/ml. The method was linear (r2>0.99), precise (RSD<2%), accurate and well within acceptable ICH limits.

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INTRODUCTION

Regulatory authorities all over the world require data on the genotoxic potential of new drugs, as part of the safety evaluation process. The pre-clinical studies are generally conducted to obtain the basic toxicological profile of new chemical entities (NCE). The toxicological data are used to evaluate the safety and efficacy of a NCE, which will help in predicting the drug's likely risk/benefit assessment in the New Drug Application (NDA) process. Genotoxicity assays have become an integral component of regulatory requirement.

Genotoxins can be carcinogens, or cancer-causing agents, mutagens (mutation-causing agents) or teratogens (birth defect-causing agents). In most cases, genotoxicity leads to mutations in various cells and other body systems. Mutations can lead to a host of other problems, from cancer to a wide variety of other disorders. Mutations can arise in many different forms; genetic information can be duplicated, deleted or inserted. Therefore these impurities must be accurately identified, measured and controlled during various stages of pharmaceutical development. In most cases, these toxic impurities can be effectively purged out or removed during early stages of development of drug substance. When purging is not achievable, GTIs must be monitored on a routine basis at or below the "Threshold of Toxicological Concern" (TTC).

The best way to avoid toxicity resulting from administration of a drug is to focus on something which can raise an alarm to the possible genotoxicity of a drug based on certain SAR analysis and focusing on the functional groups attached. This brings in the concept called – 'Structural Alerts' which was first elucidated by Ashby and Tennant [1]. This concept was so accurate that different regulatory bodies like EMEA and FDA accepted the use of in-silico methods to assess genotoxic substances based on structural alerts. Several structural alerts as proposed by Ashby and Tennant play a key role in genotoxicity assessment. 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 [2-6]. 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.

The process involved in the assessment of risk of genotoxic impurity is a multi-step one, which starts with the identification and ends with finalization of risk and has the following sequence [7].

Step 1:

Involves the identification of potential impurities in drug substances and drug products by reviewing the synthetic process including synthetic materials, reagents, intermediates, known impurities, drug substances and product degradants.

Step 2:

Involves conduction of SAR evaluation by in-silico methods.

Step 3:

Involves search for any structural alerts. If not found then considered as general impurity and if found then proceeded for next step.

Step 4:

Here assessment of the magnitude/significance of risk of potential carryover of impurities is performed in drug substances and drug products. If found insignificant then no action is taken.

Step 5:

If the risk is determined to be significant in step 4 then quantification of the level of impurity is done or safety testing is performed by Ames test.

Step 6:

Here based upon the prescribed limits for threshold of toxicological concern (TTC) in the guidelines, risk assessment is finalized. If found non genotoxic, then treated as a general impurity.

Step 7:

Here final decision is made based upon the acceptable TTC values as per the guidelines for the genotoxic impurity. Thus, if the level of genotoxic impurity is less than the TTC value then it is considered suitable for clinical use and if the level is more, then strategy are formulated to achieve acceptable limits by modification of the synthetic pathway and conduction of in vivo genotoxicity testing.

Minodronic acid is a compound represented by the following structure (Fig.1), which has excellent bone resorption inhibitory activity, anti-inflammatory activity and analgesic- antipyretic activity. It is also useful in the treatment of diseases involving bone resorption, such as Paget's disease, hypercalcemia, bone metastasis of cancer, osteoporosis, as well as progress in the bone resorption caused by inflammatory joint diseases such as rheumatoid arthritis [8-9].The maximum dose of Minodronic acid is 50 mg once for a four week period. From the evaluation study of genotoxic and carcinogenic impurities in the synthetic scheme of Minodronic acid, through the application of an appropriate structure activity relationship (SAR) process, using commercial systems such as SARAH and DEREK, an intermediate 2, 6-Diaminopyridine (Fig.2) has been considered as the principal and potential genotoxic impurity as per structural alert. There is no reported method for the quantification of this potential genotoxic impurity at low levels in Minodronic acid.

Development of an analytical method using hyphenated techniques have become a break-through in the quantification of potential genotoxic impurities at ppm levels. Different analytical instruments used in pharmaceutical industry for the detection of impurities are HPLC-UV, GC-FID and NMR with different sensitivity levels capable of detecting impurities at low levels. The detection levels at very low levels such as sub ppm levels can be enhanced with the combination of mass spectrometry with other analytical techniques like LC-MS, GC-MS and ICP-MS etc. All these instruments have high sensitivity as well as high selectivity to differentiate impurities and deal with compounds of low molecular weight and volatile impurities. In the present work, highly sensitive and reliable, LC/MS method was developed and validated for the quantification of the impurity 2, 6-Diaminopyridine (Fig.2) in Minodronic acid. The proposed method is direct, sensitive and robust which involves SIM mode with electrospray ionization to achieve very low LOD and LOQ for quantification of 2, 6-Diaminopyridine in Minodronic acid.

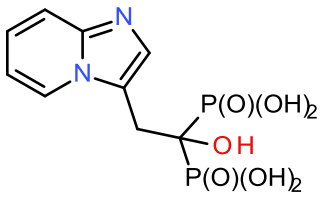

Fig.1: Minodronic acid.

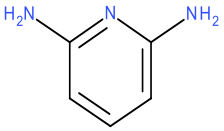

Fig.2: 2, 6-Diamino pyridine.

MATERIALS AND METHODS

LC-MS grade ammonium acetate 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). Samples of Minodronic acid and its potential genotoxic impurity 2, 6-Diaminopyridine were obtained from the synthetic chemistry section, Natco Research Centre, and Hyderabad, India.

Instrumentation

The LC-MS method development and validation was done using Agilent 1200 series HPLC system connected with Agilent mass spectrometer G6120B Single Quad, equipped with electrospray ionization in positive mode.

LC-MS chromatographic conditions:

The analysis was carried out using ZIC HILIC (150 x 4.6) mm with a flow rate of 0.6 ml/min. The mobile phase used was a mixture of 10mM Ammonium acetate+0.1% Acetic acid and acetonitrile using isocratic programme of Mobile phase-A:Mobilephase-B:30:70. The column temperature was maintained at 30°C and the injection volume was 5µl. Mass spectrometer was operated in electrospray ionization with positive ion mode with a capillary voltage of 3500V. The fragmentor was set at 70, gain was 1, the drying gas flow was 12 L/min with a temperature of 330° C and nebulizer pressure was 55 psig. Under these conditions 2, 6-Diaminopyridine in Minodronic acid was quantified by SIM mode.2, 6-Diaminopyridine was monitored at m/z 110.2(protonated).

Preparation of standards and test sample solutions:

The standard stock solutions of impurity was prepared approximately at 10 ng/ml in diluent. The Minodronic acid test samples were typically prepared at 0.5mg/ml in the diluent medium.

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 2, 6-Diaminopyridine in Minodronic acid. Different stationary phases and mobile phases were used and finally the desired chromatographic separation was achieved on a ZIC HILIC, 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 acetate+0.1% Acetic acid as mobile phase-A and acetonitrile as mobile phase-B using a isocratic programme of Mobile phase-A: Mobile phase-B in the ratio of 30:70.

Method validation:

The method has been validated for the quantification of 2, 6-Diaminopyridine in Minodronic acid 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 a known concentrations of 2, 6-Diaminopyridine and established the minimum level at which the 2, 6-Diaminopyridine can be reliably detected. The LOD is 1.0 ppm and LOQ is 3.0 ppm obtained for the impurity.

System precision and system suitability:

The precision and system suitability was performed by injecting six replicates of the reference solution (30ppm of the impurity with respect to the test sample concentration). The %RSD for the peak areas obtained was calculated. The data presented in Table 1 establishes System suitability and system precision. The Reference solution chromatogram for the impurity is represented in Figure-3.

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

S.No	Peak area for 2,6-Diaminopyridine
1	71048.938
2	69638.867
3	69829.547
4	69932.680
5	70013.828
6	69644.664
Mean	70018.09
SD	527.02
% RSD	0.75

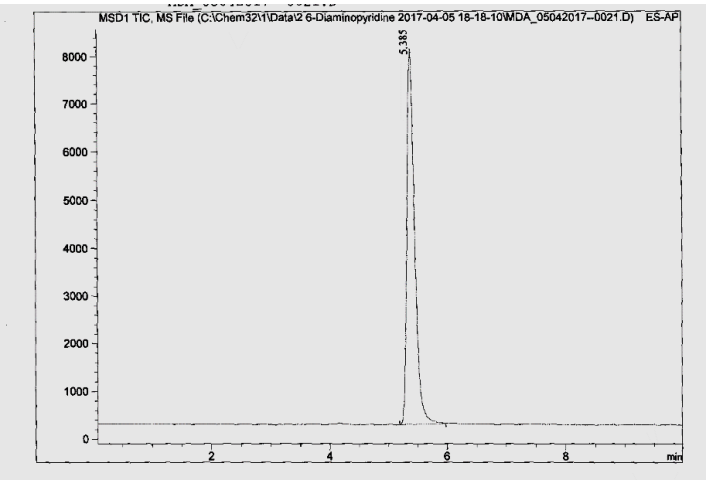

Fig.3: **Reference solution (30 ppm) chromatogram.**

Specificity:

The specificity of the optimized method was performed by injecting a stock solution of 2, 6-Diaminopyridine to check resolution between the 2, 6-Diaminophenol and Minodronic acid under the same conditions mentioned in LC-MS chromatographic conditions. Summary of retention time and m/z value for Minodronic acid and 2, 6-Diaminopyridine are mentioned in Table 2.

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

Compound	**Retention Time(Min)**	**Mass(m/z) value** (+ve)
2,6-Diaminopyridine	5.3	110.2
Minodronic acid	11.8	323.2

Linearity:

Linearity of the method was checked by preparing the solutions at 7 concentration levels from LOQ to 150% of specification limit (3, 7.5, 15, 22.5, 30, 36 and 45 ppm). The mean responses recorded for impurity 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 Fig.4 and data is represented in the Table 3.

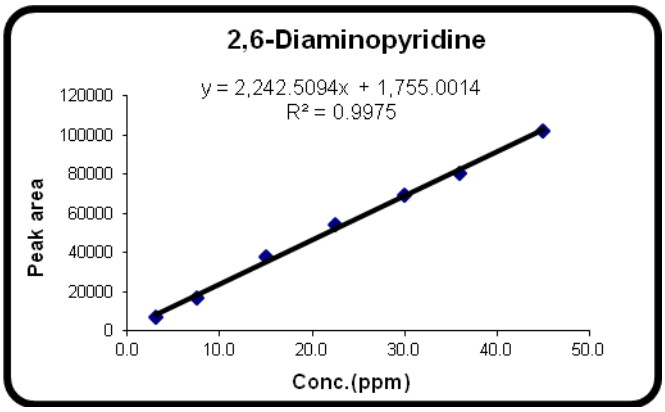

Fig.4 Linearity graph for 2, 6-Diaminopyridine.

Table 3: Linearity data.

Conc.(ppm)	**2,6-Diaminopyridine peak area**
3 (LOQ)	7133
7.5	17125
15	37768
22.5	54277
30.0	69640
36.0	80722
45.0	102179
Slope	2242.5094
R^2	0.9975

Accuracy:

Accuracy of the method was evaluated by using four solutions containing Minodronic acid spiked with the impurity at LOQ, 50%, 100% and 150% of the specification limit (30 ppm). Each concentration level was prepared in triplicate. The percentage recovery results obtained for 2, 6-Diaminopyridine are listed in Table.4. A representative spiked chromatogram for test sample and Test+ LOQ level is shown in Fig.5.

Table 4: Summary of accuracy study for 2, 6-Diaminoyridine.

TEST+SPIKED (n=3)	% Recoveries for 2, 6-Diaminoyridine
Test + LOQ spiked (3 ppm)	101.9
Test + 50% spiked (15 ppm)	111.6
Test + 100% spiked (30 ppm)	102.2
Test + 150% spiked (45 ppm)	101.2

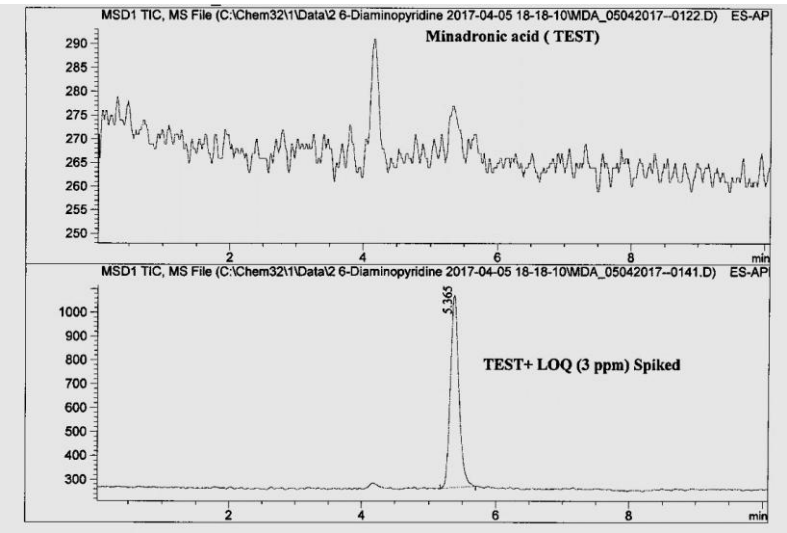

Fig.5: Test and Test+ LOQ spiked chromatogram.

CONCLUSION

The proposed LCMS method is simple, sensitive and accurate to quantify potential genotoxic impurity 2, 6-Diaminopyridine at ppm level present in Minodronic acid. The validated parameters are well within the limits and this method is found suitable for routine quality control test of Minodronic acid.

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