Research Article

Detection and Quantification of Trace Level of Ethylenediamine in Morpholine and its Impact on the Quality of a Pharmaceutical Candidate

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ABSTRACT

The main purpose of proposed study was to identify and quantify ethylenediamine in morpholine and its impact on the quality of a pharmaceutical candidate. The drug substance impurity was determined to be a diamide dimer, resulting from a low level impurity (ethylenediamine) in one of the raw materials (morpholine). At the beginning ethylenediamine was not detected in morpholine using normal GC conditions however later ethylenediamine was detected and quantified at lower level by derivatizing with benzaldehyde. Morpholine was used as reagent as well as solvent throughout. As a result, it elucidated the formation of diamide impurity in the drug at higher level. Hence these studies describe the importance of understanding root causes of impurity formation to enable proper control strategies.

Keywords: Morpholine, Ethylenediamine, Impurities, Identification, Characterization, Validation.

INTRODUCTION

The identification and quantification of impurities in Active Pharmaceutical Ingredients (API) and pharmaceutical products is a very intensive activity performed at many levels of the drug discovery pipeline and beyond. An impurity in a drug substance as defined by the International Conference on Harmonisation (ICH) Guidelines is any component of the drug substance that is not the chemical entity defined as the drug substance.[1,2] The safety of a drug substance is dependent not only on the toxicological properties of the active drug substance, but also on the impurities that it contains. Therefore, identification, quantification, and control of impurities in the drug substance and drug product, are an important part of drug development and regulatory assessment[3]. The nature and the quantity of these impurities are governed by a number of different factors, including synthetic route of the drug substance, reaction conditions, quality of the starting material of the drug substance, reagents, solvents, purification steps, excipients, drug product manufacturing processes, packaging, and storage of the end product[4-10]. As the structures of impurities are sometimes unknown, several spectroscopic and micro-chemical techniques have been developed which require minute quantities of material and readily enable the structural elucidation of the impurity. To assure the purity of drugs, quality of starting material is equally important[11-13]. The starting material should be appropriately characterised, with a well-defined impurity profile. A starting material can be defined as a raw material, intermediate, or a drug substance that is used in the production of a drug substance and that is incorporated as a significant structural fragment into the structure of the drug substance[14].

Morpholine, a key raw material used in the manufacture of Deepta Laboratories drug 'D' which is under phase 2 studies. Morpholine and its derivatives are widely used in pharmaceuticals, rubber, pesticides, dyes, paints and other fields[15]. Morpholine is six-membered heterocyclic compound featuring both amine and ether functional groups. Morpholine undergoes most chemical reactions typical for other secondary amines, though the presence of the ether oxygen withdraws electron density from the nitrogen, rendering it less nucleophilic (and less basic) than structurally similar secondary amines such as piperidine[16].

Morpholine is extensively used in drug synthesis. For example, it is a building block in the preparation of the antibiotic linezolid, the anticancer agent gefitinib and the analgesic dextromoramide[17-18].

In research and in industry, the low cost and polarity of morpholine lead to its common use as a solvent for chemical reactions. During the process development, an unknown impurity was formed in the drug candidate due to the presence of an impurity in the key raw material morpholine.

The synthetic scheme of drug formation is shown in scheme 1. The percentage of impurity in morpholine was at trace level however higher level of impurity was observed in the drug candidate. This article is intended to provide a scientific exploration on identification, characterisation and origin of this impurity. This work also provides recommendations on the importance of controlling the impurities in the starting material at trace level.
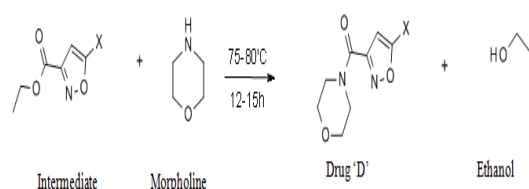
Scheme 1: Synthetic scheme of Drug formation

MATERIALS AND METHODS

Materials and chemicals

HPLC grade acetonitrile and trifluoroacetic acid were obtained from Merck (Germany). Deionized water was prepared using a milli-Q plus water purification system from Millipore (Bedford, MA, USA). AR grade benzaldehyde and ethylenediamine were procured from Sigma Aldrich. LR grade morpholine's were purchased from Sigma Aldrich, CDH and Spectrochem respectively. Dimethyl sulphoxide-d6 (for NMR) was purchased from Sigma - Aldrich Corporation (St. Louis, MO, USA).

Analytical conditions

Ultra High performance liquid chromatography

The UHPLC system was equipped with binary gradient pumps, auto sampler and auto injector (Model 1290, Make Agilent USA) connected to photo diode array detector controlled with chemstation software. The chromatographic separations were achieved on Waters Acquity BEH shield RP$_{18}$ column (100 mm length × 2.1 mm ID with 1.7µm particle size, Waters corporation, MA, USA). Mobile phase A consisted, 0.03% of trifluoro acetic acid in water and mobile phase B consisted 0.03% of trifluoro acetic acid in acetonitrile in gradient mode (TminA:B) $T_0$65:35, $T_{1.9}$50:50, $T_5$40:60, $T_8$15:85, $T_{8.1}$65:35, T_{12}65:35. The column temperature was maintained at 40 °C and the detection was carried out at 283 nm. The flow rate was set to 0.5 mL/min.

Gas chromatography (GC)

The GC system was equipped with an auto sampler and auto injector (Model 7890A, Make Agilent USA) connected to a flame ionisation detector with chemstation software. The chromatographic separations were achieved on Agilent DB-5 column (30m x 0.32mm ID, 0.25 µm film thickness). The carrier gas used was helium with a flow rate 1.0 mL/minute. The temperature profile was 50 °C held for 3 min, and then increased 20 °C/min to 250 °C, held for 3.5 min. The injector was operated in split mode (25:1) and the injector temperature was 240 °C. The detector temperature was 280 °C, and the injection volume was 1 µL.

Mass spectrometry (MS)

Gas chromatography mass spectrometry analysis was performed with an Agilent 5973 system equipped with electron ionisation (EI) source. The column was connected to a mass selective detector run under standard EI+ conditions (70 eV). The data was recorded

using GC mass lync software. The high resolution mass spectrometer consisted of an Agilent 1290 series high performance liquid chromatography system and 6530 Q-TOF MS with mass hunter software. All samples were analyzed on the Q-TOF mass spectrometer equipped with an ESI source for accurate mass values. The instrument was operated in positive ion mode and mass spectra were recorded in the range m/z 50 - 1000. The capillary voltage was maintained at 4500V with the end plate offset at 500 V. The gas flow to the nebulizer was set at a pressure of 40 psi, while the drying temperature and the drying gas flow rate were 250 °C and 12.0 L/min, respectively.

NMR spectroscopy

The ^{1}H spectra were recorded using a Bruker 400 MHz spectrometer (Bruker, Fallanden, Switzerland) equipped with a 5 mm BBO probe and a z-gradient shim system. The ^{1}H spectra were recorded with 1s pulse repetition time using 30^0 flip angle. Samples were dissolved in dimethyl sulphoxide-d6. The ^{1}H chemical shift values were reported on the δ scale in ppm relative to DMSO-d$_6$ (2.50 ppm).

Sample preparation of drug substance for UHPLC and UHPLC/MS analysis

A test preparation of 0.1 mg/mL of API sample was prepared. This solution is used for both UHPLC and UHPLC/MS analysis.

Sample preparation of morpholine for GC and GC/MS analysis

A test preparation of 100 mg/mL of morpholine sample was prepared. This solution was used for the initial GC analysis. A test preparation of 100 mg/mL of morpholine sample in acetonitrile was treated with 30µL of benzaldehyde and the mixture was sonicated for 10 minutes. This solution was used for the quantification of ethylenediamine in morpholine. A standard preparation of 0.1 mg/mL ethylenediamine in acetonitrile was treated with 30µL of benzaldehyde and the mixture was sonicated for 10 minutes. This solution was used as a standard for the quantification of ethylenediamine in morpholine (1000 ppm with respect to sample concentration).

RESULTS AND DISCUSSION
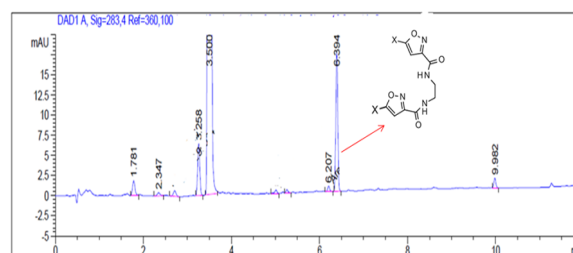
Detection of impurity

Figure 1: The analytical UHPLC chromatogram of drug substance

The UHPLC analysis of the drug substance sample had shown the presence of an unknown impurity at the level of 0.7 % area. Drug substance and the unknown impurity were eluted at a retention time of 3.5 min and 6.4 min respectively. The UHPLC chromatogram of drug substance was shown in Figure 1.

Identification of impurity

To get preliminary structural insight, accurate mass analysis was carried out on the drug substance and the impurity. The elemental formulae generated by molecular formulae generator for the impurity was $C_{36}H_{35}Cl_2N_6O_6$ (M+H) having m/z 716.19169 (M) with a measurement error of 1.43 ppm, Figure 2. Based on the molecular formulae generated for the molecular ion, the structure of the impurity was proposed. Logically this impurity could form by the reaction of final intermediate and ethylenediamine. The impurity was named as diamide dimer and the proposed structure was depicted in Figure 3. Based on the proposed structure of impurity by accurate mass analysis, diamide dimer impurity was independently synthesized and used for further structural confirmation by NMR and mass analysis. Synthesized impurity was analysed by UHPLC method as described under section 2.2 and the relative retention time observed was exactly same as that of impurity observed in the sample.
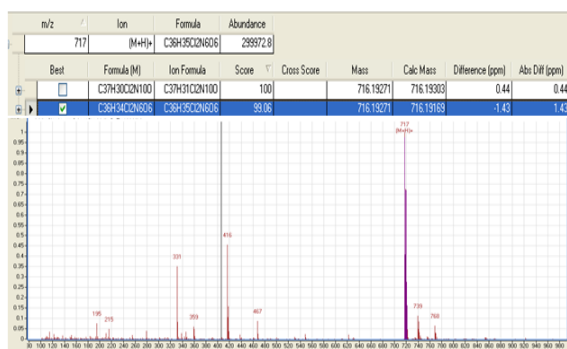
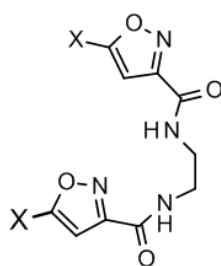

Figure 2: Accurate mass analysis

Figure 3: Diamide dimer impurity

Investigation of origin of the impurity

During lab experiments, we have used morpholine procured from a supplier, central drug house (CDH) and the percentage of diamide dimer impurity was consistently below 0.2 % area. During scale up run, we have procured morpholine from spectrochem however unexpectedly, impurity percentage observed was 0.7 % area. The structure of the diamide dimer impurity

undoubtedly indicated that it could form only if two equivalents of final intermediate react with one equivalent of ethylenediamine. Morpholine used from different sources in the process of drug substance gave different levels of diamide dimer impurity. Results are summarized in Table 1.

Table 1: % of diamide dimer impurity in drug substance using morpholine from different sources

Source	% area (In-Process)	% area (Final API)
CDH	0.7	Below 0.2
Sigma Aldrich	2	0.4
Spectrochem	4.5	0.7

Source of ethylenediamine was attributed to morpholine used in the process. The GC analyzes of morpholine from three different sources were comparable and ethylenediamine content was below 0.1% area in all the samples. The detection limit was 0.1% area and this was mainly because of the poor response of ethylenediamine under GC conditions. The level of ethylenediamine content in morpholine was low, higher formation of diamide dimer impurity in the reaction leads to uncertainty that the root cause of this impurity may be from other sources. In order to understand the formation of ethylenediamine in the reaction, we have performed several experiments using morpholine from spectrochem with different reaction conditions. However, in all the experiments the level of diamide dimer was constant. Therefore, it was concluded that the root cause of ethylenediamine formation is not from the reaction conditions and it may be from morpholine itself. As a result, an alternate strategy was adopted to estimate ethylenediamine in morpholine by derivatization.

Derivatization of ethylenediamine with benzaldehyde

Amines are generally known to be very difficult to analyze by capillary gas chromatography due to their basic character. The amino group introduces a large dipole in the molecule and generally this dipole is responsible for a strong interaction with silanol groups and siloxane bridges in the stationary phases, which often results in nonlinear adsorption effects, appearing as strong tailing peaks in the chromatogram. The best way to prevent the interaction of the strong dipole is to deactivate the column or to derivatize the amine in such a way that the interaction is minimized. A series of columns were available with deactivation technology include Rtx-5 and Rtx-35 amine columns. However, in order to analyze basic compounds at low concentration, this strategy may not be suitable. Derivatization technique will be favored if it is accurate and robust and no secondary effects such as lower recoveries and change of matrix. Moreover, it may improve the sensitivity of the peak using FID detector.

Studies show that schiff base-type reactions are best suitable to condense primary amines with a carbonyl compound. Benzaldehyde was selected as derivatizing agent and the condensation reaction with this reagent

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proceeded rapidly at room temperature. Moreover the reagent is very selective to ethylenediamine. Morpholine samples from different sources were analyzed with this method and the derivative gave very good response in GC.

GC chromatogram is depicted in Figure 4. The reaction scheme is shown in scheme 2 and the results are summarized in Table 2. GC/MS analysis has performed in order to confirm the derivatized product and the mass spectrum is shown in Figure 5.
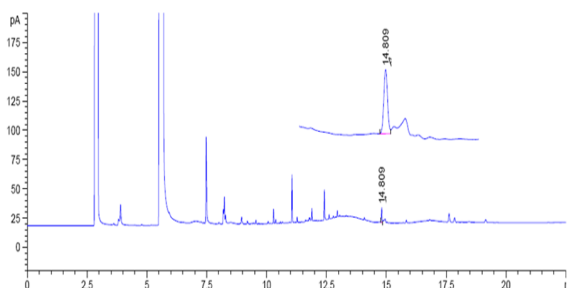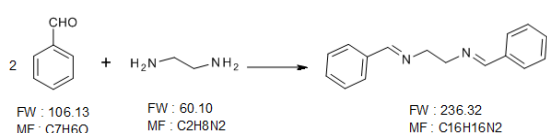

Figure 4: GC chromatogram of ethylenediamine derivatized product

Scheme 2: Diimine formation

Table 2: Ethylenediamine content in Morpholine

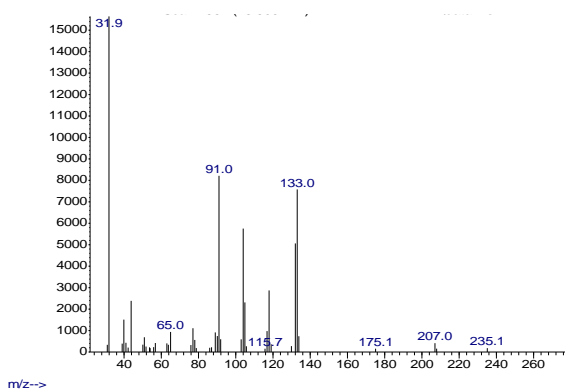| Source	Ethylene diamine (% w/w)
CDH	0.006
Sigma Aldrich	0.029
Spectrochem	0.081

Figure 5: Mass spectrum of ethylenediamine derivatized product

The data pointed out that when the ethylenediamine content in morpholine was high, the level of diamide dimer impurity was also shown high in the drug substance. It has to be noted that morpholine was used in the process both as a reactant as well as reaction medium. A total of five relative volumes of morpholine were used in the process and the surplus use of morpholine gave justification for the formation of higher amount of diamide dimer impurity in the reaction.

Therefore ethylenediamine present in morpholine at about 0.006% gives diamide impurity in drug substance at the level of 0.7 % area. This study supports our assumption that level of diamide dimer impurity formed in the process is mainly dependent on ethylenediamine content in morpholine. Therefore proper check is required in morpholine for ethylenediamine to control the impurity at API stage.

Method Validation

The newly developed GC method was partially validated for sensitivity, linearity, precision and accuracy according to ICH guidelines[19]. Method validation results are summarized in Table 3.

Table 3: Method validation summary report

Parameter	Derivatized ethylenediamine
System suitability	
RT	14.8
N	157469
T	1.03
Linearity	
r	0.9996
Slope	3691.7
Intercept	1.7982
Detection limit (%)	0.001
Quantitation limit (%)	0.003
Precision (QL)	
% RSD (n 6)	7.9
Repeatability (intra day)	
% RSD (n 6)	4.8
Intermediate precision (inter day)	
% RSD (n 6)	4.5
Accuracy at QL level (n 3)	
Amount added (%)	0.003
Amount recovered (%)	0.0032
% Recovery	106.7
Accuracy at 80% level (n 3)	
Amount added (%)	0.08
Amount recovered (%)	0.0875
% Recovery	109.4
Accuracy at 100% level (n 3)	
Amount added (%)	0.10
Amount recovered (%)	0.1071
% Recovery	107.1
Accuracy at 120% level (n 3)	
Amount added (%)	0.12
Amount recovered (%)	0.1303
% Recovery	108.6

Sensitivity

The limit of detection and limit of quantitation were determined for the derivatized ethylenediamine impurity. The LOD and LOQ for the impurity was estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively by injecting a series of diluted solutions with known concentration. The limit of detection and the limit of

quantitation were about 0.001 % and 0.003 % respectively. Precision study was also carried at the LOQ level by injecting six individual preparations and the relative standard deviation for LOQ concentration for the impurity was below 10 %.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. The solutions were prepared at six concentration levels from LOQ to 0.15 % with respect to sample concentration. (i.e. LOQ, 0.05 %, 0.08 %, 0.10 %, 0.12 % and 0.15 %) was subjected to linear regression analysis with the least squares method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The correlation coefficient obtained was greater than 0.999 for the impurity. The range of the method was from LOQ to 0.15 % for the impurity.

Precision

Precision of the method was studied for method precision and intermediate precision. Method precision was checked by injecting six individual preparations of ethylenediamine (0.1%) in morpholine derivatized with benzaldehyde. In the intermediate precision study, the similar procedure of method precision was carried out by a different day. % RSD of areas of derivative was within 10.0, confirming good precision at low level of the developed analytical method.

Accuracy

The accuracy of the method was evaluated in triplicate at LOQ, 80% level, 100% level (0.1%) and 150% level (0.15%). The percentage recovery of ethylenediamine derivatized in morpholine has been calculated. Chromatogram of ethylenediamine spiked in morpholine derivatized with benzaldehyde depicted in Fig. 3.

CONCLUSION

In the present study, an unknown impurity was detected by UHPLC in a pharmaceutical candidate. The source for the formation of this impurity was from the key contributory raw material morpholine. The impurity in morpholine was identified as ethylenediamine and quantified after pre column derivatization. The impurity pharmaceutical candidate was identified as diamide dimer impurity and formed by the reaction between ethylenediamine and final intermediate. The new GC method demonstrated that the amine impurities can be quantified at low level using derivatization techniques. The method was validated as per ICH guidelines. Newly developed GC method was found to be simple, sensitive and cost effective. This study indicated that the significance of controlling trace level of impurities in starting materials particularly when it uses as reaction solvent and reagent.

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