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Characterization and Quantitation of N-Nitroso Duloxetine Impurity in Duloxetine Hydrochloride Drug Substance

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The nitrosamine family of chemicals is a large group of carcinogens found in human diets and other environmental media. In animal experiments, it has been shown that N-nitroso compounds can cause cancer, hence the presence of nitrosamine impurities are important to evaluate and need to be quantified. This work shows the content of N-nitroso duloxetine in duloxetine (DXT) hydrochloride, before proceeding to the quantification of impurity, the impurity standard must be well characterized by analytical techniques and it places a vigorous role, *i.e.* N-nitroso DXT molecular weight conformed by mass spectral data and the atoms connectivity was confirmed by NMR spectral data (^{1}H, ^{13}C, COSY, HSQC and HMBC) and thermal analyses (DSC and TGA) were also performed. In N-nitroso DXT, two sets of signals were typically observed, indicating the presence of asymmetric N-nitroso DXT, concluding with drift functional theory (DFT) for *E*-, *Z*-isomers related to the nitroso group in N-nitroso DXT. The medical agency recommends a specified limit of 0.83 ppm for the acceptable intake (AI) and the maximum daily dosage. The determination of N-nitroso DXT was performed by liquid chromatography mass spectrometry (LC-MS) followed by electron spray ionization with triple quadrupole.

Keywords: N-Nitroso duloxetine, Impurity, NMR, LC-MS.

INTRODUCTION

In August 2004, the Food and Drug Administration (FDA) approved duloxetine for the treatment of major depressive disorder. It is a type of antidepressant that works by preventing the reuptake of serotonin and norepinephrine in the brain. In addition to its use as an anti-depressant and also it was approved for treating pain resulting from peripheral diabetic neuropathy and in some countries also for stress urinary incontinence, there are no known problems associated with long term use of duloxetine [1-4]. Globally its usage is more hence quality of duloxetine supply to the play vigorous role, some of the process related impurities of duloxetine notified and monitored [5,6], one of the impurity is N-nitroso DXT; duloxetine contains 2º amine in chemical structure so possibility to form N-nitroso DXT to react with nitrite/nitrate in acidic media is shown in Fig. 1.

FDA issued new guidance [7] for immediate implementation, recommended acceptable intake limits for NDSRI guidance for industry. In a drug product, Nitrosamine Drug Substance Related Impurities (NDSRIs) are generally formed through the nitrosation of APIs (or API fragments) containing secondary or tertiary amines when exposed to nitrosating agents such as residual nitrites in excipients. Many NDSRIs that have been identified in recent drug products lack carcinogenicity and mutagenicity data (typically from animal studies) from which an acceptable intake can be derived. It is believed that NDSRIs can form in a substantial number of drug products because of their chemical structure; however, it is unknown whether all or some of these compounds are mutagenic carcinogens.

Mutagenicity is the change in DNA sequence that can cause abnormalities and diseases. Carcinogenicity is the capacity to cause cancer. Identification of cancer involves evaluating the results of human epidemiologic studies, long-term bioassays in experimental animals and other data relevant to an evaluation of carcinogenicity. As a result of the lack of mutagenicity data and robust carcinogenicity data, establishing an acceptable intake limit for NDSRIs is challenging.

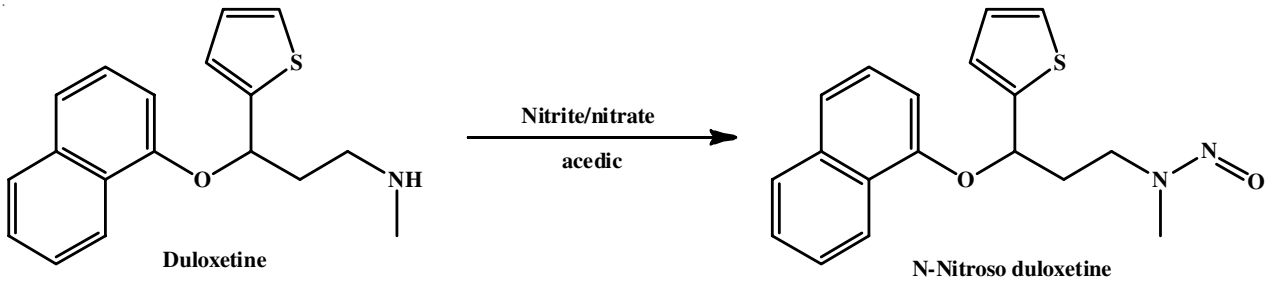

Fig. 1. Formation of N-nitroso duloxetine from duloxetine

It relies on the structure-activity relationship (SAR) concepts described in recent scientific publications for N-nitrosamine compounds to categorize carcinogenic potency. In addition, 84 N-nitrosamines were used with rat TD_{50} values provided by the carcinogenic potency database (CPDB)14 and/or has low carcinogenicity database (LCDB) 14 [8]. The approach assumes that the α-hydroxylation mechanism of metabolic activation is responsible for the mutagenic response and the highly potent cancerous response reported for many N-nitrosamines as a consequence of their usage [9].

It is expected that structural features that affect the favourability of the activation mechanism or that increase the clearance of the nitrosamine by other biological pathways will have a corresponding effect on the carcinogenic potential. A prediction of mutagenic potential and carcinogenic potency of N-nitrosamine can therefore be made based on its structural features [10]. It includes information about the carcinogenic potency categorization approach (CPCA) for setting nitrosamine limits based on robust scientific knowledge about carcinogenic potency.

EXPERIMENTAL

N-Nitroso duloxetine and duloxetine hydrochloride (DXT) drugs were procured from Cheminor Life Sciences, Hyderabad, while the HPLC grade acetonitrile, methanol, water, ammonia, formic acid and DMSO-d_6 were purchased from Merck, India Pvt. Ltd.

Acceptable intake (AI) limit: According to the EMA recommendations, the recommended AI limits for N-nitroso DXT matched with the theoretical approach as per CPCA, and this chemical structure (Fig. 2) is consistent with the published EMA standards of 100 ng/day [11].

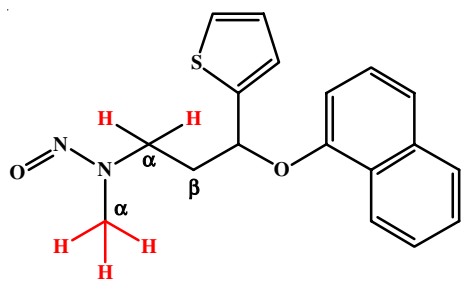

Fig. 2. Hypothetical approach on N-nitroso duloxetine

Hydrogens on α-carbon: (2, 3)	+1
Chain length on one side >5	+1
N-Nitroso group containing	+1
Sulfur atom aryl group bonded to β-carbon	–1
Total score = 1+1+1-1 = 2	
Potency score = 2	

Therefore, it falls under group 2, and the recommended daily intake of acceptable intake (AI) is 100 ng. The conversion of AI limit into ppm varies by product and is calculated based on a drug's maximum daily dose (MDD) as reflected in the drug label [ppm = AI (ng/day)/MDD (mg/day)] Duloxetine MDD 120 mg/day.

$$\text{Specification limit} = \frac{100}{120} = 0.83 \text{ ppm}$$

LC-MS method conditions: The LC-MS/MS analysis was performed with method conditions like: Samples were injected 50 µL into a LC-MS system (LCMS-Sciex triple quadrupole 4500) and separated on a octadecyl-C18 column (Inertsil ODS-3V, 150 mm × 4.6 mm, ID: 5.0 µm, GL Sciences, Japan) at 60 ºC column oven temperature. The mobile phase consisted of buffer H_2O with 0.1% formic acid and 0.1% of NH_3 solution (assay of NH_3 is about 25%) (A), methanol (B), the following HPLC gradient was used with (30:70 v/v) of mobile phase A and B, the flow rate was set to 0.8 mL/min. The N-nitroso DXT was eluted at 12.56 min. The HPLC was coupled to a triple quadrupole MS with an ESI source. The MS instrument was operated in positive mode. The mass spectrometer was run in ESI using multiple reaction monitoring (MRM) to monitor the mass transitions was chosen to be *m/z* 344.100 → 183.00. The subsequent mass settings were used: ion source: 5500, Temperature: 450 ºC, CAD-7, Curtain gas: 30, GS1-55, GS2-50, CXP-9, CE-10,EP-10, DP-40 and valco valve used to divert the DXT and other unwanted peaks to waste and will help to reduce the contamination.

NMR analysis: All the NMR measurements were performed on a Bruker 500 MHz spectrometer using tetramethyl silane (TMS) as reference. The concentration of all the solutions used for the measurements was about 20-30 mg of compounds in the 0.6 mL of DMSO-d_6.

Differential scanning calorimetry (DSC) analyis: DSC analysis was made on a Mettler Toledo, Model: DSC1, using aluminum sealed pans. A constant nitrogen flow (50 mL/min) was maintained to provide a constant thermal blanket within the DSC cell. The instrument was calibrated with high purity indium and zinc standards. Sample of about 10 mg was used and then the scans were recorded between 0 ºC and 250 ºC at a constant heating rate of 10 ºC/min.

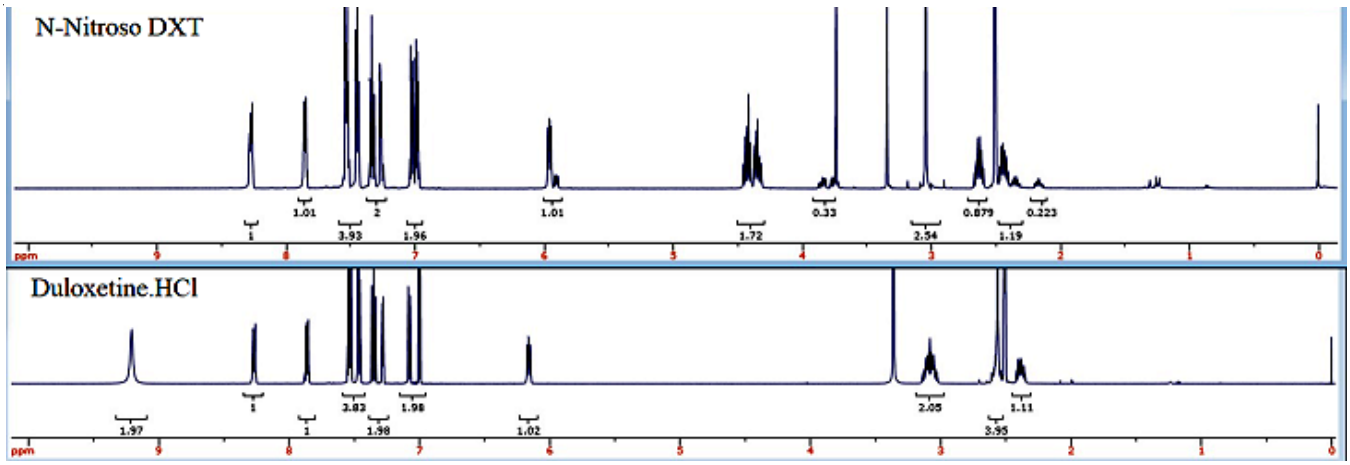

Fig. 3. Overlay of ^{1}H NMR spectrum of duloxetine. HCl and N-nitroso duloxetine

Thermogravimetric analysis (TGA) analyis: TGA analysis was performed on a Perkin-Elmer, Pyris 1 TGA thermal analyzer. The scan was recorded between 25 ºC and 850 ºC, under a 20 mL/min nitrogen flow and at a constant heating rate of 10 ºC/min.

RESULTS AND DISCUSSION

In order to estimate N-nitroso DXT with specification limit 0.83 ppm, the impurity chemical structure was well characterized before proceeding to estimation of N-nitroso DXT impurity.

Chemical structure of N-nitroso DXT (E,Z-isomers): The overlay diagram (Fig. 3) of DXT and N-nitroso DXT explains the ^{1}H NMR chemical shifts of N-nitroso DXT at 4.3 ppm and 5.9 ppm, N-H group was disabled, it is specifying N=O group substituted on Nitrogen atom. In HPLC (Fig. 4) and ^{1}H NMR (Fig. 5) analyses of N-nitroso DXT, two sets of signals were typically observed indicating the presence of asymmetric N-nitroso DXT. However, few reports on the NMR assignment of asymmetric N-nitrosamine isomers have also been reported [12,13]. Because the N-N bond in the asymmetrical N-nitroso DXT is hindered from rotation (Fig. 6), this may result in strong variations in anisotropic effects of the asymmetrical N-nitroso DXT. In terms of a double bond, the two conformers are similar to the E/Z isomers [12,13].

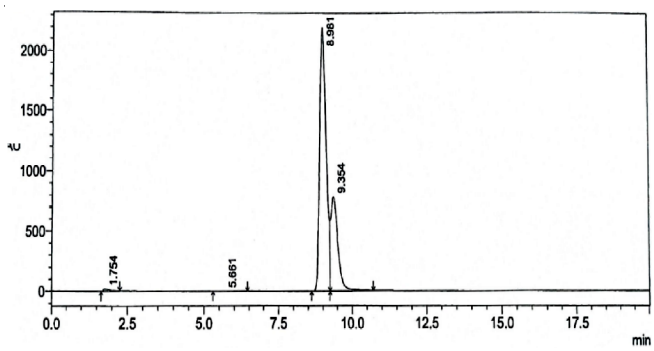

Fig. 4. HPLC chromatogram of N-nitroso duloxetine

N-Nitroso DXT chemical structure conformation by mass spectrometry: In the systematic way of structure elucidation, the +ve ESI-MS spectrum of the N-nitroso DXT (Fig. 7) exhibits sodium adduct molecular ion peak at *m/z* 349.10, then the molecular ion of N-nitroso DXT was found to be at *m/z* 326.41. The mass difference between duloxetine and N-nitroso DXT impurity was found to be 29 amu. The HR-MS was performed to evaluate the molecular formula and double bond equivalence (DBE). The positive HR-MS spectrum showed protonated molecular ion at *m/z* 327 corresponding to molecular formula $C_{18}H_{18}N_2O_2S$. The odd number of protonated molecular ion suggest that impurity contains even number of

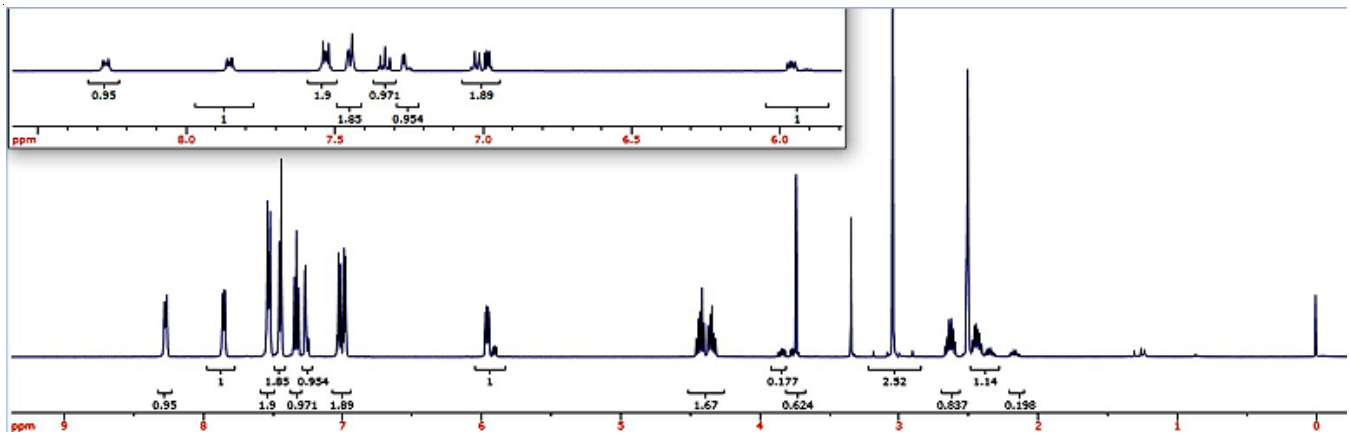

Fig. 5. ^{1}H NMR spectrum of N-nitroso duloxetine

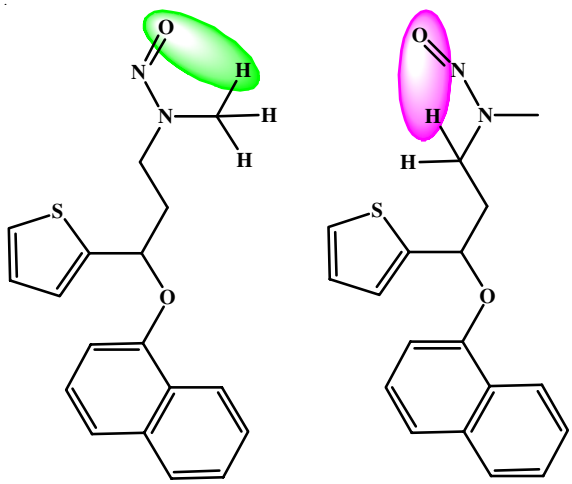

Fig. 6. Possible conformers of N-nitroso duloxetine

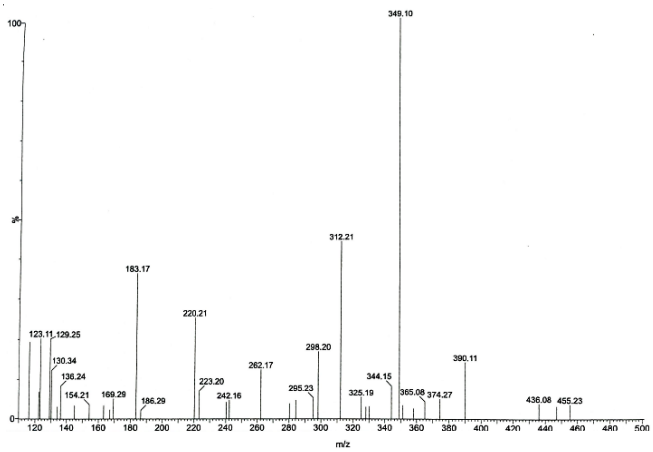

Fig. 7. Mass spectrum of N-nitroso duloxetine

nitrogen atoms (according to nitrogen rule), when compared with the molecular formula of duloxetine, there was a difference of HNO. An additional DBE value shown in the impurity data was compared with duloxetine. The difference can be rationalized in terms of the possibility of nitroso group incorporation was observed. Based on this data, the chemical structure of impurity was recognized as N-methyl-N-(3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propyl) nitrous amide.

N-Nitroso DXT chemical structure conformation from NMR spectroscopy: When the ^{1}H NMR spectra of N-nitroso DXT and DXT [11] were compared, it was found that there were considerable differences between the two compounds. In ^{1}H NMR spectrum of N-nitroso DXT, one N-H proton (Fig. 3) in downfield region was not observed. These signals confirmed the presence nitroso moiety in the N-nitroso DXT impurity structure. The proposed chemical structure was further supported by the overlay of ^{1}H NMR spectrum for duloxetine and N-nitroso DXT, ^{1}H NMR, ^{13}C NMR and 2D NMR spectral data. The proton NMR spectrum showed signals at δ 2.407-2.669 (7H), 4.319-4.459 (2H), 5.947-5.973 (1H), 6.971-7.048 (2H), 7.243-7.548 (6H) and 7.845-8.283 (2H) ppm corresponding to 18 protons. The detailed information for the ^{1}H NMR and ^{13}C NMR spectra (Fig. 8) can be seen in Table-1 and the number assignment of N-nitroso DXT chemical structure is shown in Fig. 9. Further to confirm the exact structure of impurity, the 2D NMR COSY (Fig. 10) shows proton connectivity through contours diagonal correlation, from HSQC (Fig. 11) observed that one methine signal corresponding to one proton, two methylene corresponds to 4 protons and one methane signal corresponding to three protons. The proton in aliphatic region showing signal at δ 6.0 ppm corresponds to C4 which appeared at δ 72.167 ppm, signal at δ 3.5 ppm corresponds to C2, which appeared at δ 49.664 ppm, signal at 3.0 ppm corresponds to C1 which appeared at δ 36.545 ppm. In 2D NMR HMBC (Fig. 12), the methine proton at δ 5.947 ppm showed the correlations for carbon C2, C3, C5 and C8 positions at 36.545 ppm, 49.664 ppm, 125.681 ppm & 152.263 ppm, methelene proton at 4.319 ppm showed the correlations for carbon 31.375, 36.545 and 72.167 ppm.

Thermal studies: The DSC curve of duloxetine (Fig. 13a) shows the onset melting point at 55.24 ºC. The N-nitroso DXT showed an endothermic peak was observed at 60.38 ºC due to melting, followed by an exothermic peak with an enthalpy of 70.866 J/g (Fig. 13b). There was weight loss of N-nitroso duloxetine is 0.713% up to 105 ºC and after decomposition of the material was observed.

Estimation of N-nitroso DXT by LC-MS method

Preparation of standard solution: The solvent mixture of acetonitrile:water (20:80 v/v) was used for the preparation

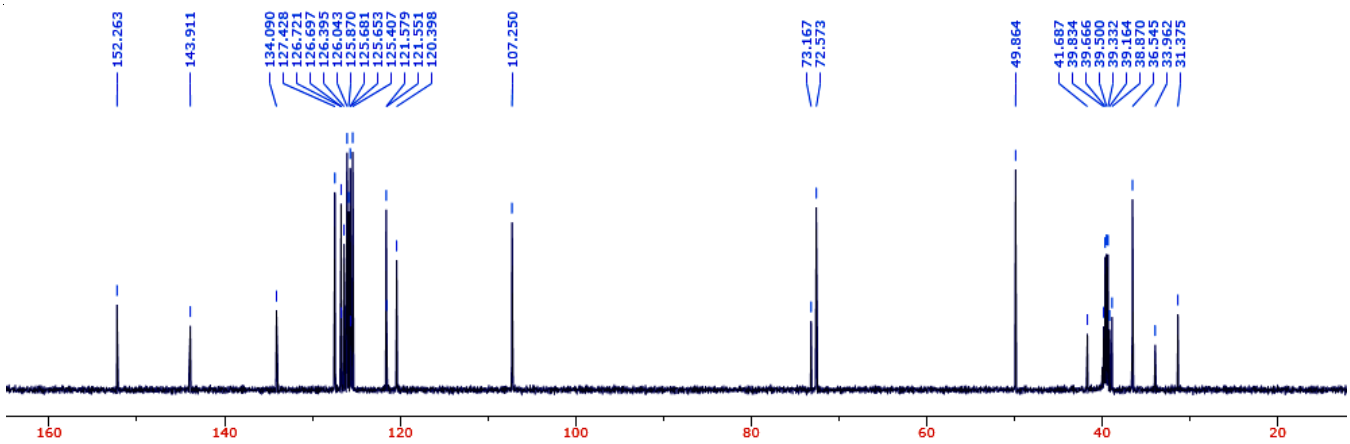

Fig. 8. ^{13}C NMR spectrum of N-nitroso duloxetine

TABLE-1
NMR ASSIGNMENT FOR N-NITROSO DXT CHEMICAL STRUCTURE

Position	Number of protons	^{1}H chemical shift	^{13}C chemical shift	COSY	HSQC	HMBC
1	3H	3.05	31.375	–	31.375 (CH_3)	C-2
2	2H	4.319-4.459	49.664	2.407-2.669	49.664 (CH_2)	C-1, 3, 4
3	2H	2.407-2.669	36.545	4.319-4.459, 5.947-5.973	36.545 (CH_2)	C-2,4
4	1H	5.947-5.973	72.167	2.407-2.669	72.167 (CH)	C-2,3,5,9
5	–	–	143.911	–	–	–
6	1H	6.971-6.994	126.697	7.315-7.353	126.721 (CH)	C-7,8
7	1H	7.315-7.353	125.653	6.971-6.994, 7.243-7.272	125.653 (CH)	C-6,8
8	1H	7.243-7.272	125.681	7.315-7.353	125.681 (CH)	C-5,6,7
9	–	–	152.263	–	–	–
10	1H	7.013-7.040	107.250	7.434-7.457	107.250 (CH)	C-9,10,11
11,12	2H	7.434-7.457	121.579, 125.681	7.013-7.040	121.579, 125.681 (CH)	C-10
13,18	–	–	134.090	–	–	–
14	1H	7.845-7.863	127.428	7.512-7.548	127.428 (CH)	C-15,16
15,16	2H	7.512-7.548	126.721, 126.395	7.845-7.863, 8.263-8.283	126.721, 126.395 (CH)	C-14,17
17	1H	8.263-8.283	121.579	7.512-7.548	121.579 (CH)	C-15,16

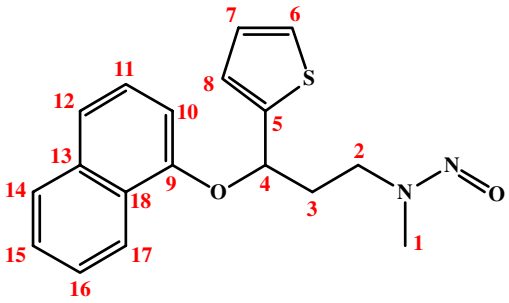

Fig. 9. Numbering assignment of N-nitroso duloxetine

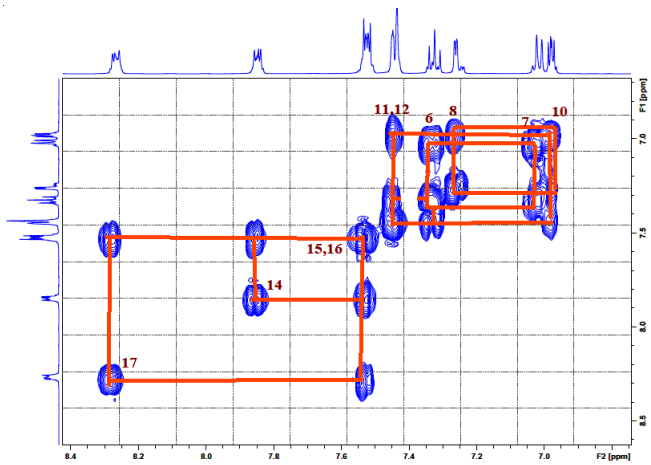

Fig. 10. COSY spectrum of N-nitroso duloxetine

of the standards. Accurately weighed 20 mg of N-nitroso duloxetine impurity standard in 20 mL of volumetric flask (stock-1) dissolved up to the mark with diluent, 1 mL of stock-1 into 100 mL volumetric flask (stock-2) containing some diluent and make up to mark with diluent and then 0.1 mL of stock-2 into 10 mL volumetric flask (stock-3), made up to mark with diluent and cyclomixed, finally standard prepared with 0.42 mL of stock-3 into 10 mL volumetric flask containing diluent on the basis of test sample with respect to 5 mg/mL, the latter N-nitroso duloxetine solution corresponds to 0.83 ppm and diluent was used as blank.

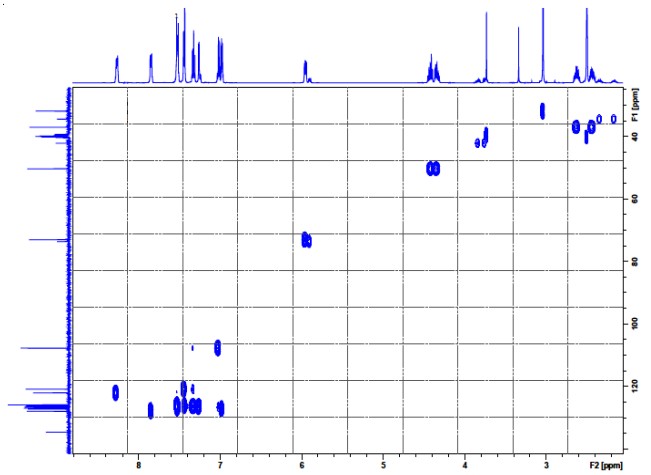

Fig. 11. HSQC spectrum of N-nitroso duloxetine

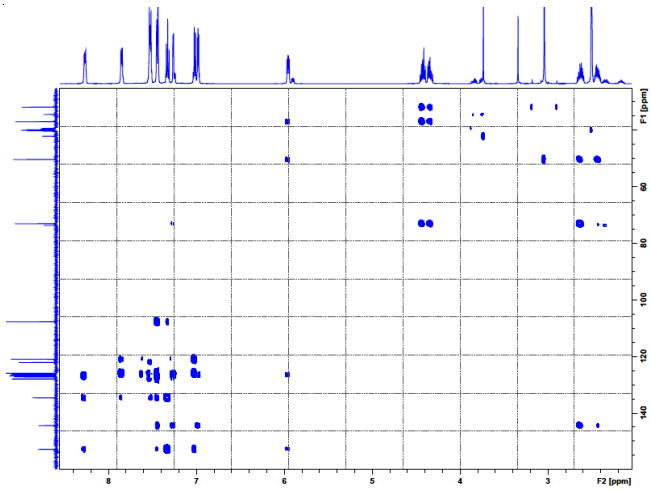

Fig. 12. HMBC spectrum of N-nitroso duloxetine

Sample preparation: Weighed and transfer about 50 mg of drug substance into 15 mL of polypropylene tube dissolved in 10 mL of diluent and centrifuged at 4000 rpm for 10 min, filtered the solution through 0.2 µm PVDF filter into HPLC vial.

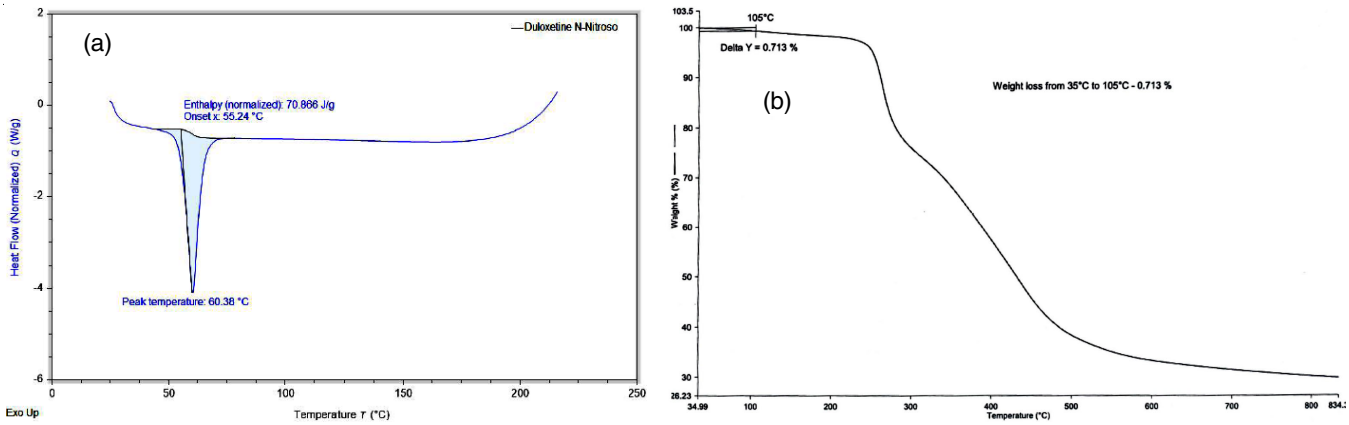

Fig. 13. (a) DSC and (b) TGA thermogram of N-nitroso duloxetine

The method was validated according to below mentioned parameters; system suitability (Table-2) proved system was in good condition, blank and standrd solution shown in (Figs. 14 and 15) followed by LOD, LOQ establishment, LOD (0.040 ppm) measurement of lowest concentration of the analyte that can be detected but cannot be quantified and its signal to noise ratio more than 3. LOQ solution (0.080 ppm) concentration should be 10% of specification limit, it states lowest concentration of analyte that can be quantified with an acceptable precision and accuracy and signal to noise ratio more than 10, EMA guidelines [11] provides the option for in the case of a single nitrosamine impurity, as long as it can be shown that levels of the single mutagenic impurity in the drug substance are consistently less than 10% from specification level then go for omission test.

TABLE-2
SYSTEM SUITABLETY OF N-NITROSO DXT STANDARD SOLUTION

Standard solution	Impurity area
Injection-1	3143322
Injection-2	3119821
Injection-3	3098611
Injection-4	3165447
Injection-5	3195365
Injection-6	3178321
Average	3150147.83
Standard deviation	36591.6329
% of RSD	1.2

Selectivity is the ability of an analytical method to ensured interference of the analyte in the presence of other components in a sample. This was demonstrated by analysis of blanks, system suitability and test sample. Established the solution stability from standard solution, test solution and spiked test sample solution at 100% level over a period of 15 h at 2-8 ºC.

Precision was evaluated by injection of six replicates of sample solutions that were prepared by spiking drug substance test samples at LOQ, 100% and 150%. Recovery was evaluated by spiking samples with N-nitroso duloxetine at LOQ, 50%, 100% and 150% of specification level and comparing the analyte peak area against a pure standard of the same concentration. The analyte could be fully recovered (111.8% at 50% level 107.9 % at 100% and 106.8% at 150%), which indicated that no additional matrix effect was observed (Table-2). In duloxetine hydrochloride test samples, N-nitroso duloxetine impurity was detected between 0.3 and 0.4 ppm, it was subtract in spiking studies from accuracy, precision tests and calculated, for LOQ precision sample content was more than LOQ hence LOQ solution spiked to blank solution.

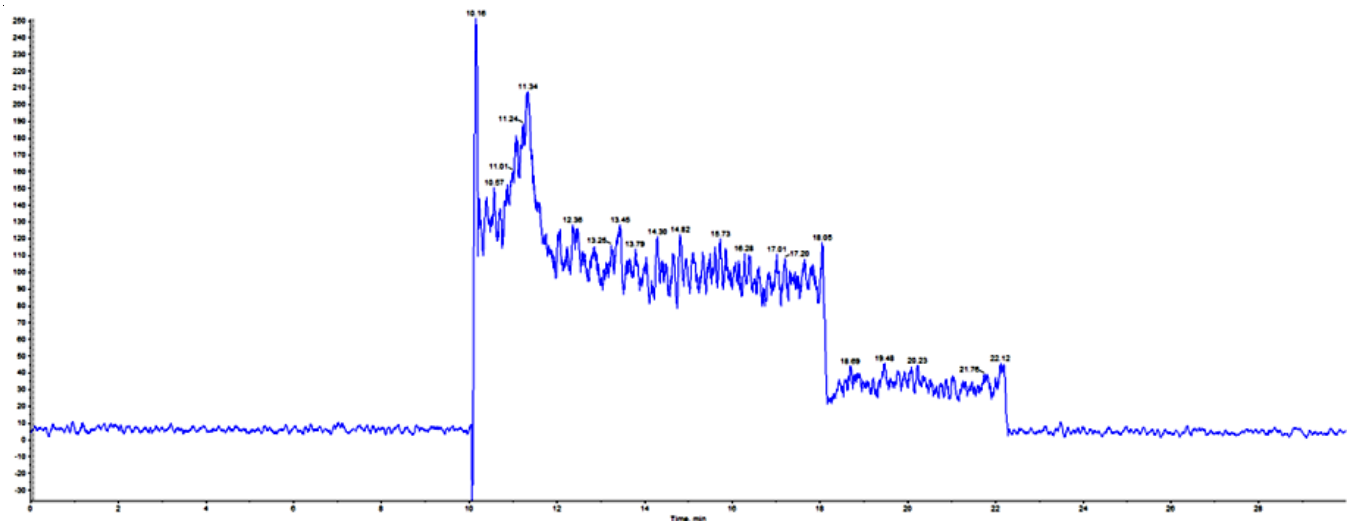

Fig. 14. Blank chromatogram of N-nitroso duloxetine

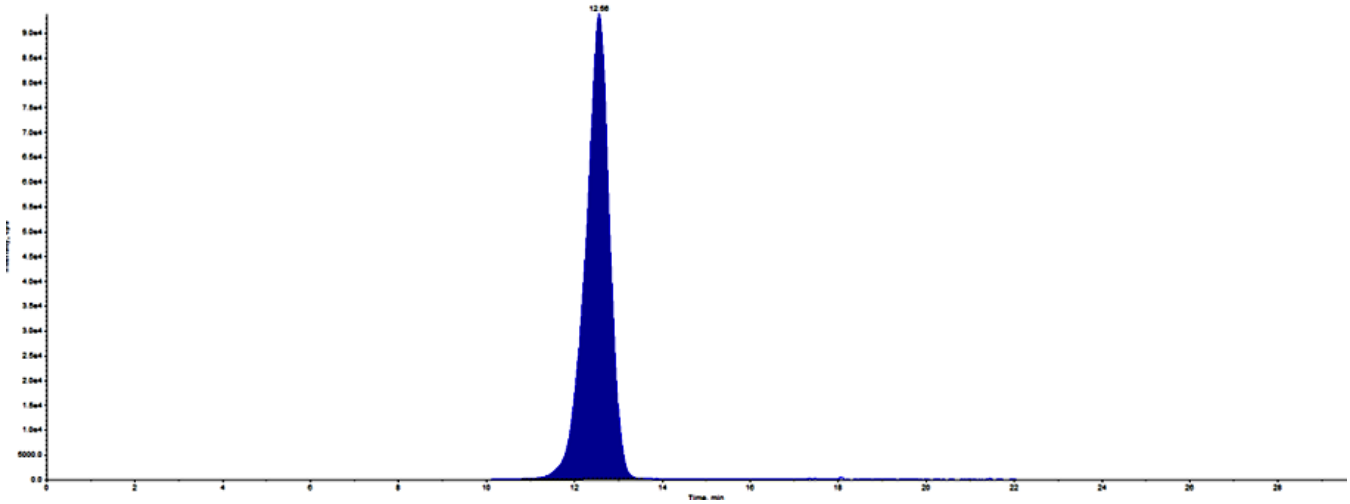

Fig. 15. Standard solution chromatogram of N-nitroso duloxetine

Range is defined as the range of concentration in which method is linear, precise and accurate, for range, data was considered from linearity, precision and accuracy results are shown in Table-3.

TABLE-3
RANGE RESULTS FROM LOQ TO 150%

Level	Precision (% of RSD)	Accuracy (%Recovery)	Linearity
LOQ solution	0.6	NA	0.9996
25% solution	NA	NA	
50% solution	NA	106.0	
100% solution	0.9	109.2	
125% solution	NA	NA	
150% solution	1.7	105.0	
Acceptance criteria	RSD should be not more than 15.0%	The recovery should be between 70 to 130	Correlation coefficient should not be less than 0.99

Method precision and intermediate precision are the analytical method well-defined as proximity in the repetitive measurements. It is established by using six different preparations of test sample spiked at specification level along with duplicate preparations of the test sample. The ruggedness of an analytical method intermediate precision also performed with different analyst, different day and different column, cumulative (method precision and intermediate precision) % RSD of the N-nitroso duloxetine impurity content (Table-4) was within the limit (≥ 25).

The robustness condition at column oven temperature and column flow 10% variation, robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage, it was performed various parameters like column flow rate at 0.72 mL/min, 0.88 mL/min and column Oven temperatures at 54 ºC and 66 ºC even these various parameters also system suitability, test sample and 100% spike was within acceptable limits.

From the above experimental data on the various method validation parameters, it is proved that this method which was designed to determine N-nitroso duloxetine impurity content by LCMS, it is precise, accurate, linear, selective, rugged and robust. Solution stable up to 15 h and robust and range from LOQ to 150%.

TABLE-4
CUMULATIVE % RSD FROM METHOD PRECISION AND INTERMEDIATE PRECISION

Injection ID	Method precision	Intermediate precision
1	0.919	0.919
2	0.901	0.914
3	0.909	0.914
4	0.918	0.913
5	0.913	0.911
6	0.925	0.911
Mean (for n = 12)	0.9139	
STD DEV	0.0058	
% of RSD	0.63	

As per need the drug supply in to the market, regulatory bodies was given exception in AI and outlined in Q&A20, EMA guidelines [11,14] the less-than lifetime (LTL) concept or the use of interim limits may be considered by the lead authority and national competition authorities (NCAs) on a temporary basis in order to inform market actions and at the same time ensure availability of medicines. Authorisation Holder (MAH) are expected to establish and implement corrective and preventive actions (CAPAs) in authorized medicines without any delays in order to ensure patients safety and product quality. Nevertheless, it is recognized that implementation of CAPAs may require some time before the MAH is able to mitigate the presence of the identified N-nitrosamine below the established acceptable intake. Therefore, in order to avoid unnecessary risk of supply disruptions, a harmonized approach promoting the establishment of interim limits in a streamLined way is agreed. The approach is applicable to all authorized products that have CAPA implementation timeline of up to 3 years from the establishment of the AI (nevertheless MAHs are expected to expedite CAPAs implementation) and basis on LTL approach N-nitroso DXT limit for drug product specification limit can be use up to 5.583 ppm (100 × 6.7 = 670).

$$\text{Acceptable intake (AI) limit} = \frac{6.7 \times 100}{120} = \frac{670}{120} = 5.583 \text{ ppm}$$

Treatment duration	Up to 12 months	> 12 Months
Interim limit	13.3 × AI	6.7 × AI

As per test sample of drug substance, N-nitroso DXT content detected between 0.3 and 0.4 ppm. As for DXT drug product, If detected content exceeds the specification limit 0.83 ppm or AI value (100 ng/day) that could be due to nitrite/nitrate impurities present in excipients in ppm, which N-nitroso DXT can form during manufacturing or shelf-life storage. It is possible to consider the specification limit up to 5.583 ppm instead of 0.83 ppm as interim limit [11].

Conclusion

In this study, the chemical structure of impurity confirmed by LC-MS and NMR spectroscopy, E,Z-isomers of N-nitroso DXT was explained by drift functional theory (DFT) and LC-MS method for the estimation of N-nitroso duloxetine was developed and ensured that the proposed method was sufficiently sensitive to control the impurity at trace level. The method was validated and fulfilled as per EMA guidelines for analytical method validation and in addition to its simplicity and ease of use, this method is also easily adaptable to quality control for routine analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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