DETERMINATION OF GENOTOXIC IMPURITIES N-NITROSAMINE'S IN EFAVIRENZ DRUG SUBSTANCE USING RP-HPLC TECHNIQUE

ABSTRACT

Highly sensitive method for the determination of genotoxic N-Nitrosamine's in Efavirenz using RP-HPLC method have been developed. N-Nitrosamine's were determined by RP-HPLC method using Zorbax SB C18 (150x4.6mm, 3.5µ) column as stationary phase. Column temperature maintained 25°C, injection volume 20 µL, flow rate 1.0 mL/min and sample cooler temperature 5°C and run time was 15 minutes. The mixture of 0.1% formic acid buffer and methanol in the ratio of 50:950 (v/v) was used as mobile phase. The method was validated as per International Conference on Harmonization guidelines. Limit of quantitation (LOQ) was found in 0.3 µg/mL for N-Nitrosamine's.

Key words: Efavirenz, determination of genotoxic impurities, Limit of quantitation and liquid chromatography.

Abbreviations:

(NDMA = N-Nitrosodimethylamine, NDEA = N-Nitrosodiethylamine, NDIPA= N-Nitrosodiisopropylamine)

1.0 Introduction

Synthesis of drug substances often involves the use of reactive reagents and hence, these reagents may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose [1]. These limits generally fall at low mg/mL levels and hence conventional HPLC, GC methods (or final drug substance methods) are not suitable for their determination. Hyphenated techniques like GC–MS and LC–MS combine physical separation capabilities of chromatography (GC or HPLC) with the mass analysis capabilities of mass spectrometry and have high sensitivity and specificity over conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances [2].

Efavirenz is chemically (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3, 1-benzoxazin-2-one **(Figure 1).** It is a white powder form and used as antiretroviral agent, for the

treatment of HIV infection. It has an empirical formula of $C_{14}H_9ClF_3NO_2$ and molecular weight of 315.675. Efavirenz belongs to a class of antiretroviral drugs known as non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV-1) [3].

In the manufacturing process of Efavirenz, N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA) and N-Nitrosodiisopropylamine (NDIPA) may exist as impurities in Efavirenz drug substance.

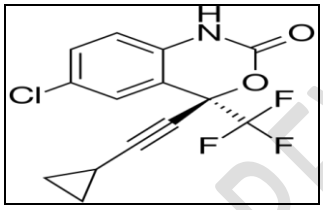
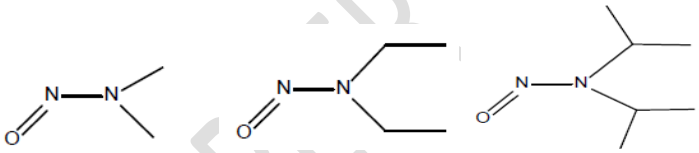


Figure 1. Chemical structure of Efavirenz



N-Nitrosodimethylamine **Nitrosodiethylamine** **N-Nitrosodiisopropylamine**

Figure 2. Nitrosamines impurities structures

Based on maximum daily dose of Efavirenz (600 mg/ day). The acceptable concentration for NDIPA was authorized by a European Medicines Agency on 20 August 2019 [4] to the level of 0.177 ppm, corresponding to its maximum daily dose of 26.5 ng/day. The acceptable concentrations for NDMA and NDEA were provided by European Medicines Agency on 14 February 2019 [5] to the level of 0.64 ppm and 0.177 ppm, corresponding to their maximum daily dose of 96.0 ng/day and 26.5 ng/day respectively.

Generally, most of the reported methods for nitrosamine impurities analyses in scientific literature utilize Gas Chromatography separation and that too specifically for NDMA. Moderate to high polarity stationary phases are used in these separations with carbowax or analogous material [6–8] . Alternatively, High Performance Liquid Chromatography (HPLC) on reverse

phase columns have been mentioned for NDMA determination by Ultraviolet (UV) using diode array detection (DAD) at wavelength of 230 - 233 nm [9–11] .

However, no method was reported for the determination of Nitrosamines in Efavirenz. Hence, the present work is aimed towards the development of rapid, specific and robust methods for the determination of Nitrosamines in Efavirenz at trace level concentration.

2.0 Experimental

2.1. Chemicals and reagents

N-Nitrosodimethylamine, N-Nitrosodiethylamine and N-Nitrosodiisopropylamine purchased from sigma-Aldrich., Mumbai, India. Formic acid and Methanol were procured from Merck, India.

2.2 Preparation of solutions

Preparation of 0.1% formic acid buffer

Transferred 1.0 mL of formic acid into a 1000 mL of water and mixed well.

Preparation of mobile phase

Prepared a mixture of 0.1% formic acid buffer and methanol in the ratio of 50:950 (%v/v). Filtered through 0.45 membrane filtered and sonicated to degas.

Preparation of diluent

Methanol was used as diluent.

Preparation of NDMA and NDIPA standard stock solution-I

Accurately weighed and transferred 20 mg of each impurity NDMA and NDIPA standards into a 20 mL volumetric flask containing 5 mL diluent and made upto the mark with diluent.

Transferred 0.75 mL of above solution into a 100 mL volumetric flask containing 25 mL diluent made upto the mark with diluent.

Preparation of NDEA standard stock solution-II

Accurately weighed and transferred 20 mg of NDEA standard into a 20 mL volumetric flask containing 5 mL diluent and make up with diluent.

Transferred 0.75 mL of above solution into a 100 mL volumetric flask containing 25 mL diluent made upto the mark with diluent.

Preparation of standard solution (0.3 ppm)

Transferred 1.0 mL of standard stock solution-I and standard stock solution-II in to a 25 mL volumetric flask containing 25 mL diluent and made upto the mark with diluent.

Preparation of test Solution

Weighed accurately 10 mg of the test sample into a 10 ml volumetric flask and added 7.0 mL of diluent and made upto the mark with diluent and mixed well. (1mg/ml).

Transferred 0.1 ml of the above solution into a 10 ml volumetric flask and added 7.0 mL of diluent and made upto the mark with diluent and mixed well. (0.01mg/ml)

2.2. Preparation of spiked sample

Methanol was used as diluent in this method. NDMA, NDEA and NDIPA stock solutions were prepared by dissolving 20 mg each individually in 20 mL of diluent. Transferred 0.75 mL of this solution into 100 mL volumetric flask containing 25 mL diluent and made upto the mark with diluent. Further diluted 1.0 mL of this into 25 mL with diluent. The mixture solution, 0.3µg/mL with respect to 0.01mg/mL of Efavirenz, was prepared by diluting the appropriate volumes of above stock solutions with diluent as above. A blend solution was also prepared by spiking 0.3µg/mL of Nitrosoamines to 0.01mg/mL of Efavirenz and is used for method development.

2.3. Optimization of Chromatographic conditions

After series of trials, the chromatographic conditions are accomplished with the Zorbax SB C18 150 x 4.6mm, 3.5µ column was used as stationary phase. The mixture of 0.10% formic acid buffer and methanol in the ratio of 50:950 (v/v) was used as mobile phase. The flow rate of the mobile phase was kept at 1.0 mL/min. The injection volume was set as 20 µL. Column oven temperature and auto sampler temperature were set as 25°C and 5°C ,wavelength 240 nm. respectively.

3.0 Validation of Analytical Method

Validation was performed as per FDA guidelines for the developed method.

System Suitability/System Precision

Established system suitability / system precision by injecting standard solutions for six times and calculated % RSD for peak areas of NDMA, NDEA and NDIPA.

Specificity

Specificity was demonstrated by injected blank solution, standard solution, sample solution, spiked sample and individual impurities and analyzed as per the test method. It was observed that known impurities are not co eluting with each other and main analyte peak.

Method Precision

Method precision was demonstrated by prepared six samples by spiking of impurities at specification level and analyzed as per the test method.

Intermediate precision

Carry out the intermediate precision study on different day with different analyst using the fresh preparations.

Limit of detection (LOD) & Limit of Quantitation (LOQ)

Detection limit have been established for NDMA, NDEA and NDIPA. DL solution were prepared. DL solution of NDMA, NDEA and NDIPA peak were finalized by using the worst found signal to noise ratio for each peak was greater than 3 in each injection.

Quantitation limit have been established for NDMA, NDEA and NDIPA Based on the concentration obtained from DL solutions, the QL solutions were prepared. QL solution of NDMA, NDEA and NDIPA peak were finalized by using the worst found signal to noise ratio for each peak was greater than 10 in each injection.

Quantitation Limit (QL) & Precision at QL

This is the measurement of lowest concentration of NDMA, NDEA and NDIPA that can be quantified with acceptable precision.

Linearity

The linearity is determined by injecting the solutions in duplicate containing known impurities and Efavirenz and impurities ranging from LOQ to 150% of the specified limit. Perform the regression analysis and determine the correlation coefficient.

Accuracy

The accuracy of the test method was demonstrated by preparing recovery samples LOQ level to 150% of level. The recovery samples were prepared in triplicate for each concentration level.

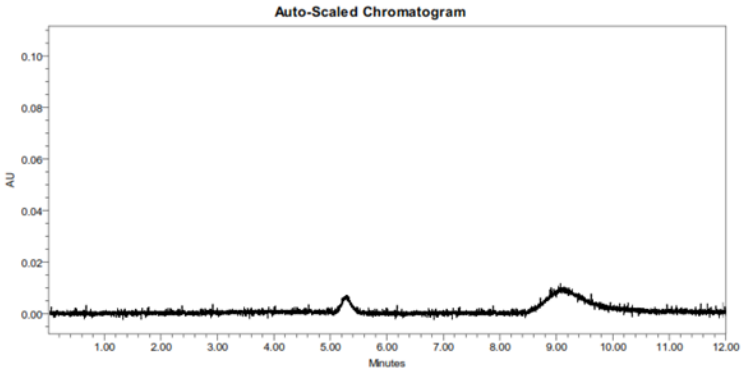


Figure 3. Typical chromatogram of Blank

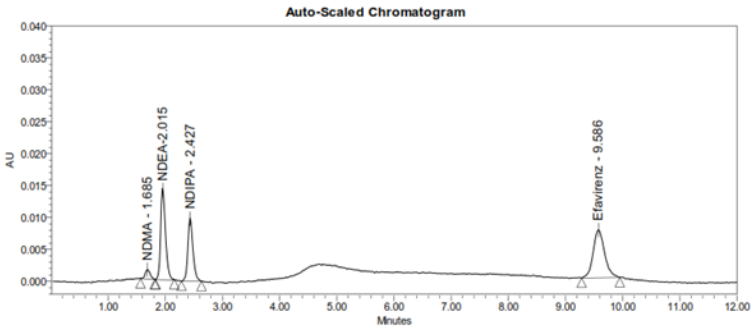


Figure 4. Nitrosoamines spiked chromatogram of Efavirenz

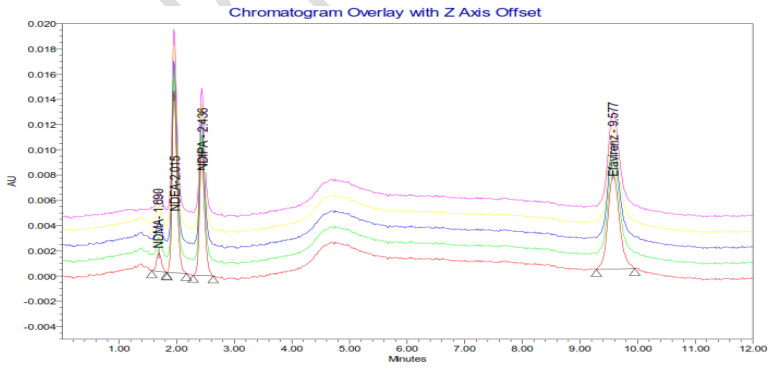


Figure 5. Overlay spiked chromatogram

Table 1. Validation results of Efavirenz for the determination of NDMA, NDEA and NDIPA impurities

Parameter	NDMA	NDEA	NDIPA
System precision (%RSD)	6.4	8.5	9.2
LOD (µg/mL)	0.03	0.02	0.03
LOQ (µg/mL)	0.09	0.06	0.09
Precision at LOQ (RSD, %)	3.4	18.7	5.2
Method Precision (%RSD)	1.3	3.4	2.7
Intermediate precision (%RSD)	0.9	3.9	3.0
Linearity range (µg/mL)	0.093-0.464	0.062-0.461	0.088-0.439
Correlation coefficient	1.000	1.000	1.000
Slope	60226.74	14285.21	13713.59
Intercept	-444.56	90.44	-146.75
Accuracy (recovery, %)			
Accuracy at LOQ	106.7	112.3	117.6
Accuracy at 100%	107.3	105.9	114.2
Accuracy at 150%	107.9	104.4	113.2

4.0 Results & Discussion

The specificity of the developed LC-MS method was indicated by Nitrosoamines solutions (0.3µg/mL each) with respect to 0.01mg/mL of Efavirenz.

For selectivity, the chromatograms were recorded for standard and sample solutions of Efavirenz and its Nitrosoamines. There is no interference of blank at Efavirenz and impurities peaks. Selectivity studies reveal that the peaks are well separated from each other.

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For Precision studies six ~~(6)~~ replicate injections were performed. %RSD was determined from the peak areas of Efavirenz and its Nitrosoamines. The acceptance limit should be not more than 15.0% and the results were found to be within the acceptance limits.

The precision of the method was checked by injecting six different preparations at specification level with respect to the test sample concentration. The % RSD for the results obtained from method precision study were within the limit.

The intermediate precision of the method was also verified on six different preparations at specification level with respect to the test sample concentration different days in the same laboratory. The % RSD for the results obtained from intermediate precision study were within the limit.

The cumulative %RSD for the results obtained from method precision study and intermediate precision study together were within the limit.

The limit of detection (DL) and limit of quantitation (QL) for Efavirenz and its Nitrosoamines were injected separately and S/N ratios were recorded. It was observed that the S/N values of NDMA, NDEA and NDIPA obtained from QL & DL level were within the limit.

Linearity test solutions for Nitrosoamines were prepared individually at six concentration levels in the range of LOQ to 150% of the specification level 0.3µg/mL. LOQ and sixth levels were injected six times and other four levels were injected thrice. The average peak areas versus concentrations were subjected to least-squares linear regression analysis. The derived correlation coefficients were above 0.9999 indicating the best fitness of the linearity curves of the developed method.

Standard addition experiments (% recovery) were conducted in triplicate preparations to determine accuracy of the method at LOQ level and recoveries of all the genotoxins were determined. The recoveries were found to be in the accepted range

Efavirenz at trace level concentration have been developed and validated as per ICH guidelines. The efficiency of the method was ensure by the specificity, precision, Linearity and accuracy. Hence, the method well suit for their intended purposes and can be successfully applied for the release testing of Efavirenz into the market.

5.0 Conclusion

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RP-LC method that can quantify genotoxic Nitrosoamines in Efavirenz at trace level concentration have been developed and validated as per ICH guidelines. The effectiveness of the method was ensured by the specificity, precision, linearity and accuracy. Hence, the method well suit for their intended purposes and can be successfully applied for the release testing of Brinzolamide into the market.

References

1. European Medicines Agency, Guideline on the Limits of Genotoxic Impurities, CPMP/SWP/5199/02, EMEA/CHMP/QWP/ 251344/2006 (2007).

2. N.V.V.S.S. Raman, A.V.S.S. Prasad, K. Ratnakar Reddy, Strategies for the identification, control and determination of genotoxic impurities in drug substances: A pharmaceutical industry perspective, J. Pharm. Biomed. Anal. 2011; (55): 662–667.

3. http://drugbank.cd/drugs/DB00625

4. Temporary interim limits for NMBA, DIPNA and EIPNA impurities in sartan blood pressure medicines. 2020.

5. Assessment report. Committee for Medicinal Products for Human Use (CHMP). 2020.

6. Empl MT, Kammeyer P, Ulrich R, Joseph JF, Parr MK, Willenberg I, et al. The influence of chronic l-carnitine supplementation on the formation of preneoplastic and atherosclerotic lesions in the colon and aorta of male F344 rats. Archives of Toxicology. 2015;89(11):2079–2087.

7. Crews C. The determination of N-nitrosamines in food. Quality Assurance and Safety of Crops & Foods. 2010;2(1):2–12.

8. Seyler TH, Kim JG, Hodgson JA, Cowan EA, Blount BC, Wang L. Quantitation of Urinary Volatile Nitrosamines from Exposure to Tobacco Smoke. Journal of Analytical Toxicology. 2013;37(4):195–202.

9. Jurado-Sánchez B, Ballesteros E, Gallego M. Screening of N-nitrosamines in tap and swimming pool waters using fast gas chromatography. Journal of Separation Science. 2010;33(4-5):610–616.

10. Li W, Chen N, Zhao Y, Guo W, Muhammd N, Zhu Y, et al. Online coupling of tandem liquid-phase extraction with HPLC-UV for the determination of trace N-nitrosamines in food products. Analytical Methods. 2018;10(15):1733–1739.

11. Al-Kaseem M, Al-Assaf Z, Karabeet F. A Rapid, Validated RP-HPLC Method for the Determination of Seven Volatile N-Nitrosamines in Meat. Pharmacology & Pharmacy. 2014;05(03):298–308.