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Determination of Genotoxic Impurity n-acetyl sulfanilic acid methyl ester in Darunavir Drug Substance using RP-HPLC

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Abstract **-** *A highly sensitive method for the determination of genotoxic impurities, such as N-acetyl sulfanilic acid methyl ester in Darunavir using RP-HPLC, has been presented in the present paper. Quantification of N-acetyl sulfanilic acid methyl ester content in Darunavir samples by HPLC with UV Detector. The N-acetyl sulfanilic acid methyl ester was determined by RP-HPLC method using Zorbax SB-Phenyl (250 x 4.6mm, 5µm) column as stationary phase. Flow rarely was 0.8 mL/min, Column temperature maintained 30°C, sample cooler temperature 5°C, Injection volume 25µL, and run time was 25mintues. Mobile phase-I was used as milli-Q water, and Mobile phase-B was used as acetonitrile, methanol, and water in the ratio of (80:10:10 v/v/v). The method validation has been carried as per International Conference on Harmonization guidelines (ICH). Limit of quantitation (LOQ) and Limit of detection (LOD) was found 0.374ppm and 0.125ppm for N-acetyl sulfanilic acid methyl ester.*

Keywords **-** *Genotoxic impurity, Darunavir, RP-HPLC method, Validation and Limit of quantitation, and Limit of detection.*

I. 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 µg/mL levels. HPLC, GC methods (or final drug substance methods) are suitable for their determination. Their applications are oriented towards the potential identification and quantitation of trace levels of impurities in drug substances [2].

The chemical name of [(1R,5S,6R)-2,8-dioxabicyclo[3.3.0]oct-6-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methyl propyl)amino]-3-hydroxy-1-phenyl- butan-2-yl] carbamate Corresponding to the molecular formula $C_{27}H_{37}N_3O_7S$. It has a relative molecular mass of 547.67 g/mol. Darunavir sold under the brand name Prezista among others, is an antiretroviral medication used to treat and prevent HIV/AIDS. It is generally recommended for use with other antiretrovirals [3-4]. It is often used with low doses of ritonavir or cobicistat to increase darunavir levels.

Darunavir is a white to off-white, hygroscopic powder. Its solubility in organic solvents varies significantly, and it is very slightly soluble in an aqueous solution (solubility increases with decreasing pH). Its pKa has been found to be 11.43, and its partition coefficient was found to be 1.89. Therefore, the particle size is likely to be important to the rate and possibly to the extent of absorption of Darunavir. It contains 5 chiral centers. However, the manufacturing process leads, in a consistent way, to the single enantiomer 3R, 3aS, 6aR, 1S, 2R. The absolute configuration has been confirmed by X-ray diffraction analysis. Under commercial synthesis conditions, Darunavir is isolated as a crystalline ethanolate (1:1 solvate). It can exist as a non-solvated amorphous form and as a hydrate form as well. Investigations of conditions under which inter-conversion between the different polymorphs occur showed that the hydrate form can be formed under conditions of high relative humidity and that both solvates can be converted into the amorphous form when subject to heat and/or extremely low relative humidity.

In the manufacturing process of Darunavir, N-acetyl sulfanilic acid methyl ester is used as a reagent, and hence genotoxic N-acetyl sulfanilic acid methyl ester may exist as an impurity in Darunavir drug substance.

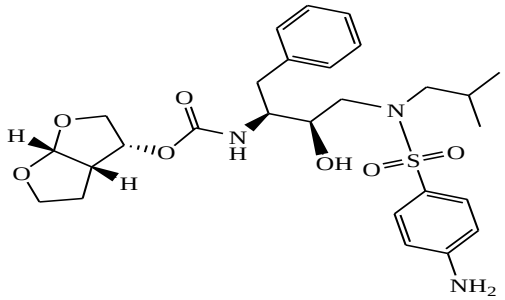

Figure 1. Chemical structure of Darunavir

A. Impurity structure:

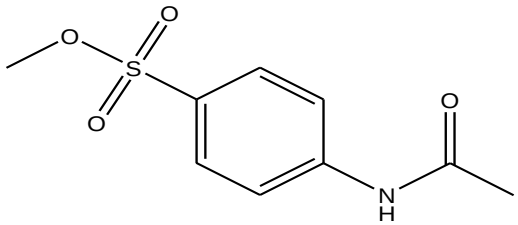

Figure 2 Chemical structure of N-acetyl sulfanilic acid methyl ester

In literature, no analytical method was reported for the determination of N-acetyl sulfanilic acid methyl ester in the Darunavir drug substance. Hence the author was aimed towards the development of rapid, specific, and robust methods for the determination of N-acetyl sulfanilic acid methyl ester in Darunavir drug substance at trace level concentration. The method validation has been carried as per International Conference on Harmonization guidelines (ICH) [5].

II. Experimental

Chemicals and reagents

N-acetyl sulfanilic acid methyl ester purchased from Sigma-Aldrich., Mumbai, India. Methanol and acetonitrile were procured from Merck, India.

Mobile phase-A:

Milli-Q water was used as mobile phase-A.

Mobile phase-B:

A mixture of acetonitrile, methanol, and water in the ratio of (80:10:10 %v/v/v). Filter through 0.45µ membrane filter paper.

Preparation of diluent:

Milli-Q water was used diluent.

Preparation of N-acetyl sulfanilic acid methyl ester stock solution:

Weighed and transferred 10.165 mg of N-acetyl sulfanilic acid methyl ester impurity into a 100mL volumetric flask. Added 20 mL of diluent, dissolved and made up to volume with diluent, and mixed well. Further transferred 0.62 mL of this solution into 20 mL volumetric flask containing about 10 mL of diluent then made up to volume with diluent and mixed well.

Standard solution:

Transferred 100µLof N-acetyl sulfanilic acid methyl ester impurity stock solution into a 5mL volumetric flask containing about 2mL of diluent. Mixed well and made up to the mark with diluent. This solution is equivalent to 1.25ppm of N-acetyl sulfanilic acid methyl ester with respect to 50.0mg/mL of sample solution.

Preparation of sample spiked solution:

Weighed 250mg of the Darunavir into a 5mL volumetric flask. Dissolved in 3mL of diluent and added 100µL of N-acetyl sulfanilic acid methyl ester impurity stock solution. Mixed well and then made up to the mark with diluent.

Chromatographic conditions:

RP-HPLC analysis was carried out on Agilent-1200 (Agilent Corporation, USA) wavelength 210 nm. Zorbax SB-Phenyl (250 x 4.6mm, 5µm) column was used as stationary phase. Mobile phase-I was used as milli-Q water, and Mobile phase-B was used as acetonitrile, methanol, and water in the ratio of (80:10:10 v/v/v). The flow rate of the mobile phase was kept at 0.8 mL/min. The injection volume was set as 25µL. The column oven temperature and autosampler temperature were set as 30°C and 5°C, respectively.

III. RESULTS AND DISCUSSION

A. Method development

A blend solution containing N-acetyl sulfanilic acid methyl ester impurity and Darunavir was run at 0.6mL/min flow rate. Darunavir eluted too extended, and hence the flow rate of the mobile phase was decreased from 0.6 mL/min to 0.8 mL/ min. In this condition, Darunavir eluted at an optimum retention time, but the retention time of N-acetyl sulfanilic acid methyl ester impurity was drastically increased. Hence, the elution order was observed from the chromatogram **(Fig.5.0).** Darunavir solution spiked with N-acetyl sulfanilic acid methyl ester impurity (1.25µg/mL).

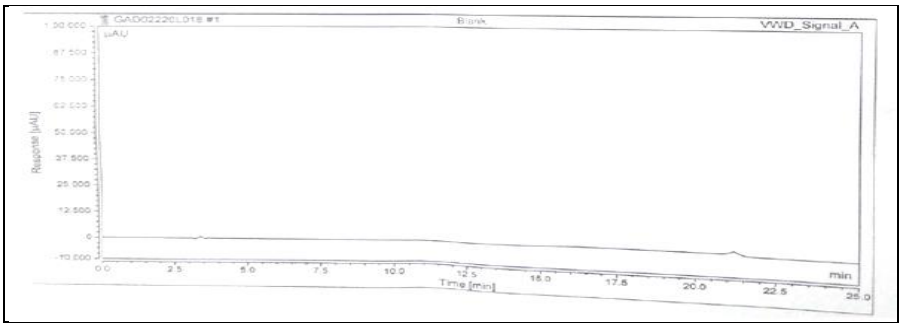

Figure 3 typical chromatogram of Blank

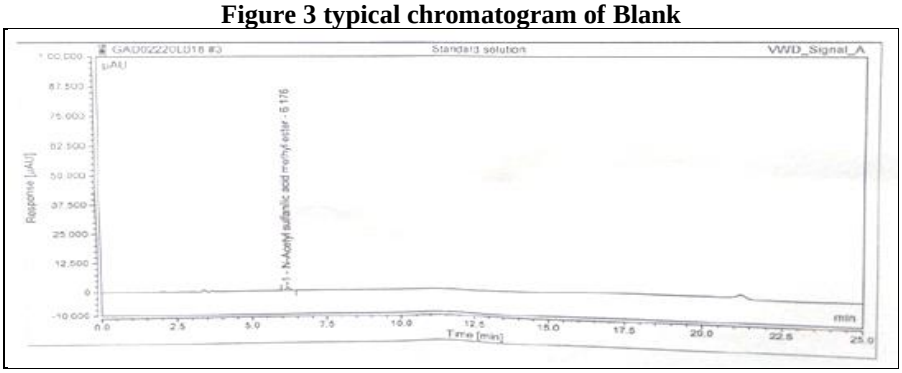

Figure 4 typical chromatogram of Standard

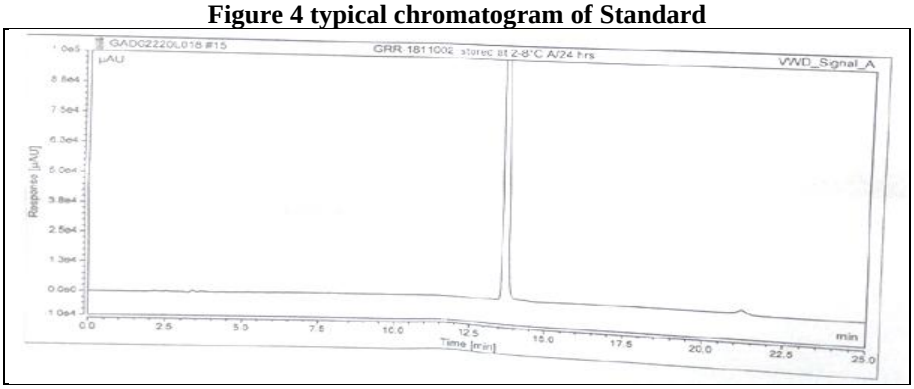

Figure 5 typical chromatogram of Darunavir sample

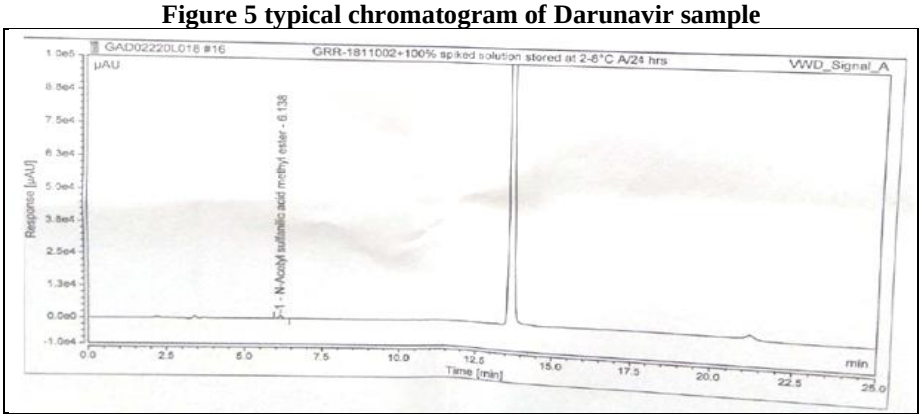

Figure 6 Spiked N-acetyl sulfanilic acid methyl ester chromatogram of Darunavir

B. Method validation

The developed method was validated as per ICH guidelines [5] in terms of specificity, the limit of detection (LOD), the limit of quantitation (LOQ), precision, linearity, accuracy, and system suitability, and the data are presented in Table 1. The specificity of the developed LC method was indicated by N-acetyl sulfanilic acid methyl ester impurity solution (1.25µg/mL each) with respect to 50mg/mL of Darunavir was injected separately, and S/N ratios were recorded. These solutions were further diluted to achieve the signal-to-noise

(S/N) ratios at about 3 and 10 for determining LOD and LOQ, respectively, for both methods. The precision of the methods was checked by injecting LOQ solutions six times. The value of RSD for an area of N-acetyl sulfanilic acid methyl ester impurity was calculated.

Table 1. Validation data of Darunavir for the determination of N-acetyl sulfanilic acid methyl ester impurity

Parameter	N-acetyl sulfanilic acid methyl ester impurity
LOD (µg/mL)	0.125
LOQ (µg/mL)	0.374
Precision at LOQ level (RSD, %)	1.2
Precision at sixth level (RSD, %)	0.4
Linearity (µg/mL)	0.374-1.872
Correlation coefficient	0.9998
Slope	7787.43
Intercept	248.08
Accuracy at LOQ (recovery, %)	96.7
Preparation-1	96.7
Preparation-2	98.3
Preparation-3	95.2

The intermediate precision of the method was also verified on six different days in the same laboratory using the LOQ level solutions. The low RSD values ensured the precision of the developed method. Linearity test solution for N-acetyl sulfanilic acid methyl ester impurity was prepared individually at six concentration levels in the range of LOQ to 150% of the specification level 1.25µg/mL. LOQ and sixth levels were injected six times, and the 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 0.999, indicating the best fitness of the linearity curves of the developed method. Standard addition experiments were conducted in triplicate preparations to determine the accuracy of the methods at the LOQ level, and recoveries of all the genotoxins were determined. The recoveries were found to be in the accepted range. The system suitability of the method was ensured by getting the %RSD less than 10.0 for six injections of the N-acetyl sulfanilic acid methyl ester impurity in the RP-HPLC method at the specification level. Darunavir at trace level concentration has been developed and validated as per ICH guidelines.

successfully applied for the release testing of Darunavir into the market.

IV. CONCLUSION

The proposed RP-LC method that can quantify genotoxic N-acetyl sulfanilic acid methyl ester impurity in Darunavir at trace level concentration has been developed and validated as per ICH guidelines. The effectiveness of the method was ensured by specificity, precision, and accuracy. Hence, the method is well suited for their intended purposes and can be

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