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RESEARCH ARTICLE

# DEVELOPMENT, VALIDATION & STRESS DEGRADATION STUDIES OF DARUNAVIR BY REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHGY (RP-HPLC)

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**Abstract:** A simple, selective, rapid, precise and economical reverse phase high pressure liquid chromatographic method has been developed for the estimation of Darunavir in pharmaceutical dosage form. The mobile phase consisted of 80:20% (v/v) of Methanol & 0.1% ortho-phosphoric acid was used and operated on isocratic mode. The flow rate is 1.0 mL/min. Chromatographic determination of Darunavir was performed on Agilent Polaris $C_{18}$ column (150 X 4.6 mm id, ODS 2,5µm). The wavelength of detection is 265nm. The injection volume is 20µL. The retention time of Darunavir is 2.42 ± 0.01minutes. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification and solution stability. The influence of Acid, Alkaline, Oxidative stress, Photolytic stress condition on Darunavir was studied. Results indicated completed degradation in Alkaline medium. The proposed method has been successfully used for the estimation in tablet dosage forms.

**Key words:** Darunavir; HPLC, Tablet formulation.

## Introduction

Darunavir ethanolate (DRV) is an oral anti-retroviral agent which selectively inhibits the cleavage of Human immunodeficiency virus (HIV-1) encoded Gas-polyproteins in infected cell, thereby preventing the formation of mature virus. Darunavir ethanolate is chemically [(1S,2R)-3-[[(4-amino phenol) sulfonyl](2-methyl propyl)amino]-2-hydroxy-1-(phenylmethyl)propyl]-carbamic acid (3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ester monoethanolate [1] (fig. 1). Darunavir was designed to form robust interactions with the protease enzyme from many strains of HIV, including strains from the treatment-experienced patients with multiple resistance mutations to Protease inhibitors.[2] It blocks HIV protease, an enzyme which is needed for HIV to multiply. According to in vitro experiments, DRV was active against HIV-1 with PI-resistance mutations and against PI-resistance clinical isolates [3-4]. This drug is effective in patients experienced in anti-retroviral treatment, such as those carrying HIV-1 strains which are resistance to more than one PI [5]. The use of advanced instrumentation techniques for the analysis of drugs has been discussed elsewhere [6]. Literature survey revealed that different analytical methods have been reported for the determination of DRV in plasma using liquid chromatography coupled with tandem mass spectroscopy [7]; simultaneous determination of DRV with other anti-retroviral agents in plasma[8-9]. Few HPTLC method for determination of darunavir in rat plasma and in tablet dosage form its application to pharmacokinetics studies [10]. Infrared Spectroscopy method for determination of Darunavir in tablets [11]. Few methods had been developed for determination of Darunavir by HPLC [12-16] and Spectrophotometric method [17]; and electrophoretic method for the separation of DRV[18]. Reported HPLC methods require more time for sample analysis resulting in lesser throughput. Therefore, the present work involves the development of a rapid RP-HPLC method for estimation of Darunavir ethanolate in bulk and tablet dosage form. Validation as per USFDA & ICH guidelines [19], [20] is done along with stress degradation study. The aim of the present study is to develop a simple, precise and accurate reversed-phase HPLC method for the development and validation of Darunavir in bulk drug samples and in pharmaceutical dosage form.

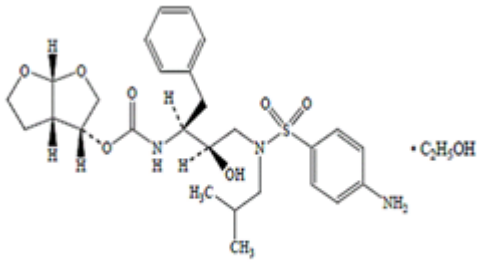

**Fig. 1: Structure of Darunavir ethanolate**

## 2. EXPERIMENTAL

### *2.1 Reagents and Chemicals*

Methanol (HPLC grade, Merck Ltd), Milli-Q water, Darunavir ( Reference standard purchased from Sigma Aldrich, USA), 0.1% Ortho-Phosphoric acid (GR Grade, SD Fine Chem Ltd). All other chemicals are of the highest grade commercially available unless otherwise specified.

### *2.2 Instrumentation*

The chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC solutions software.

### *2.3 Chromatographic Conditions*

The mobile phase consisted of 80:20 % (v/v) of Methanol & 0.1% Ortho-phosphoric acid operated on isocratic mode. The flow rate is 1.0 ml/min. Chromatographic determination of Darunavir was performed on Agilent Polaris $C_{18}$ column (150 X 4.6mm id, ODS 2,5µm). The wavelength of detection is 265. The injection volume is 20µL.

### *2.4 Preparation of standard solutions, Calibration standards & Quality Control Samples*

Stock solution of Darunavir (5mg/mL) was prepared separately in a volumetric flask and labelled accordingly. Suitable dilutions of Darunavir were prepared using 50:50 % v/v Methanol & Milli-Q water as diluents solutions. A linear Calibration curve containing eight zero standards were prepared using diluent solution in the concentration range of 10.3-101.27µg/mL. The linear standard calibration standard sample is then transferred into the auto sampler for analysis. Samples for Specificity (Sample with Drug; Blank Sample were also prepared accordingly).

For the preparation of quality control samples, a separate stock containing approximately the same concentration of the drug substance is prepared and labelled as quality control stock. From this stock, quality control samples were prepared at three concentration levels namely LQC (23.20 µg/mL), MQC (50.64 µg/mL), HQC (73.65 µg/mL) so as to obtain low, median and high concentration quality control samples. The performance of the linear calibration curve is then evaluated using quality control samples.

### *2.5. Assay*

The assay of tablets containing Darunavir is done using the procedure given in Indian Pharmacopoeia for tablets. Briefly, twenty tablets, each containing 600 mg of Darunavir as labelled claim were weighed and finely powered; a quantity of powder equivalent to 600 mg of Darunavir was transferred to a 20mL volumetric flask. To this 10mL of methanol was initially added and vortexed thoroughly. The final volume is made up to volume with methanol. The final solution was mixed well. This mixture is then carefully filtered using 0.45µm membrane filter. The filtrate is then taken and suitably diluted and injected for analysis. The assay content was evaluated using the regression equation of linear calibration curve.

### *2.5 Method Validation*

#### *2.5.1 System Suitability*

System suitability tests are an integral part of liquid chromatographic method in the course of optimizing the conditions of the proposed method. They are used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters of these tests are column efficiency (number of theoretical plates), tailing factor of chromatographic peak. The system suitability was assessed by six replicate analysis of the drug at a concentration of 50.64 µg/ml. The acceptance criterion is ± 1 % for the per cent coefficient of the variation for the peak area and retention times for the drug.

#### *2.5.2 Detection and Quantitation Limits (Sensitivity)*

Limits of detection (LOD) and quantification (LOQ) (**Fig-3**) were estimated from both linearity calibration curve method and signal to noise ratio method. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantification limit was defined as the lowest concentration level that provided a peak area with signal to noise ratio higher than 5, with precision (%CV) and accuracy with (±) 20%.

#### *2.5.3 Linearity (Calibration curve)*

A linear relationship was evaluated across the range of the analytical procedure with minimum of six concentrations. A series of combination standard dilutions were prepared over a concentration range of 10.36 to 101.27 µg/mL. The linearity was evaluated by linear regression

analysis, which was calculated by least square method (**Fig. 4**).

*2.5.4 Precision and Accuracy*

Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability refers to the use of the analytical procedure within the laboratory over the shorter period of the time that was evaluated by assaying the QC (Quality control) samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days). Accuracy of the assay method was determined for both intra-day and inter-day variations using triplicate analysis of the QC samples.

*2.5.5 Specificity*

Specificity of the method was determined by comparing the Blank sample with that of the sample containing Darunavir (**Fig-5**). A less than 20% interference of the peak area at the retention time of the drug in the blank sample is taken as acceptance criteria for the analyte. Sample Specificity is also observed in the degradation study of the drug. None of the degraded products must interfere with the quantification of the drug.

*2.5.6 Stability*

The stability of the drug is determined by placing the MQC samples for the short term stability by keeping at room temperature up to12 hours and then comparing the obtained peak area with that of the similarly prepared fresh sample. Further, auto-sampler stability for up to 24 hrs was studied and established.

*2.5.7 Stress Degradation Studies*

For Stress Degradation Analysis, 1 mL aliquots (in duplicate) of samples containing MQC level concentration are treated separately with 100 µL of 0.1N HCl (Acid stress), 0.1N NaOH (Alkaline stress), 5% v/v Hydrogen Peroxide (Oxidative Stress), for 24 Hrs. Samples for Photolytic stress are placed in a transparent glass vial & placed in a UV chamber for 24 Hrs. Samples are then injected for analysis. The results of analysis are then compared with similarly prepared fresh samples.

## 3. RESULTS AND DISCUSSION

*3.1 Method Development and Validation*

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Functional group analysis revealed the presence of acidic character to the molecule. Therefore we evaluated the chromatographic behaviour at different pH values ranging from pH 3.0 to pH 6.5 using various columns like Hypersil-BDS- C18, Symmetry C18, Ymc-pack C18, Ymc-packpro, Spherisorb C18, Phenomenex C18 have been tried with different buffer salts such ammonium Formate, ortho phosphoric acid, di- potassium hydrogen orthophosphate, in combination with acetonitrile, methanol and tetrahydrofuran. However less tailing and high theoretical plates are obtained with Agilent Polaris ODS column C18 150 X 4.6 cm 5µm column. Mobile phase composition consisted of (80:20 v/v) of Methanol and 0.1% Orthophosphoric acid operated on isocratic mode. The flow rate of the method is 1.0 ml/min. Calibration standards were prepared in diluents solution containing 50:50 % v/v of methanol and Milli-Q water. The wavelength of detection is 265 nm. The column temperature is maintained at ambient condition. At the reported flow rate, peak shape was excellent, however increasing or decreasing the flow rate resulted in unacceptable tailing factor and poor peak shape. Hence 1.0 ml/min was optimized flow rate decreasing the consumption of the mobile phase, which in turn proves to be cost effective for long term routine quality control analysis. A typical chromatogram showing the separation of the drug is given in **Fig-2**.

**3.2 Method Validation**

*3.2.1 System Suitability*

The % RSD of the peak area and the retention time for both drug and internal standard are within the acceptable range (**Table-1**). The efficiency of the column was expressed as the number of theoretical plates for the six replicate injections and the USP tailing factor was 4195 and 1.26 ± 0.05.

*3.2.2 Determination and Quantification Limits (Sensitivity)*

Limit of Detection (LOD) and Limit of Quantification(LOQ) were calculated based on calibration curves. They were expressed as LOD = (3.3× σ)/S; LOQ = (10× σ)/S (where σ is the standard deviation of the y-intercepts of the regression line and m is the mean of the slope of the calibration curve). **Fig-3** represents the chromatogram of limit of detection and limit of quantification. The method is found to be sensitive which can be determined from the data obtained from the (**Table-2)**.

*3.3.3 Linearity*

Linear relationship were observed at the concentration of 10.36 to 101.27 µg/mL for DRV were analyzed. Peak areas and concentrations were subjected to least square regression analysis to calculate regression equation. The peak area of DRV were found to be linear in the range concentrations. The linearity was demonstrated in triplicate. All the standards were found to be within the range of 95 – 105 %. The results are tabulated in Table 3 and Table 4.

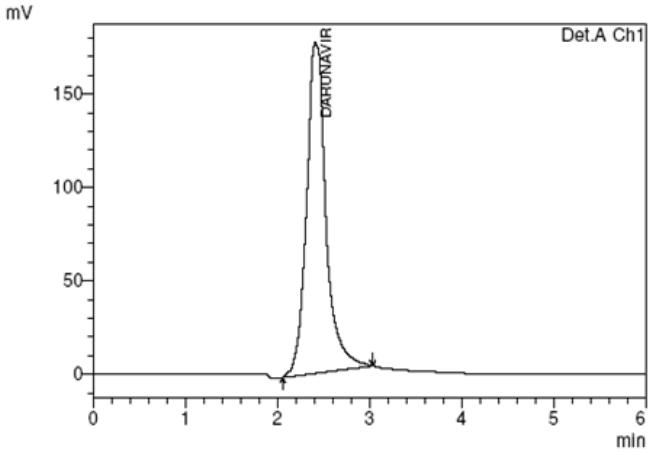


Fig. 2: Typical Chromatogram of Darunavir

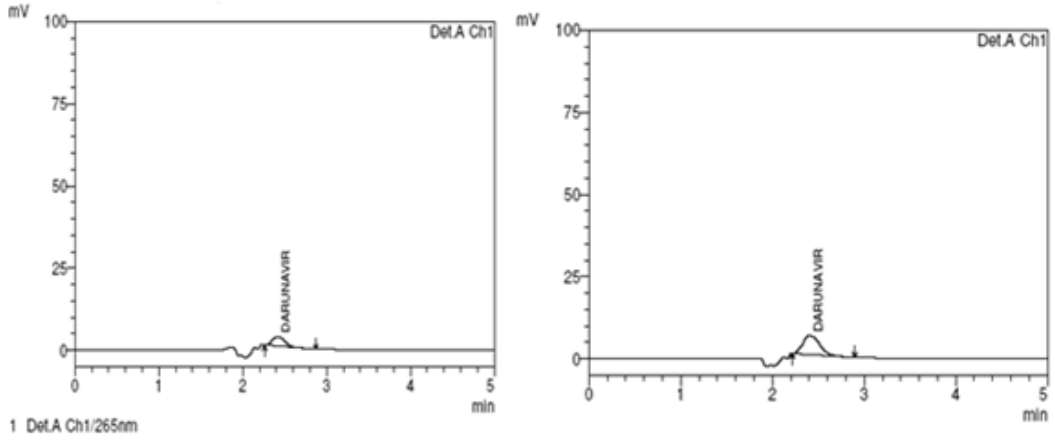


Fig. 3: Chromatograms shown below indicate Limit of Detection (LOD) and Limit of Quantification (LOQ)

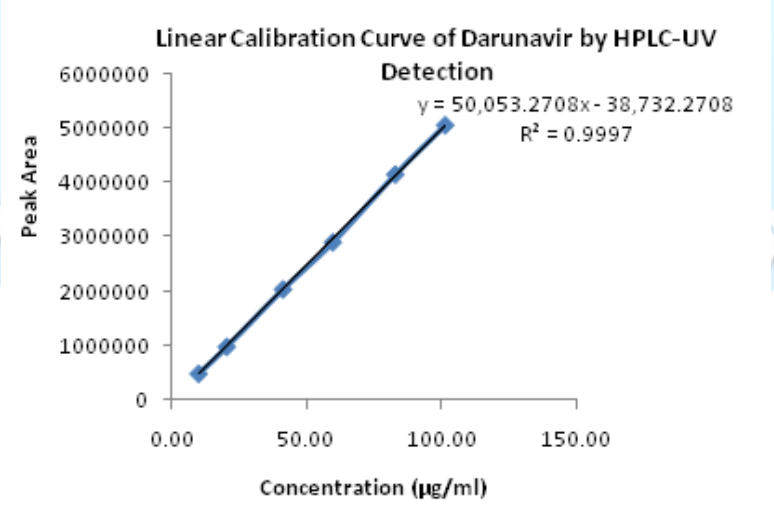


Fig. 4: Linearity Calibration Curve of Darunavir

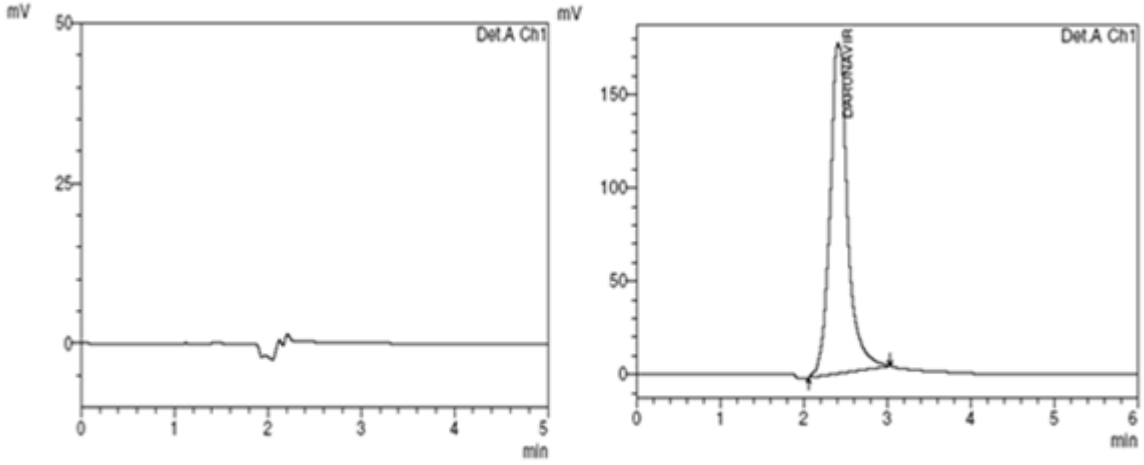


**Fig. 5: Comparison of Blank Chromatogram to that of sample containing Darunavir standard**

**Table 1. System Suitability test for Darunavir**

| S.No | Retention Time | Peak Area | Theoretical Plates | Tailing factor |
|---|---|---|---|---|
| 1 | 2.43 | 2623947 | 4292 | 1.18 |
| 2 | 2.41 | 2613500 | 4161 | 1.27 |
| 3 | 2.42 | 2616504 | 4085 | 1.25 |
| 4 | 2.41 | 2623343 | 4066 | 1.27 |
| 5 | 2.41 | 2692873 | 4396 | 1.28 |
| 6 | 2.42 | 2741876 | 4172 | 1.32 |
| **Mean** | **2.42** | **2652007.17** | **4195.33** | **1.26** |
| **Std.Dev** | **0.01** | **53101.24** | **126.68** | **0.05** |
| **%CV** | **0.34** | **2.00** | **3.02** | **3.66** |

**Table 2. Sensitivity of Darunavir by HPLC**

| LOQ | | |
|---|---|---|
| **S.No** | **Retention Time** | **Peak Area** |
| 1 | 2.40 | 72983 |
| 2 | 2.41 | 72863 |
| 3 | 2.4 | 72712 |
| **Mean** | **2.40** | **72852.67** |
| **Std.Dev** | **0.01** | **135.79** |
| **%CV** | **0.24** | **0.18** |

| LOD | | |
|---|---|---|
| **S.No** | **Retention Time** | **Peak Area** |
| 1 | 2.41 | 34599 |
| 2 | 2.41 | 34953 |
| 3 | 2.41 | 34611 |
| **Mean** | **2.41** | **34721** |
| **Std.Dev** | **0.00** | **201.00** |
| **%CV** | **0.00** | **0.57** |

**Table 3. linearity data of Darunavir**

| S.No | Concentration (µg/mL) | Peak Area |
|---|---|---|
| 1 | 10.36 | 500053 |
| 2 | 20.71 | 992527 |
| 3 | 41.43 | 2041199 |
| 4 | 59.84 | 2900522 |
| 5 | 82.86 | 4137794 |
| 6 | 101.27 | 5035870 |

**Table 4. Statistical data of Linearity for Darunavir by RP-HPLC method**

| Parameter | Value |
|---|---|
| Absorption maximum (nm) | 265 |
| Linearity range (µg/mL) | 10.36-101.27 |
| Regression equation | Y = 50053x - 38732 |
| Correlation coefficient | $R^2 = 0.9997$ |
| Slope | 50053 |
| Intercept | 38732 |

**Table 5. Precision and Accuracy studies of Darunavir**

| | Intra-day Precision | | Inter-day Precision | | Accuracy(%) |
|---|---|---|---|---|---|
| Concentration taken (µg/mL) | Peak Area | % RSD | Peak Area | % RSD | |
| 23.20 | 1131380 | 1.25 | 1137155 | 1.76 | 101.26 |
| 50.64 | 2554351 | 1.0 | 2544958 | 0.38 | 101.93 |
| 73.65 | 3728566 | 1.41 | 3732659 | 0.72 | 102.31 |

**Table 6. Room Temperature Stability of Darunavir (n = 6)**

| Sample ID | Retention time | Peak area | % RSD |
|---|---|---|---|
| Fresh sample | 2.41 | 1024121 | 1.17 |
| Stability sample | 2.40 | 1081526 | 1.47 |

**Table 7: Stress Degradation study data for Darunavir**

| Degradation Condition | Peak Area | Peak Area (%) |
|---|---|---|
| Acidic hydrolysis (0.1% HCl) | 2464581 | 98.18 |
| Oxidation (3% $H_2O_2$) | 2539196 | 101.15 |
| Photolytic degradation (UV light) | 2535741 | 101.02 |
| Control sample | 2510107.5 | - |

**Table 8. Robustness Data**

| Parameters | Optimized | Used | Peak Area | Retention Time | USP plate count | Tailing |
|---|---|---|---|---|---|---|
| Flow rate (± 0.1) | 1.0 mL | 0.9mL | 2856209 | 2.69 | 5232 | 1.29 |
| | | 1.0 mL | 2617782 | 2.41 | 4104 | 1.26 |
| | | 1.1 mL | 2329011 | 2.19 | 3862 | 1.35 |
| Mobile Phase (± 5%) | 80:20 (%) | 75:5 | 2578119 | 2.62 | 5378 | 2.13 |
| | | 80:20 | 2644240 | 2.14 | 4325 | 1.26 |
| | | 85:15 | 2678010 | 2.31 | 3928 | 1.15 |

***3.3.4 Accuracy and Precision***

Accuracy and precision calculated for the QC samples during the intra-day and inter-day run are given the (**Table-5)**. The intra-day (day-1) and inter-day accuracy ranged from 98.00 to 102.00%. The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria.

***3.3.5 Specificity***

Specificity of the method was observed by injecting the blank and working standard solutions of darunavir. The specificity was determined by comparison of the blank chromatogram with that of the Standard chromatogram (**Fig. 5**).

***3.3.6 Room Temperature Stability***

Stability studies were done for short term stability up to 12hrs on the bench top for MQC levels conditions. Stability is calculated as the ratio of the mean peak area of the stability sample to the mean peak area of the fresh sample and expressed as the percentage (n=6). The room temperature stability was found to be 105.60%. The results are tabulated in Table-6.

***3.3.7 Stress Degradation***

The stress studies involving acid, light (UV) and oxidation revealed that Darunavir was not fully degraded. However in alkaline conditions (0.1N NaOH), the drug was instable and the degradation peak eluted earlier accompanied with a drastic peak distortion and increased tailing. Except for alkaline conditions, the drug content was within 95 –105 % for all stress conditions indicating the stability and specificity of the analytical method to differentiate the degradation peaks (**Table-7**).

**3.3.8 Robustness study**

Robustness is the measure of method capacity to remain unaffected by deliberate small changes in the chromatographic conditions. The experimental conditions were deliberately altered to evaluate the robustness of the method. The impact of flow-rate (1.0 ± 0.1 ml/min), and effect of mobile-phase composition (± 5%) on chromatographic parameters such as retention time, theoretical plates, and tailing factor, were studied (**Table-8**). There was no significant variation due to the variation of mobile phase composition or flow rate variation.

**4. CONCLUSION**

In this work, a simple, rapid, accurate and precise RP-HPLC method developed for determination of Darunavir in bulk and tablet dosage forms. The method was validated fully as per ICH guidelines and validation acceptance criteria were met in all cases. Hence the present RP-HPLC method was specific and stability indicated and can be suitable for routine quality control analysis for raw materials, formulations and dissolution studies

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**Conflict of Interest**

Authors do not have any conflict of interest in this research work.

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