Development of Ion pair Chromatography Method for Assay of Telaprevir by Response Surface Methodology

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ABSTRACT

Response surface methodology approach has been utilized for the assay of Telaprevir in pure and formulation using ion-pair chromatography. In this, A risk assessment approach (Control-Noise-Experiment) has been used for identifying the risk factors, i.e. Percentage of Organic Modifier (% Acetonitrile), Buffer's pH and flow rate of the method. The Central Composite design was applied to optimize the critical method parameters (CMPs) and to find out the Design space (DS) of the method. The coefficient of correlation (R^2), %CV and Lack of fit are utilized for the evaluation of method responses (Retention time and Asymmetric factor Evaluation of model is justified by two diagnostic plots (normal probability plot of residuals and plot of residuals vs predicted values). The mobile phase is Acetate Buffer (20mM) pH 4.4: Acetonitrile (35:65) with 0.9 ml/min of Flow rate. The separation has taken placed in the Eclip Plus C-18 column (250 × 4.6 mm, 5μm) at 268 nm. The retention time of Boceprevir was found to be 4.6 min. The validation of the optimized method has performed according to ICH guideline. The method has been successfully used for routine analysis of the Telaprevir throughout the life cycle of the product.

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INTRODUCTION

Worldwide about 130,000,000 people have been affected by the Hepatitis C virus (HCV), with a burden of 366,000 deaths per year. In the Western world, HCV infection is a vital reason for liver disease (Gentile *et al.*, 2009). Telaprevir is the first direct-acting antiviral drug approved in 2011 (Rao *et al.*, 2015). It acts as NS3/4A serine protease inhibitor (Clinical pharmacology and biopharmaceutics review, 2011).

The IUPAC name for Telaprevir is (1S,3aR,6aS)-2-[(2S)-2-({(2S)-2-cyclohexyl-2-[(pyrazin-2-ylcarbonyl)amino]acetyl}amino)-3,3-dimethylbutanoyl]-N-[(3S)-1(cyclopropylamino)-1,2-dioxohexan-3-yl]-3,3a,4,5,6,6a-hexahydro-1H-cyclopenta[c]pyrrole-1-carboxamide.The molecular formula of Telaprevir is $C_{36}H_{53}N_7O_6$ and its molecular weight is 679.85. It is white to an off-white powder with 0.0047 mg/mL water solubility (Clinical pharmacology and biopharmaceutics review, 2011).

The methods published so far for Telaprevir quantification in plasma and dosage formulations are

based on liquid chromatography coupled to mass spectroscopy and UV detectors. For example, Reddy et al. has reported the HPLC method who separated Telaprevir on the Acquity UPLC-BEH C-18 column using a diode array detector (Reddy *et al.*, 2015). Aouri et al. have developed an LCMS method where the separation was achieved on Hypercarb® 148 3 μm, 2.1 mm x 149 100 mm column maintained at 80°C using thermostated (Aouri *et al.*, 2013). Panda et al. have published QbD method developed by HPLC on the Enable-C18G column (Panda *et al.*, 2016). Chenet al. reported the LC-MS/MS method on a Waters XBridgeTM BEH Shield C18 column (Chen *et al.*, 2014), Heinz et al. has separated Telaprevir on LUNA C18(2)-HST column and detected using a UV-VIS photodiode array detector. The column was protected using the 18 pre-column and maintained at 40°C using a column thermostat (Heinz *et al.*, 2015). Panda et al. performed method development on Phenomenex Luna® C-18 column (Panda *et al.*, 2017), and Penchala et al., used Accucore C18 column for chromatographic separation (Penchala *et al.*, 2013). Tempestilli et al. developed the HPLC-UV method to quantify using X TerraVR RP18 column for chromatographic separation (Kumar *et al.*, 2015). Corrien et al. published an assay method for the determination of Telaprevir in dried blood using liquid chromatography and tandem mass detector using Phenomenex NX C18 column (Verweij-Van Wissen CPWGM *et al.*, 2015). In underdeveloped and developing countries like India, Bangladesh, and Nepal use of a dedicated column for a single compound is not cost-effective. This problem has received limited attention in the literature. So there is a demand for developing an effective and reliable technique using the C18 column (L1 packing) for cost-effective regular analysis.

Due to the poor peak-shape of Telaprevir chromatogram and inadaptability of the ion suppression technique because of the use of a mobile buffer phase of pH>8, Higher pH is not compatible with most of the standard reversed-phase columns (Jost and Hauck, 1983). In these situations, ion pair chromatography is the best option available. Here in this present work, we used 1-Octanesulphonic acid salt monohydrate as an Ion pair reagent in the mobile phase for HPLC based assay of Telaprevir.

This work aims to develop a simple isocratic Ion pair chromatography method using a common C18 column with L1 packing by applying the response surface methodology.

The present study is planned to be carried out in three phases, (i) Development of Ion pair chromatography method using C18(L1 packing) (ii) Optimization of the chromatographic condition by Central Composite Design (CCD) to find out Method Operable Design Region (MODR) and finally to detect Design Space (DS) and (iii) Validation of the method according to ICH guideline.

Experimental

Chemical and reagents

Telaprevir was procured from MNS Laboratories Pvt Ltd. Sodium Acetate Trihydrate (Analytical Grade) was purchased from Ranbaxy laboratories ltd, Mumbai, and HPLC grade Methanol was purchased from Thermo Fischer Scientific. Ultra-pure water (HPLC grade) was obtained from a Milli-Q Plus 185 water purification unit.

Instruments

All experiments for the method development and validation were performed on Shimadzu Ultra Flow Liquid Chromatography (UFLC) system equipped with LC-20AD pump with a PDA detector. The signal was processed and integrated using LC Real-time Analysis software.

Initial Chromatographic Condition

The initial separation was taken placed on Eclip plus C-18 column (250 $\times$ 4.6 mm, 5 μm) with 20 μl injection during 10 minutes run time 1.0ml/min flow rate. The mobile phase considered was 20mM Sodium acetate Buffer (pH 4.5): Methanol (33:67% v/v). The mobile phase was added with 5mM 1-Octanesulphonic acid salt monohydrate (IP reagent). The signals are monitored at 268nm in PDA.

Statistics

The obtained results were subjected to Central Composite Design (CCD) using Design-Expert® 11 Software Trial Version.

Preparation of standard and sample solution

Preparation of primary Stock solution (1000μg/ml)

10mg of Telaprevir was dissolved in 10ml of acetonitrile to obtain the strength of 1000μg/ml

Preparation of working standard (20 μg/ml)

The stock solution was diluted accordingly to obtain the strength of 20μg/ml solution of Telaprevir.

Standard Curve construction

For calibration, standard Telaprevir solutions were made by diluting working standard with acetonitrile to get 5, 10, 15, 20, 25, 30, 35 & 40 μg/mL (Figure 1). QC samples of the low, medium and high concentrations (10, 20 & 30 μg/mL) were prepared in the same solvent.

Table 1: Control-Noise-Experimentation (CNX) approach

Critical Method Parameter	Critical Method Attributes		Initial Risk assessment Scores	C,N,X	Experimental Strategy
	Retention Time	Asymmetric Factor			
Isocratic Binary Parameter	2	2	40	C	Calibrated
Flow Rate	10	10	200	X	DOE
Stationary Phase	5	5	100	C	New Column
Particle size	2	2	40	C	Optimum
Dimension	2	2	40	C	Standard
Temperature	5	5	100		Ambient
Buffer pH	10	10	200	X	DOE
% Acetonitrile	10	10	200	X	DOE
Solvent Grade	5	5	100		HPLC grade
Injection Vol	2	2	40	C	20μL
Flow Cell temp	5	5	100	C	400 C
PDA	5	5	100		Standard

Note: Low Risk-1, Medium Risk-5 and High Risk- 10
Total = (10 X First CMA) + (10 X Second CMA). X-Experiment,N-Noise and C-Control

Table 2: Experimental Design

Std	Run	Factor 1 A:%ACN %	Factor 2 B:Buffer pH pH	Factor 3 C:Flow rate ml/min	Response 1 Retention Time(R1) Rt	Response 2 Asymmetric Factor(R2) As
17	1	67	4.5	1	4.6	1.15
19	2	67	4.5	1	4.6	1.15
6	3	72	4	1.5	2.8	1.18
9	4	58.591	4.5	1	5.7	1.1
13	5	67	4.5	0.159104	17.02	1.02
10	6	75.409	4.5	1	4.02	1.05
1	7	62	4	0.5	11	0.96
20	8	67	4.5	1	4.6	1.15
5	9	62	4	1.5	3.5	1.15
2	10	72	4	0.5	8.3	1.02
15	11	67	4.5	1	4.6	1.15
8	12	72	5	1.5	2.8	1.02
7	13	62	5	1.5	3.5	1.11
14	14	67	4.5	1.8409	3.2	0.956
4	15	72	5	0.5	9.1	0.83
3	16	62	5	0.5	4.6	0.8
11	17	67	3.6591	1	5.2	0.625
18	18	67	4.5	1	4.6	1.15
12	19	67	5.3409	1	8.3	0.83
16	20	67	4.5	1	4.6	1.15

Table 3: Final Chromatographic Condition

Parameters	Optimized Values
Mobile Phase	Sodium Acetate Buffer (pH 4.4 & IP reagent: 5mM) : Acetonitrile
Mobile Phase Ratio	35:65
Buffer pH	4.4
Diluent	Acetonitrile
Flow Rate	0.9mL/min
Lamda max	268 nm
Column i.d	Eclip Plus C-18
Injection vol	20μl
Column temperature	Ambient
Detection Type	PDA
Run time	10 min

Initial Risk Assessment

In the present studies, high-risk variables (Table 1) are identified and assessed with the help of Control-Noise-Experimentation (CNX) approach (Raman et al., 2015). Three critical method parameters viz. % of acetonitrile, flow rate, and pH of Buffer were identified and imperilled to response surface methodology (Design of experiment) to find a design space of the developed method.

Experimental Design

In experimental design, the number of experimental runs is constructed to achieve or identified true optimum points. Therefore, the effective variable on the HPLC method's efficiency was optimized by using a central composite design and a quadratic model was constructed between the attributes (Retention time & Asymmetric factor) and the independent (% organic modifier, mobile phase pH & Flow Rate) variables (Box and Wilson, 1951; Hashemi et al., 2010).

For an experimental design to get a bias-free response, a 20μg/ml of Telaprevir was used for all runs. The developed model is used for the main and interactive study of variables. The analysis of variance (ANOVA) was found to be significant ($p < 0.05$) while framing the polynomial equation. The parameters like lack of fit, coefficient of correlation (R^2) and %CV are used for model fitting (Sivakumar et al., 2007; Khodadoust and Ghaedi, 2013). The fitness of the model is investigated and justified by diagnostic plots, such as the residual plot & normal probability plot (Olivero et al., 1995; Stalikas et al., 2009). The interaction study was carried out using 2D and 3D plot to understand the interaction between critical method parameters and attributes (Choisnard et al., 2003).

The Design Space (DF) was found out by using Derringer's desirability function(DF) (Hadjmohammadi and Sharifi, 2012; Panda et al., 2015).

Validation

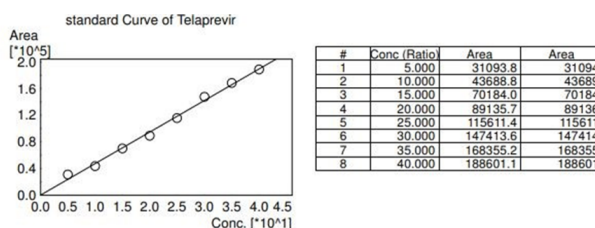Validation of the method has been performed as per ICH guideline Q2 (R1) (Procedures, 1996).
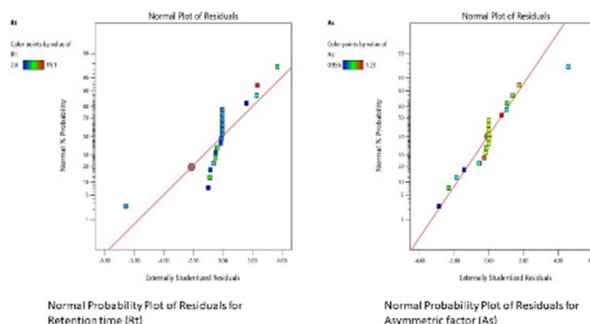

Figure 1: Standard Curve of Telaprevir

Figure 2: Normal probability plot for Retention Time (Rt) and Asymmetric factor (As)

RESULTS AND DISCUSSION

Optimization of Chromatographic Condition

The separation of Telaprevir is optimized by experimental factors such as buffer and organic modifier of the mobile phase, detection wavelength and flow rate of the elution.

Table 4: Validation Report

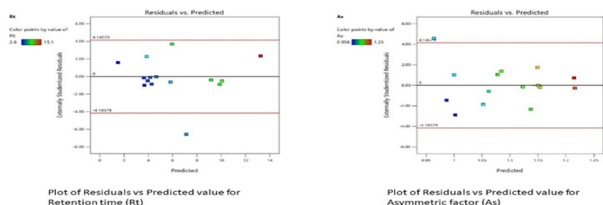| Assay Parameter	Result	Acceptance Criteria
Specificity	Retention Time of Telaprevir is observed to be 4.6 min with Peak Purity Index: 0.9999.	The excipient compounds must not interfere with the analysis of the targeted analyte.
Linearity	The standard linearity for Boceprevir was generated from 5μg/mL to 40 μg/mL R2 was found to be 0.9970 with y = 4638.92x - 655.79 And Standard error was found to be 2540.6741	The correlation coefficient for five concentration levels will be $\geq$0.997 for the range of 80 to 120% of the target Concentration.
Range	10μg/mL to 30 μg/mL range was used for the Accuracy and Precision study. %RSD was less than 2.	The acceptable range will be defined as the concentration interval over which linearity and accuracy are obtained per the above criteria, and in addition, that yield a precision of $\leq$3% RSD.
Accuracy	For drug substance: Average %assay at three concentration level (50%, 100% & 150%) for Telaprevir were found to be 86.5%, 95.75% & 98.3% respectively. And % RSD were found to be 2.20, 0.520 & 0.779 for three different level. For Accuracy of Drug Product (Boceprevir tablet) Average Recovery (%) and Standard Deviation (SD) Values at Each level (80%,100%,120%) for Boceprevir were found to be 97.88$\pm$0.452, 98.44$\pm$0.958 and 98.25$\pm$0.249, respectively.	The Relative Standard Deviation (RSD%) for the individual recovery result at each level, not more than 2.0%. The average recovery at different concentration levels: 95.0-105.0%.
Precision	The % RSD for the repeatability study was found to be 0.263 for Retention Time and 0.956 for Peak Area of Telaprevir, respectively. Therefore, the precision of the analytical method was found to be within acceptable limits.	The %RSD for repeatability, not more than 2.0
LOD	1.43 μg/mL	Complies the report
LOQ	4.41 μg/mL	Complies the report
%Assay	98.78%	98% to 102%

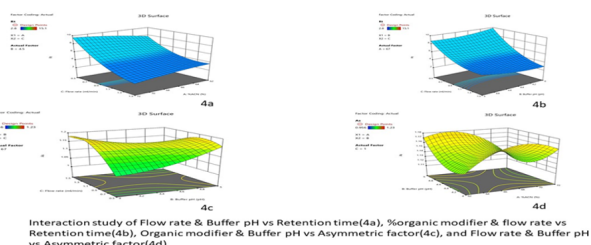**Figure 3: Plot of residuals vs predicted values for Retention Time (Rt) and Asymmetric factor (As)**

Interaction study of Flow rate & Buffer pH vs Retention time(4a), %organic modifier & flow rate vs Retention time(4b), Organic modifier & Buffer pH vs Asymmetric factor(4c), and Flow rate & Buffer pH vs Asymmetric factor(4d)

Figure 4: 3D- plot for interaction study of % of Acetonitrile and Flow Rate and pH

5a: Design Space for Retention Time (Rt)

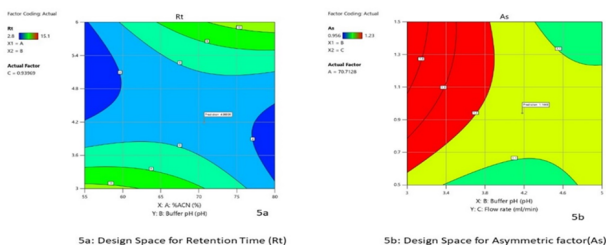5b: Design Space for Asymmetric factor(As)

Figure 5: Design space by using% of Organic Modifier, pH of Buffer and Flow Rate
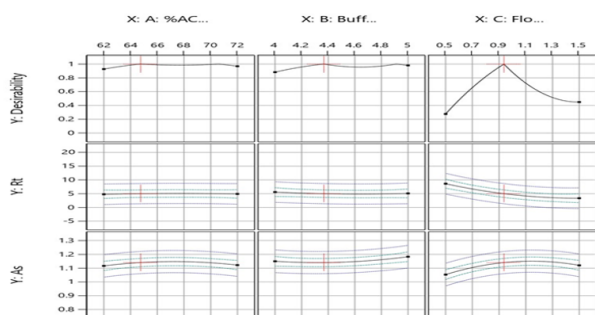

Figure 6: Derringer's desirability plot for optimizing % of Organic Modifier, pH of the Buffer and Flow Rate
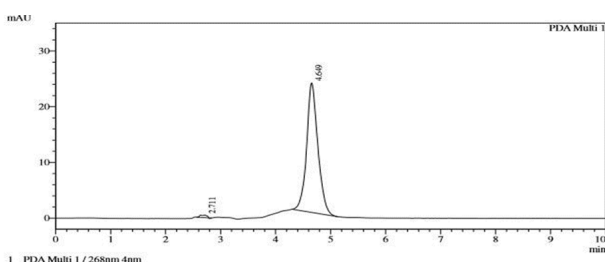

Figure 7: Final Chromatogram of Telaprevir

The optimized separation was achieved with Sodium acetate buffer and acetonitrile among different applied solvent systems. Ion ion-pair (IP) reagent 1-Octanesulphonic acid salt monohydrate is added to the mobile phase to achieve better separation.

Final optimized conditions were: Eclip Plus C-18 column with flow rate 1mL/min mL at 268 nm, 20mM Sodium Acetate Buffer (pH 4.5 & 1-Octanesulphonic acid salt monohydrate: 5mM), and acetonitrile in the ratio of 33:67

Effect of Concentration of Ion-pair reagent

In this study, 0.5mM to 10mM 1-Octanesulphonic acid salt monohydrate concentration in the mobile phase was studied for optimisation. The best concentration for separation and retention was found to be 5 mM.

Design of Experiment

The Design of the experiment is constructed to explore a better understanding of dependent and independent factors to achieve the best separation using central composite design (CCD). [15,16] In CCD, all experiments are performed in randomized order to minimize the effects of uncontrolled variables, as shown in Table 2.

Using this design interaction study was evaluated to optimized quadratic effects. The experimental results of the CCD have been fitted with coded expressions for Retention time.

(R1) and Asymmetric factor (R2) as expressed in

$Rt=4.65+0.0787A+0.0376B-2.86C+0.4625AB-0.8375AC+0.5875BC-0.2302A^2+0.4381B^2+1.34C^2$

$As=1.15-0.0047A+0.0005B+0.0112C-0.0200AB-0.0175AC-0.0550BC-0.0283A^2+0.0230B^2-0.0591C^2$

A = % CAN, B = Buffer pH, C = Flow rate, Rt = Retention Time, As = Asymmetric factor

The effects, as well as interactions of the dependent and independent variables, are evaluated following ANOVA using Design expert 11. The P-value (P<0.05) for R1 & R2 is observed to be 0.002 & 0.0006, indicating the significance of the factorial effect at a 95% confidence level. The Model F-values of 7.20 for R1 & 10.24 for R2, respectively, implies the model is significant (Hashemi *et al.*, 2010; Sivakumar *et al.*, 2007).The fit of the polynomial model equation is expressed by the coefficient of determination R^2, as shown in the result with 0.8663 and 0.9021 for the corresponding values of Retention time and Asymmetric factor, whereas 0.8087 and 0.8140 represented the same for the adjusted R^2 values. When adjusted R^2 values are $\geq$ 0.80, the relation between the fitted model and

experimental data is found to be good (Hashemi *et al.*, 2010; Sivakumar *et al.*, 2007). "The adequate precision value is a measure of the signal (response) to noise (deviation) ratio". A ratio greater than four is desirable (Hashemi *et al.*, 2010; Sivakumar *et al.*, 2007). In this study, the ratio is found to be 10.8811 for R1 & 10.6190 for R2, indicating the model is significant for the separation process. The value of 3.03 in the case of the parameter of %CV for all models is in agreement with previous literature, hence indicate reasonable reproducibility (Hashemi *et al.*, 2010; Sivakumar *et al.*, 2007).

The Model Responses R1 and R2 are evaluated using diagnostic plots, (i) a normal probability plot of residuals and (ii) plot of residuals vs predicted values. The close observation of a normal probability plot of residuals in Figure 2 tells that the residuals are fall on a straight line. Hence it is concluded that the distribution of errors is normal and the model fits the data adequately (Khodadoust and Ghaedi, 2013; Olivero *et al.*, 1995). It is observed in the plot of residuals vs predicted values in Figure 3 that there is no obvious pattern in the residual versus predicted response. The plot also exposes an almost equal distribution of residues above and below the x-axis predicting the suitability of the model. Due to regularity and continual adjustment of the residuals, the fitted model for the R1 and R2 may be accepted (Khodadoust and Ghaedi, 2013; Olivero *et al.*, 1995).

Interference study

3D-response surface plots are used to analyse for identifying the interaction(s) among the dependent and independent variables (Stalikas *et al.*, 2009; Choisnard *et al.*, 2003) and shown in Figure 4.

The 3D-response surface plot showed a linear increasing Retention time value with a decrease in buffer pH. At the higher pH of the buffer and lowest flow rate, optimum Retention time is shown. It is also observed a linear decreasing retention Time with increasing the Organic Modifier as well as flow rate. The asymmetric factor showed a linear increasing value with an increase in the pH as well as a % organic modifier. The 3D-response surface plot also showed a linear increasing asymmetric factor value with an increase of the flow rate and buffer pH.

Design Space and Desirability Function

Design Space (DS) is created using the modelling software Design Expert Trial Version. Two-dimensional charts are created by taking three factors (% of Acetonitrile, pH & Flow rate) and represented in Figure 5. The shaded blue and yellow region of the 2D contour plots depict the design space for retention time as well as the asymmetric factor, which defines the robust region of the method where results are within designated criteria (?Hadjmohammadi and Sharifi, 2012).

Optimizing the Retention time of symmetrical peak and minimization of asymmetric factors are the main objectives of Derringer's desirability function (D). It is a technique to optimize different parameters with multiple responses (Hadjmohammadi and Sharifi, 2012; Panda *et al.*, 2015). The value of D with zero indicates a desirable range of all responses and D close to 1 indicates optimum responses with a near target value. The maximum desirability function (D = 1) is pull out from the response surface curve (Figure 6), signifying the model is excellent (Hadjmohammadi and Sharifi, 2012; Panda *et al.*, 2015). The coordinates produce the maximum desirability value at Acetonitrile 65% v/v, pH 4.4, and a flow rate of 0.9 mL min_1.

Hence, these critical method parameters have been optimized and strictly control during the development of the method. The final robust UFLC-method´s condition for Telaprevir estimation is shown in Table 3, and Chromatogram in Figure 7.

Validation of the method

The method has been validated by applying the working point conditions according to ICH guideline Q2(R1) with respect to selectivity, linearity, range, accuracy, precision, the limit of detection, and quantitation (Procedures, 1996). The summary of the validation report is shown in Table 4 **.**

CONCLUSIONS

In this work, the QbD approach has been successfully implemented to develop a robust method for the estimation of Telaprevir in API and formulation. To initiate the method development by QbD approach, the physiochemical properties of Telaprevir was considered in the selection of input variables for the Design of Experiment using Central Composite Design. Because of the narrow concentration range of Telaprevir, the concentration was not considered as a quantitative variable in this design. So, mobile phase pH, % organic modifier mobile phase & flow rate were considered as qualitative variable and were controlled. Each step of the Analytical QbD process has been studied to find out the Design Space. Response surface plots graphically illustrated the major effects of mobile phase pH, % organic modifier mobile phase and flow rate on the separation. Using the QbD approach, the robustness of the method is already available before going for

validation. The method was also validated for accuracy and precision, and the result was satisfactory. The method has been found to be cost-effective, precise, accurate, and linear at concentrations ranging from 5 μg/ml to 40 μg/mL for Telaprevir using Eclip plus C-18 column in 10 minutes runtime.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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