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NEW ANALYTICAL METHODS FOR THE DETERMINATION OF NEW ANTI-VIRAL DRUG FAVIPIRAVIR: A POTENTIAL THERAPEUTIC DRUG AGAINST COVID-19 VIRUS, IN BULK AND DOSAGE FORMS

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Simple, accurate and robust analytical methods have been developed and validated for the determination of favipiravir (FVPR) by RP-HPLC and UV spectroscopy techniques as per the ICH guidelines. In the RP-HPLC method for FVPR determination, the mobile phase was ammonium acetate buffer pH 6.5 in pump A and methanol in pump B. The C18 (Sunfire) 5 μm, 4.6 × 250 mm column was used as a stationary phase, and the detection wavelength was at 323 nm. Under these conditions, FVPR was eluted as a sharp peak at 2.65 min and the overall time taken for each injection was 10 min. In case of the UV spectroscopy method, standard FVPR solutions were prepared with pure ethanol and scanned from 250 to 400 nm and a flourishing spectrum was obtained at 323 nm. Hence, the wavelength of 323 nm was fixed for the whole process of validation in both techniques. The limit of detection (LOD) and limit of quantification (LOQ) in the RP-HPLC method were 1.0 and 3.5 μg/mL, respectively, and the linearity was established in the 10 to 50 μg/mL range. In the UV spectroscopy method, the LOD and LOQ values were found to be 3.5 and 12 μg/mL, respectively, and the linearity was established within 20 to 60 μg/mL range. The regression coefficient was found to exceed 0.999 in both methods. The proposed RP-HPLC and UV spectroscopy techniques are simple, accurate, rugged and robust.

Keywords: Favipiravir; anti-COVID-19; method development; dosage form; RP-HPLC; UV spectroscopy.

1. INTRODUCTION

Favipiravir (FVPR) is an antiviral drug used for the treatment of all three types of influenza- A, B, and C [1]. The IUPAC name of FVPR is 6-fluoro-3-hydroxy-2-pyrazine carboxamide with molecular formula $C_5H_4FN_3O_2$ (Fig. 1). It is a pyrazinecarboxamide derivative, has a melting point from about 187 to 193°C and is soluble in organic solvents such as ethanol, DMSO and DMF, but only sparingly soluble in water. Favipiravir is a prodrug that undergoes intracellular ribosylation and phosphorylation into an active form of favipiravir ribofuranosyl-5′-triphosphate (favipiravir RTP), which can bind and inhibit viral RNA-dependent RNA polymerase (RdRp), and inhibits transcription and replication of the viral genome [2 – 4]. In addition to being active against influenza, it also exhibits antiviral activity against alpha-, filo-, bunya-, arena-, flavi-, and noroviruses, and now against COVID-19 [5, 6].

According to the literature search, there are very few articles related to the development and validation of analytical methods for the FVPR assay using HPLC. In these methods, the chromatographic separation by HPLC was obtained with a runtime of 60 min [7, 8]. Bulduk et.al. [9] developed and validated a sensitive HPLC-UV method for FVPR determination using potassium dihydrogen orthophosphate and acetonitrile as the mobile phase. This method involved a more complex mobile phase as compared to the proposed RP-HPLC method. Nguyen, et al. [10] studied the pharmacokinetics of FVPR in Ebola-infected patients by the JIKI trial method. Mikhail, et al. [11] developed HPLC and spectrofluorimetric methods for the determination of FVPR. The HPLC method involves isocratic elution of the drug with a

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complicated mobile phase of 0.02 M Brij-35, 0.15 M SDS, and 0.02 M disodium hydrogen phosphate, pH 5.0 for which the drug was eluted at 3.8 min. Megahed et. al. [12] developed a spectrofluorimetric method for the determination of FVPR. A liquid chromatography–tandem mass spectrometry method was used to develop and validate a two-dimensional isotope dilution technique for the determination of multiple antiviral drugs [13]. FVPR in human plasma was determined by LC-MS/MS method [14, 15]. The quantitation of FVPR by LC-MS/MS in human serum was developed and validated in [16]. A thorough literature survey revealed that no methods were developed and validated for the determination of FVPR by UV spectroscopic technique (Table 1). The proposed RP-HPLC and UV spectroscopic methods are simple, accurate, rugged, reproducible and cost-effective. Hence, these methods can be applied in the industrial as well as academic laboratories.

2. MATERIALS AND METHODS

2.1. Instruments

Shimadzu Prominence HPLC system was used for the development and validation of method for the determination of FVPR. The instrument contains a UV detector with deuterium lamp as a source of light, a quaternary pump, and an auto-injector. Lab solution software was used to examine the chromatograms. The pH of the buffer was adjusted with Eutech pH meter. All solvents were degassed by using the Ultrasonic sonicator bath and Axiva India, membrane filters of 0.45 µm nylon filters were used for filtration. Agilent Carry 60 UV spectrophotometer equipped with a deuterium lamp as the source of light was used for development and validation of the UV spectroscopic method. Winlab software was used for monitoring the spectrum and data processing.

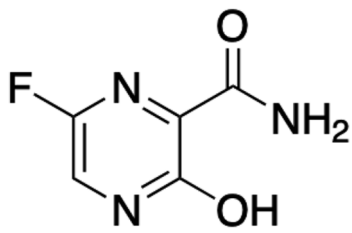

Fig. 1. Chemical structure of FVPR.

2.2. Chemicals and Reagents

The FVPR drug of more than 98% purity was provided by Karnataka Antibiotics and Pharmaceutical Ltd. (Bengaluru, India) as a gift sample. The analytical grade methanol and ammonium acetate were purchased from Merck Ltd. India. Millipore water purifier system was used for the preparation of ultra-pure water. Pure ethanol was used as diluent for development of the UV spectroscopic method.

2.3. Preparation of Mobile Phase, Standard Stock Solution and Dilutions

The mobile phase was composed of two solutions: (A) 0.1 M ammonium acetate buffer pH 6.5 and (B) methanol.

TABLE 1. Comparison of Statistical Data for Reported and Proposed Methods of FVPR Determination

Reference	Analytical method	Drug analyzed	Remarks
[7]	HPLC	FVPR	Analyzed related substances of FVPR Total run time: 60 min
[8]	HPLC	FVPR	Analyzed related substances of FVPR Total run time: 60 min
[9]	HPLC-UV	FVPR	Different mobile phase used, mixture of 50 mM potassium dihydrogen phosphate (pH 2.3) and acetonitrile (90:10, v/v)
[10]	Pharmacokinetics	FVPR	Other than HPLC-UV method developed
[11]	HPLC and spectrofluorimetry	FVPR	FVPR determined in human plasma samples. Mobile phase used as 0.02 M Brij-35, 0.15 M Sodium dodecyl sulfate, and 0.02 M disodium hydrogen phosphate, pH 5.0
[12]	Spectrofluorimetry	FVPR	Other than HPLC-UV method developed
[13]	LC-MS/MS	FVPR and other drugs	FVPR determined in human serum
[14]	LC-MS/MS	FVPR	FVPR determined in human in human plasma
[15]	UPLC–MS/MS	FVPR	Quantification of FVPR in human plasma
[16]	LC-MS/MS	FVPR	Quantification of FVPR in human serum
Proposed method	RP-HPLC and UV spectroscopy	FVPR	The proposed method used cost effective mobile phase in case of RP-HPLC method which are Pump A. Ammonium acetate and Pump B. methanol

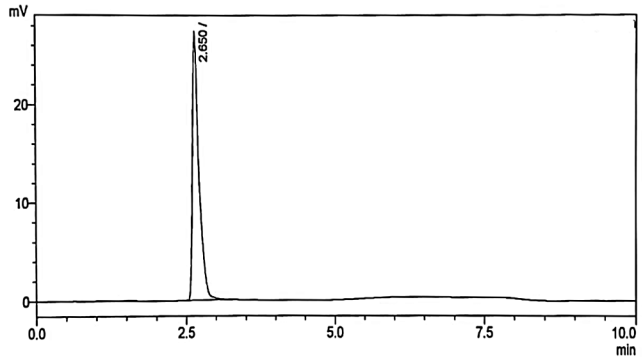

Fig. 2A. Typical chromatogram showing the elution of FVPR.

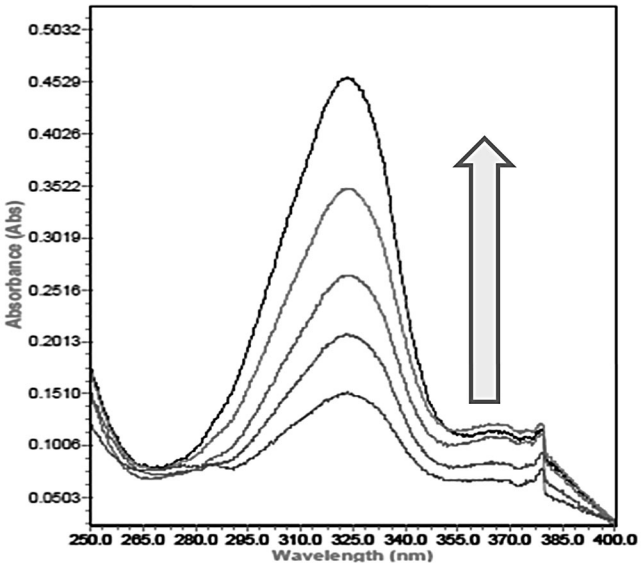

Fig. 2B. Overlaid UV spectra of FVPR at concentrations from 20 to 60 μg/mL.

The diluent was prepared by mixing the two solutions (A) and (B) in the ratio 85:15 v/v. The standard stock solution of FVPR was prepared in the solvent mixture at concentration 1000 μg/mL and then was used to prepare working solutions of the required concentrations. Similarly, different working standard solutions of FVPR were prepared to measure the various parameters of HPLC method. A similar procedure was followed for the UV spectroscopic method development and validation. Ethanol was used as the diluent in the case of UV spectroscopic method. Various working standard solutions were prepared by dilution according to the parameters of interest like linearity, accuracy, recovery, LOD, and LOQ.

2.4. Chromatographic Conditions

Chromatographic conditions play an important role in method development. The mobile phase contains ammonium acetate buffer in Pump A and methanol in Pump B. The stationary phase was Waters Sunfire C18 column, 5 μm, 4.6 × 250 mm. The mobile phase flow rate was set at 1.0 mL/min. The FVPR peak was eluted at the column oven temperature of 25°C and the wavelength of detection was fixed at 323 nm for the whole validation process. The sample injection volume was fixed at 10 μL. The FVPR retention time was found to be 2.65 min and the total run time was fixed 10.0 minutes for the whole method validation.

3. RESULTS AND DISCUSSION

3.1. Method Development

The mobile phase and stationary phase play an important role in method development. The mobile phase was optimized by performing different trials with various combinations of solution A and solution B. Sunfire C18, 5 μm, 4.6 × 250 mm column was used as a stationary phase. The sharp peak of FVPR was eluted in trials with different mobile phase ratios in a gradient time program. The mobile phase used in various trials was ammonium acetate pH 6.5 in pump A and acetonitrile in pump B and different time programs. However, the peak was not eluted properly after examining various ratios of solutions of pumps A and B. As a result, the mobile phase in pump B (i.e., acetonitrile) was replaced with methanol and the time program was fixed as shown in Table 2. With these chromatographic conditions, a peak with good baseline correction was observed and hence these conditions were used for further studies.

Typical chromatogram obtained by maintaining the above chromatographic conditions is shown in Fig. 2A. The FVPR peak was eluted at 2.65 min, the tailing factor and numbers of theoretical plates were also obtained within acceptable limits. Hence, for the entire RP-HPLC method development and validation, the mobile phase containing ammonium acetate buffer pH 6.5 in pump A and methanol in pump B was used. The flow rate of the mobile phase was set at 1.0 mL/min. In these experimental trials, the resultant FVPR sharp peak was eluted satisfactorily and these experimental conditions were in accordance with ICH guidelines. In the proposed method, the total run time was kept for 10.0 minutes for the whole RP-HPLC method development

TABLE 2. Gradient Programme for FVPR Elution by RP-HPLC

Time (min)	Pump A (ammonium acetate)	Pump B (methanol)
0.01	50	50
1.0	50	50
1.5	75	15
3.5	75	15
4.0	50	50
10.0	50	50

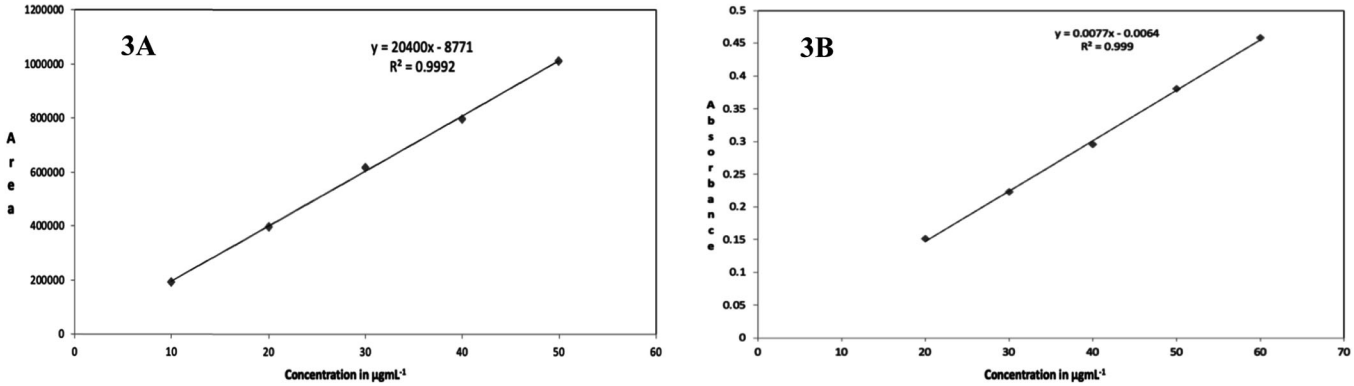

Fig. 3. Linearity graphs for FVPR determination by (3A) RP-HPLC and (3B) UV spectroscopy methods.

and validation, and 323 nm wavelength was fixed for detection of the FVPR peak. The proposed method was validated as per ICH guidelines [17]. The system suitability parameters were satisfactory and the results are given in Table 3.

The FVPR determination was also carried out by UV spectroscopy, in which the drug was scanned in the UV wavelength interval from 250 to 400 nm. FVPR exhibited the maximum absorbance at λ_{max} = 323 nm. Hence, this λ_{max} value was used for the whole method development and validation. For the determination of FVPR, various drug concentrations (depending on the parameters) were prepared by diluting with ethanol. All parameters of the method development and validation were complying with the ICH guidelines [17].

3.2. System Suitability

The FVPR peak showed a consistent retention time in the proposed RP-HPLC method, which was observed at 2.65 min and did not change much throughout the analysis. The corresponding percentage standard deviation (% RSD) of six individual spikes (analytes) was found to be less than 2.0 (i.e., 0.2% of FVPR) at LOQ concentration. The system suitability results are presented in Table 3 and the typical chromatogram is shown in Fig. 2A. The LOD and LOQ values were found to be 1.0 and 3.5 µg/mL, respectively and the *S/N* ratio at LOD and LOQ was 7 and 20, respectively. The outcomes indicated that the established method had good sensitivity and accuracy, and ensured reproducible FVPR determination as illustrated by data given in Table 4. For the UV spectroscopy method, the RSD percentage was also found to be less than 2.0. The LOD and LOQ values for FVPR were found to be 3.5 and 12 µg/mL, respectively. The outcomes have been found acceptable as presented in Table 4.

3.3. Linearity

In the HPLC method, various concentrations ranging from 10 to 50 µg/mL of FVPR standard solutions were injected and the observed regression coefficient was found satisfactory (R^2 = 0.999) for the equation $Y = bX + C$. The graph plotted as the areas under curve against FVPR concentration shows a straight line as shown in Fig. 3A. Hence, the developed method is considered validated. The results are summarized in Table 4.

For determining the graph linearity parameters in UV spectroscopic method, five different working standard solutions of FVPR with various concentrations ranging from 20 to 60 µg/mL were prepared. These solutions were scanned using the UV spectrophotometer and it was found that the absorbance of standard FVPR solutions increases linearly

TABLE 3. System Suitability Parameters

Parameter	FVPR	Limit
Number of theoretical plates	3027	NLT* 2000
Retention time (t_R), min	2.75	-
Peak asymmetry (A_S)	1.20	NMT* 2.0
% RSD	0.35	NMT* 2.0

* NLT = not less than; * NMT = not more than.

TABLE 4. Linearity Graph Parameters, Limit of Detection and Limit of Quantification

Parameter	RP-HPLC	UV spectroscopy
Linear dynamic range (µg/mL)	10 – 50	20 – 60
Regression equation (Y)[a]	–	–
Slope (*b*)	20400	0.007
Intercept (*c*)	-8771	-0.006
Correlation coefficient (*r*)	0.999	0.999
LOD (µg/mL)	1.0	3.5
LOQ (µg/mL)	3.5	12

[a] $Y = bX + c$, where X is the drug concentration (µg/mL).

TABLE 5. Recovery and Precision Testing

Concentration	Amount of drug taken	RP-HPLC		UV spectroscopy		
		FVPR	%RSD	FVPR	%RSD	Limits
60%	60 mg/mL	101.0	0.41	99.5	0.50	98 – 102%
80%	80 mg/mL	99.0	0.39	98.5	0.42	98 – 102%
120%	120 mg/mL	100.5	0.41	99.8	0.71	98 – 102%
Intra-day			0.42		0.82	NMT 2.0
Inter-day			0.35		0.77	NMT 2.0

with the concentration. The FVPR exhibited maximum absorbance at 323 nm and the resulting linearity overlay spectra are presented in Fig. 2B. The regression coefficient (R^2) was found to be more than 0.999. The linearity chart plotted as the absorbance against FVPR concentration is shown in Fig. 3B. The obtained results are summarized in Table 4.

3.4. Recovery

For determining this parameter, data were accomplished for the standard solutions of lower, middle, and upper concentrations and blank, spiked at 60, 80, and 120% against 100%. The obtained results indicated that the proposed method is accurate. The outcomes were calculated with the standard formula. The recovery data were established and found acceptable. The results are shown in Table 5. The limits of accepted recovery must occur in the range of 98 – 102%, and the obtained data fell within this range. Hence, the recovery parameter was complying with ICH guidelines and the method can be adopted in the industry as well as in academic laboratories. The recovery parameter was also estimated using the UV spectroscopy method. For this purpose, standard, solutions of lower, middle, and upper concentrations and blank spiked at 60%, 80, and 120% against 100% were scanned in the indicated wavelength range. The obtained results were found acceptable. Hence, the spectroscopic method can also be presumed for the determination of FVPR. The results are summaized in Table 5.

3.5. Precision

The results of precision testing for the proposed RP-HPLC method were good and in compliance with the ICH guidelines. The obtained results were precise and fell within the accepted range. Six individually spiked standard solutions were tested repeatedly on the same day and on different days for determining the precision parameters. The results of intra-day and inter-day performance examination showed that there were not much variations in the outcomes. The % RSD was found to be less than 2.0. Thus, the established RP-HPLC method is precise. The outcomes are tabulated in Table 5. The intra-day and inter-day precisions were also studied for the UV spectroscopy method. Stable results were obtained at three different concentrations and time intervals on the same day and on three different days, respectively, at various working concentrations. The% RSD was found to be less than 2.0 for both intra-day and inter-day results. Hence, the established spectroscopic method is precise. The results are shown in Table 5.

3.6. Robustness Studies

The robustness studies were performed using slight deviations from optimum conditions of the developed method. The standard solution of known concentration was injected at variable conditions, i.e., changes in the column oven temperature at 20, 25 and 30°C and flow rate variation on the mobile phase ranging from 0.9 to 1.1 mL/min. The outcomes are tabulated in Table 6. The obtained results were well within the acceptable range and there was no much deviation in the outcomes. Hence, the established method can be used under different conditions. Therefore, the proposed RP-HPLC method is robust. For robustness studies in the case of UV spectroscopy method, we slightly modified the actual wavelength of detection in the instrument, i.e., ±2 nm to 323 nm, and the results were found to fall within this range.

3.7. Ruggedness Studies

In studying the RP-HPLC method development in respect of its ruggedness parameters, the same chromatographic system was used on different days for analysis of the same FVPR working standard solutions with the same column. The results were monitored and minute variations in the FVPR peak area were observed, improving consistency, and there were not many variations in the reenhanced reli-

TABLE 6. Robustness Evaluation Parameters

Parameter	Variations	FVPR
		Retention time (min)
Flow rate	0.9 mL/min	2.94
	1.0 mL/min	2.65
	1.1 mlL/min	2.41
Temperature	25°C	2.76
	30°C	2.73

TABLE 7. Favipiravir Assay Results

Name of drug	Method	Label claim of market sample in mg per tablet	Obtained result in mg per tablet	% Assay	Limits
FVPR**	HPLC	200	199.7	99.85	98.0 – 102.0% *t = 2.1, *F =1.003
	UV spectroscopy	200	198.5	99.25	98.0 – 102.0% t =1.9, F = 0.998

* t = test value; t value at 95% confidence level is 2.78 for n = 5;
* F = test value; F value at 95% confidence level is 6.39 for n = 5;
** Trade name: Avigan 200 mg, manufacturer: Dr. Reddy μms Laboratories, Hyderabad, India.

ability and retention time. The percentage RSD was found to be less than 2.0 as shown in Table 3. The outcomes indicate that the established method is rugged. The same detector response was monitored on different days. The results were found to be effective and the established method can reproduce accurate and precise even on alternate days. In the UV spectroscopy method, the same detector response was monitored on alternate days by loading the known concentration of FVPR. The results were found to be well within the range. There were small changes in absorbance reading on different days with the same detector. The outcomes were found successfully well within the range. Hence, the developed method is competent enough to reproduce precise and accurate data on different days. The RSD percentage was found to be less than 2.0. Hence, both the established RP-HPLC and UV spectroscopy methods were rugged and could be adopted for drug assay in the industry.

3.8. Drug Assay Performance

Performance of the drug assay procedures was studied by following the established standard approach. The sharp peak of FVPR was obtained at consistent retention times for both standard (bulk drug) as well as test solution (formulation). When injected these solutions separately, the results were found well within the range of 98.0 to 102.0%. There was no interference of potential excipients. The excipients used for this purpose were lactose monohydrate, microcrystilline, cellulose, starch, magnesium stearate, micro powder, silica gel, pulvis talci and magnesium sulfate. It was found that these excipients did not interfere with the FVPR analysis by HPLC method. Hence, the established RP-HPLC method showed specificity for the assay of FVPR with the t-test and F test for the obtained data. The outcomes have been acceptable and are presented in Table 7. The assay was also performed using the UV spectroscopy technique according to the proposed method, in which there was no interference with tablet excipients. It was found that the selected excipients did not interfere with analysis of the FVPR dosage form, so that the developed method was considered specific enough (Table 7).

4. DISCUSSION

Most of the antiviral drug formulations have analytical methods for their determination such as HPLC and/or spectroscopic methods. FVPR is an antiviral drug which can be used to prevent COVID-19 and other influenza viral diseases. Therefore, the established RP-HPLC and UV spectroscopic methods are necessary for the determination of FVPR. The developed methods are rapid, simple, sensitive, and rugged. All parameters are complying with ICH guidelines. The established methods provide the estimation of FVPR by both RP-HPLC and UV spectroscopic techniques.

In concluding, a thorough literature survey revealed that there were no many analytical methods based on RP-HPLC and UV spectroscopic techniques for the determination of FVPR in bulk and dosage forms. The developed methods are typical of the reported RP-HPLC and UV spectroscopic methods. The other few analytical methods reported using different mobile phase and stationary phases for the determination of FVPR in bulk and formulation forms. A detailed literature survey discovered that struggles were concluded to establish gradient, rapid, simple, sensitive, rugged, reproducible, and robust analytical methods for the determination of FVPR. In the proposed HPLC method, the LOD and LOQ values were found to be 1.0 and 3.5 μg/mL, respectively, and the linearity range was established between 10 and 50 μg/mL. In the UV spectroscopy method, the drug showed maximum absorbance at 323 nm. The LOD and LOQ were found to be 3.5 and 12 μg/mL, respectively. The graph linearity was established in the range from 20 to 60 μg/mL. Both methods were simple, accurate, rugged, and robust. The proposed methods are new and unique, and can be applied in industrial practice and academic research. The results revealed that the RP-HPLC method is moderately more sensitive than the UV spectroscopy technique.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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