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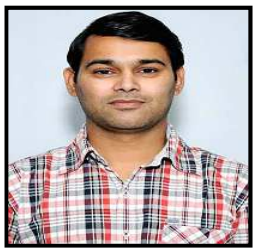

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF DOXORUBICIN AND HYDROXYCHAVICOL IN BULK AND PHARMACEUTICAL FORMULATION: IMPLICATIONS IN ROUTINE ANALYSIS

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ABSTRACT

A novel, simple, precise, and accurate reverse phase High Performance Liquid Chromatographic method for estimation of Doxorubicin and Hydroxychavicol was developed and validated according to ICH guidelines. HPLC method was developed using C18, (150mm × 4.6mm, 3.5µm) column with 1% Acetic acid in water: Methanol (45:55 v/v) as a mobile phase at a flow rate of 0.8ml/min and eluents were detected at 284 nm. The calibration curves were linear over the concentration range of 2.5 to 30ng/ml (R^2 = 1) for Doxorubicin and 5 to 60ng/ml (R^2 = 0.9998) for Hydroxychavicol. The average retention time of Doxorubicin and Hydroxychavicol was 1.17 min and 3.36 min respectively. Average percentage recoveries of Doxorubicin and Hydroxychavicol were 100.15± 0.34 and 100.55± 0.28 %, respectively. The LOQ values for Doxorubicin and Hydroxychavicol were 0.2897 and 0.4197ng/ml respectively. Intra- and inter-day precision values (% RSD) of proposed method were less than 2%.

KEYWORDS

Doxorubicin, Hydroxychavicol, Validation and RP- HPLC.

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INTRODUCTON

Cancer is the uncontrolled development of cells[1]. Various drugs may also be used for its treatment. Doxorubicin (DOX) (Figure No.1) is an anthracycline drug, initially extracted from *Streptomyces peucetius var. caesius* in the 1970's and have been used for cancer therapy since then[2-3]. It stops the growth of cancer cells by blocking an enzyme topoisomerase 2[4]. DOX is also used in combination with different anticancer drugs

to obtain best therapeutic effects and to reduce the toxicities[5-6]. When administered orally, DOX exhibits poor oral bioavailability which is around 5 %[7]. Major reason of the poor oral bioavailability of DOX is the extensive efflux via intestinal P-glycoprotein[8]. Poor oral bioavailability of DOX limits its use as an oral chemotherapeutic agent. Since long, attempts are being made to improvise the oral bioavailability of DOX[9-16]. Recently, a plant based oral bioavailability enhancer has been developed for the DOX by the Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Briefly, Hydroxychavicol (4-Allylpyrocatechol) (OH-CHV); (Figure No.2) phenolic compounds isolated from betel leaf (*piper betel*)[17-18] when administered with DOX, is found to enhance oral bioavailability of DOX consistently by 98.97%. Based on the significant findings of DOX bio enhancement by OH-CHV, an Indian patent has been filed and published for the prospective commercial use[19]. Simultaneously a pharmaceutical composition comprising DOX and OH-CHV was developed using QbD approach. Considering therapeutic and commercial importance of both the drugs, it was envisaged that development of simple RP-HPLC method for simultaneous estimation of DOX and OH-CHV will be worth, it would be useful in routine analysis of DOX and OH-CHV composition in near future.

MATERIAL AND METHODS

Chemicals and Reagent

DOX and OH-CHV was obtained from TCI chemicals (India) Pvt. Ltd. All the chemicals and reagent used were of at least analytical grade. HPLC grade methanol and water were used for the proposed study.

Instruments

Chromatographic analysis was performed using an Agilent HPLC system that consisted of a G1311C quaternary HPLC pump (Agilent Technologies, Palo Alto, CA), G1329B auto sampler system (Agilent Technologies) and G1315F variable wavelength detector (Agilent Technologies). HPLC grade water was obtained from "Extra pure" water purification system (Lablink). Mobile phase was degassed by using Ultrasonicator (PCiAnalyticals). For weighing purpose, Vibra HT (Essae) analytical balance was used.

Optimization of RP-HPLC Method

Chromatographic conditions were optimized by injecting standard solution (15ng/ml DOX and 30ng/ml OH-CHV) into HPLC system and allowed to run in different mobile phases so as obtain optimum conditions for separation of both the drugs.

Preparation of Mobile Phase

1 % Acetic acid in water was prepared by dissolving 10 ml of Acetic acid in 1000ml of HPLC grade water. It was filtered through 0.22µm filter and degassed by ultrasonication for 10 min. HPLC grade methanol was used in combination with 1% acetic acid as a mobile phase.

Preparation of standard stock solution

Stock solutions (1mg/ml) of DOX (Stock I) and OH-CHV (Stock II) were separately prepared in HPLC grade methanol and filtered through 0.45-m nylon membrane syringe filter. The solution of DOX was protected from light using aluminium foil.

Preparation of standard calibration curve

Stock I and II were diluted suitably with methanol and mixed together to achieve 7 calibration standards (CAL STD) containing DOX and OH-CHV in combination: CAL STD-1: DOX 2.5ng/ml + OH-CHV 5ng/ml; CAL STD-2: DOX 5ng/ml + OH-CHV 10ng/ml; CAL STD-3: DOX 10ng/ml+ OH-CHV 20ng/ml; CAL STD-4: DOX 15ng/ml + OH-CHV 30ng/ml; CAL STD-5: DOX 20ng/ml + OH-CHV 40ng/ml; CAL STD-6: DOX 25ng/ml + OH-CHV 50ng/ml; CAL STD-7: DOX 30ng/ml + OH-CHV 600ng/ml. All the solutions were injected into HPLC column and the peak area of each solution was measured. The standard calibration curves of peak area vs concentration (ng) were plotted.

Method Validation

Developed method was validated as per ICH guidelines. Various analytical method validation parameters viz. system suitability, linearity, range, LOD, LOQ, accuracy, precision and stability were assessed[20-22].

System Suitability

Before performing the main analysis, the system suitability test was carried out using freshly prepared standard working solution consisting of 2.5ng/ml of DOX and 5ng/ml of OH-CHV. During the test, five replicates of above mentioned solution were analyzed for retention time, peak area and the theoretical plates. Qualification parameters for the system suitability tests were less than 2% relative standard deviation (RSD) for retention time and peak area and more than 1500 theoretical plates for both DOX as well as OH-CHV. The resolution (acceptance criteria > 3) was calculated using the following formula.

$R = 1.18 [(t2-t1)/ (W2+W1)]$

Where t1 and W1 are retention time and peak width at half height of DOX

t2 and W2 are retention time and peak width at half height of OH-CHV

Validation Parameter

Linearity

Linearity of the proposed method was calculated by using seven different CAL STDs. After analyzing CAL STDs, calibration curves representing concentration vs. peak area were plotted and linear regression analysis was performed.

Accuracy (% Recovery)

To ensure the accuracy of method, recovery studies were performed by standard addition method using 80%, 100% and 120% levels of drug concentrations. Percent recovery was calculated from the amount found and the actual amount added.

Precision

Precision of proposed method was evaluated at three different levels i.e. LQC (DOX 2.5ng/ml + OH-CHV 5ng/ml), MQC (DOX 15 ng/ml + OH-CHV 30 ng/ml) and HQC (DOX 30ng/ml + OH-CHV 60ng/ml). Intra-day and Inter-day precision was determined by analyzing the solutions at different time intervals on the same day and on three consecutive days.

LOD and LOQ

ASTM LOD and LOQ were calculated by analyzing the CAL STD-1. Chromatogram of CAL STD-1 was processed using HPLC software settings "Annotations" and values were reported.

Stability

The stability of DOX and OH-CHV solutions (Standard as well as formulation) was determined by keeping MQC and the formulation in closed volumetric flasks at room temperature for 48 hrs. The solutions were analyzed at 12 hr intervals. The % assay and the RSD values were reported.

Estimation of DOX and OH-CHV in Pharmaceutical formulation

In-house pharmaceutical formulation containing DOX and OH-CHV were prepared using excipients viz. propylene glycol, PEG-4000 and DMSO as per guidelines of IIG limits. Initially, 50mg DOX and 10mg OH-CHV was dissolved separately in 100µl DMSO and water respectively. Obtained solutions were mixed using vertex mixer and sonicated for 10 min. In the resultant solution, 500µL of propylene glycol and PEG-4000 were added and mixed thoroughly using vortex mixer. Water was added to above solution in a sufficient quantity so as to make a final volume of 10ml (pharmaceutical formulation).

One ml of pharmaceutical formulation was diluted suitably with methanol (final concentration: DOX-50µg/ml and OH-CHV-10µg/ml) and filtered through 0.45µm syringe filter. Predefined volume of solution was analyzed using pre-optimized HPLC conditions (n = 3). Contents of pharmaceutical formulation were calculated by comparing mean peak area of sample with that of the standard.

RESULTS AND DISCUSSION

Optimization of RP-HPLC Method

Resolution was considered to be the most important criteria for the method and was imperative to achieve good resolution among both the compounds. Based on pKa and solubility of both the compounds, various compositions of mobile phase were tried and best resolution was obtained with mobile phase consisting of 1% acetic acid in water and methanol in the ratio of 45:55v/v. Better resolution of the peaks with clear base line was found. Detection was carried out at 284 nm. Optimized chromatographic conditions are given in

Table No.1. Under these conditions retention time for DOX and OH-CHV were 1.17 min and 3.36 min, respectively (Figure No.3).

System suitability

During system suitability test, RSD of all parameter were calculated to evaluate the suitability of the developed method. From the results, it was found that %RSD for retention time and peak area was less than 2 and the number of theoretical plates were more than 2000 (Table No.2). Adequate resolution of 13.37 was obtained between DOX and OH-CHV using developed HPLC method.

Method validation

Linearity and Range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven point calibration curve of DOX (2.5-30ng/ml) and OH-CHV (5-60ng/ ml) were constructed. Different concentrations and peak area values are depicted in Table No.3. Calibration curve when subjected to least square regression analysis yielded an equation; y = 44168x - 6844.3 for DOX and y = 47793x - 29074 for OH-CHV with correlation coefficient 1 and 0.9998 respectively (Figure No.4 and 5). From the linearity study, it was revealed that, there is a linear relationship between response and amount of drug within the range 2.5-30ng/ml for DOX and 5-60ng/ml for OH-CHV.

Accuracy (% Recovery)

Accuracy is the closeness of test results to the true value obtained by proposed method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For DOX and OH-CHV, accuracy was determined using recovery studies. At 80, 100 and 120 % standard addition, mean recovery of DOX and OH-CHV was found to be 100.15 and 100.55 % respectively. The relative standard deviation (% RSD) was found to be less than 2 (Table No.4). From the results of accuracy studies it was concluded that the analytical technique showed good accuracy.

Precision

Precision was studied by analysis LQC, MQC and HQC STDs containing both the drugs at concentrations covering the entire calibration range**.** The results expressed in terms of % RSD for the intra- and inter-day precision study (Table No.5 and 6). Percent RSD values of intra-day precision study were found to be 0.5626 and 0.6429 for DOX and OH-CHV respectively, whereas inter-day precision was 0.6199 and 0.5942 respectively. It was concluded that the analytical technique showed good repeatability.

LOD and LOQ

Limit of detection LOD (signal-to- noise ratio of 3) and limit of quantification LOQ (signal-to- noise ratio of 10) were measured based on the signal-to noise ratio. The LOD and LOQ values for DOX were 0.0869 and 0.2897 ng/ml, respectively and these values for OH-CHV were 0.1259 and 0.4197 ng/ml.

Stability

The stability of DOX and OH-CHV in the prepared sample was determined by analyzing LQC (DOX 2.5ng/ml + OH-CHV 5ng/ml) at 1, 12, 24 and 48 h (Table No.7). During stability testing, no significant change was observed in the content of DOX and OH-CHV. Percent RSD was less than 2% indicating a good stability of the sample. Hence it was concluded that DOX and OH-CHV solutions are stable for 48 h.

Estimation of DOX and OH-CHV in Pharmaceutical formulation

Proposed validated analytical method was successfully applied to the determination of DOX and OH-CHV in pharmaceutical formulation. Figure 6 depicts typical HPLC chromatograms obtained by the analysis of pharmaceutical formulation. The results of the assay (n = 3) yielded 100.29 % for DOX and 99.89% OH-CHV. The observed concentration of DOX was found to be 50.14 ± 0.076ng/ml (mean ± SD) these values for the OH-CHV was 9.98 ± 0.016ng/ml (Table No.8). The results of the assay indicate that the method is selective for the analysis of DOX and OH-CHV without interference of the excipients.

Table No.1: The optimized chromatographic conditions

S.No	Separation variable	Optimized conditions
1	Chromatography	Agilent HPLC system
2	Column	C18, (150mm × 4.6mm, 3.5µm)
3	Mobile phase	1% Acetic acid in water: methanol(45:55 v/v)
4	Flow rate	0.8 ml/min
5	Temperature	40^0C
6	Detection wavelength	284nm
7	Retention time (DOX)	1.17 min
8	Retention time (OH-CHV)	3.36 min

Table No.2: System suitability parameters for DOX and OH-CHV

S.No	Parameter	Acceptance criteria	DOX		OH-CHV	
			Observed Value	%RSD	Observed Value	%RSD
1	Retention Time	%RSD ≤ 2%	1.17	0.202	3.35	0.070
2	Area	%RSD ≤ 2%	101183	2.47	219686	2.41
3	Theoretical plates	≥ 2000	2451	0.235	7539	0.295

Table No.3: Linearity of DOX and OH-CHV

S.No	DOX		OH-CHV	
	Conc. (ng/ml)	Peak Area	Conc. (ng/ml)	Peak Area
1	2.5	103470	5	222395
2	5	215267	10	451284
3	10	438206	20	925288
4	15	651491	30	1390279
5	20	872915	40	1864332
6	25	1097760	50	2365744
7	30	1320987	60	2852742
8	Slope	44168	Slope	47793
9	y-intercept	6844.3	y-intercept	29074
10	R^2	1	R^2	0.9998

Table No.4: Recovery studies of DOX and OH-CHV

S.No	Sample	Spiked level	Amount present (ng/ml)	Amount recovered (ng/ml)	% Recovery	Mean % Recovery	% RSD
1	DOX	80%	12	12.06	100.53	100.15	0.4321
		100%	15	14.97	99.86		
		120%	18	18.01	100.07		
2	OH-CHV	80%	24	24.21	100.88	100.55	0.5878
		100%	30	30.10	100.33		
		120%	36	36.16	100.45		

Table No.5: Intra-day precision data for DOX and OH-CHV

S.No	DOX				OH-CHV			
	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD
1	2.5	2.49	99.60	0.8758	5	5.04	100.8	1.062
2	15	14.99	99.93	0.1315	30	30.009	100	0.3627
3	30	30.40	101.33	0.6805	60	59.97	99.95	0.5040

Table No.6: Inter-day precision data for DOX and OH-CHV

S.No	DOX				OH-CHV			
	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD	Amount present (ng/ml)	Amount recovered (ng/ml)	% Assay	% RSD
1	2.5	2.49	99.60	0.9120	5	4.93	98.60	0.7501
2	15	15.02	100.13	0.4589	30	30.17	100.5	0.6090
3	30	30.36	101.20	0.4888	60	59.97	99.9	0.4237

Table No.7: Stability study of DOX and OH-CHV

S.No	Time (h)	DOX			OH-CHV		
		Amount recovered (ng/ml)	% Assay	% RSD	Amount recovered (ng/ml)	% Assay	% RSD
1	1	14.98	99.86	0.2596	30.3	0.5678	101.00
2	12	14.72	98.13	1.4589	29.76	0.2679	99.20
3	24	15.01	100.06	0.3597	29.84	0.5891	99.46
4	48	15.12	100.80	1.0597	30.28	1.5698	100.93

Table No.8: Analysis of pharmaceutical formulation

S.No	Amount present (ng/ml)		Amount recovered (ng/ml)	
	DOX	OH-CHV	DOX	OH-CHV
1	50	10	50.07	9.97
2	50	10	50.22	10.0
3	50	10	50.14	9.98
4	Average		50.14	9.98
5	± S.D.		0.076	0.016
6	% Assay		100.29	99.89

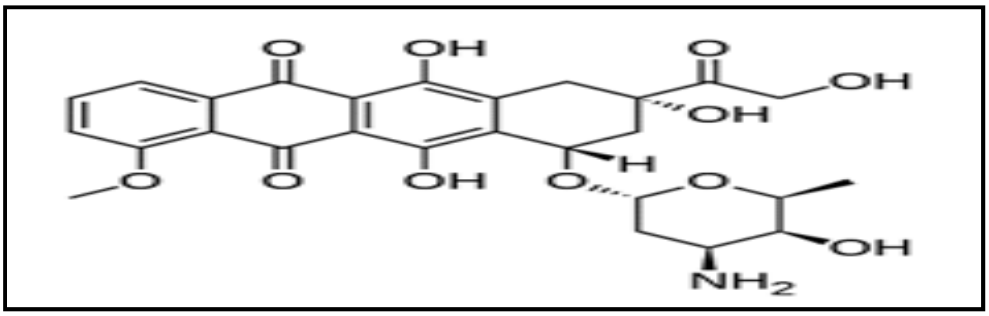

Figure No.1: Chemical structure of DOX

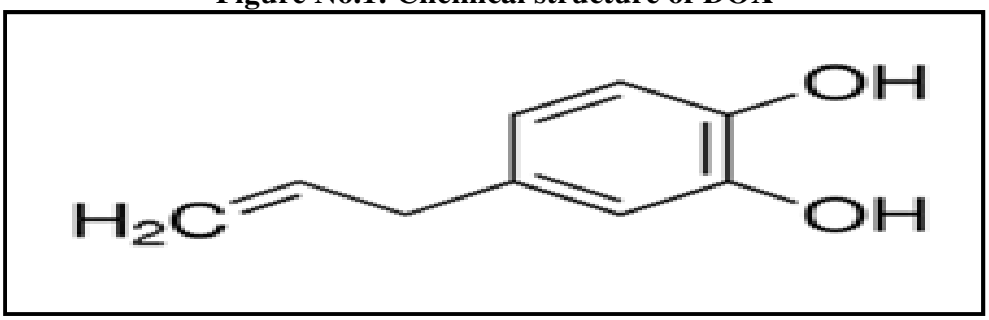

Figure No.2: Chemical structure of OH-CHV

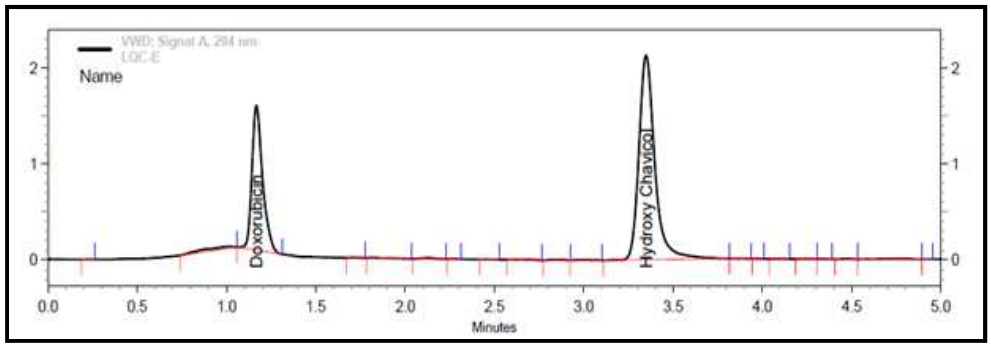

Figure No.3: A typical RPLC chromatogram of DOX and OH-CHV

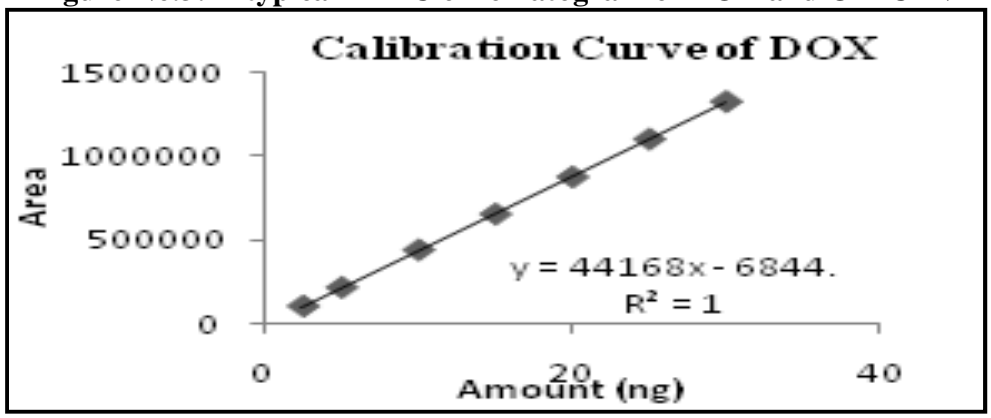

Figure No.4: Linearity curve of DOX

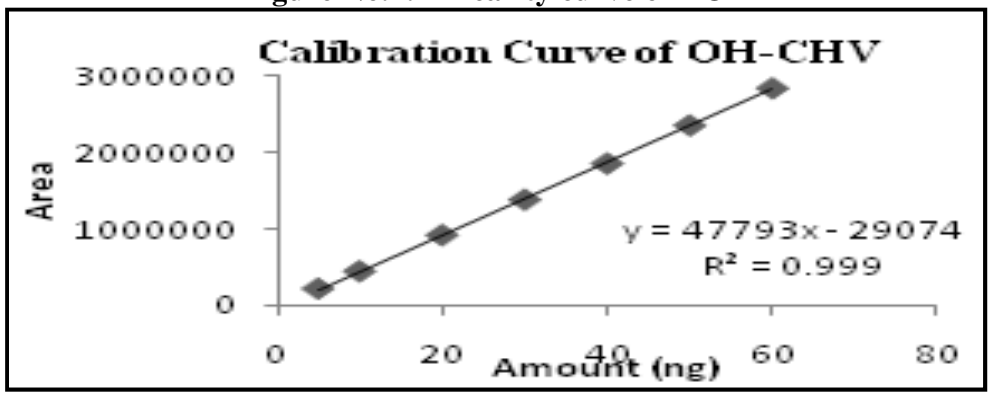

Figure No.5: Linearity curve of OH-CHV

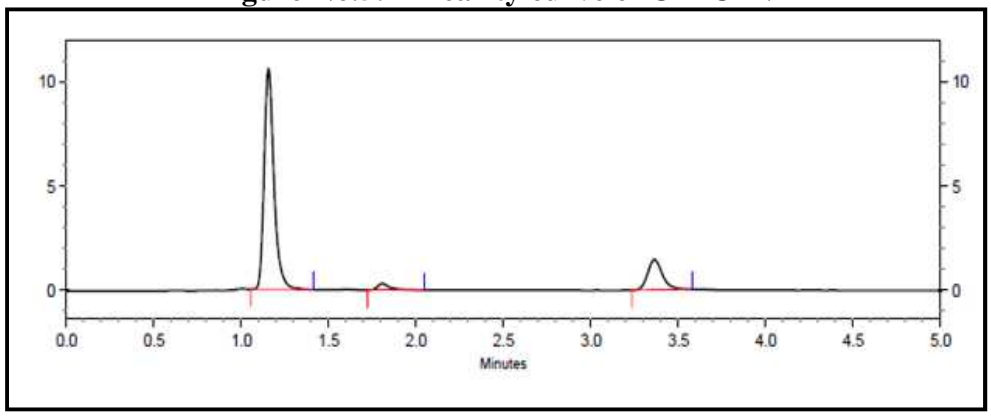

Figure No.6: A typical RPLC chromatogram of sample

CONCLUSION

An accurate, precise, simple, rapid yet sensitive RP-HPLC method was developed and validated for the simultaneous determination of DOX and OH-CHV. Further, it was found that developed method could be used for the routine analysis of pharmaceutical composition containing DOX and OH-CHV.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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