

A STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DAUNORUBICIN AND CYTARABINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: A simple, accurate, and precise method was developed for the simultaneous estimation of daunorubicin and cytarabine dosage form using reverse-phase HPLC and validated with different parameters such as accuracy, precision, repeatability, linearity, limit of detection (LOD), and limit of quantitation (LOQ) as per ICH Q2R1 guidelines.

Methods: The chromatogram was run through Agilent C18 Column of dimensions 150×4.6 mm, 5 m. Mobile phase containing 0.01N KH₂PO₄; Methanol taken in the ratio 50:50 was pumped through the column at a flow rate of 1.0 ml/min. The temperature was maintained at 30°C. The optimized wavelength selected was 240 nm.

Results: Retention times for daunorubicin and cytarabine were found to be 2.433 min and 3.045 min. The %RSD of the daunorubicin and cytarabine was found to be 0.7 and 0.4, respectively. The %Recovery was obtained as 99.96% and 100.40% for daunorubicin and cytarabine, respectively. LOD and LOQ values obtained from regression equations of daunorubicin and cytarabine were 0.08 µg/ml, 0.24 µg/ml and 0.94 µg/ml, 2.86 µg/ml, respectively. The regression equation for daunorubicin is $y=28587x+3141$ and $y=35995x+37534$ for cytarabine.

Conclusion: The method developed was simple and economical that can be adopted in regular quality control tests in industries.

Keywords: Daunorubicin and cytarabine, RP-HPLC, Validation, Stability studies.

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INTRODUCTION

Daunorubicin, also known as daunomycin, is a chemotherapy medication used to treat cancer. Specifically, it is used for acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and Kaposi's sarcoma [1]. Cytarabine, also known as cytosine arabinoside (ARA-C), is a chemotherapy medication used to treat acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and non-Hodgkin's lymphoma [2].

HPLC is an accurate and sensitive method used for the quantitative analysis of several drugs [3,4]. The literature shows a few methods for simultaneous estimation of daunorubicin and cytarabine [5-10]. The present study aimed to develop and validate an economical and effective HPLC method for the simultaneous determination with good linearity and sensitivity for both drugs which could be used in quality control analysis (Fig. 1).

EQUIPMENT AND CHEMICALS

The API daunorubicin and cytarabine were from MSN Pharma Limited, Hyderabad. The marketed formulation VYXEOUS (Cytarabine 100 mg and Daunorubicin 44 mg), MSN Pharma Limited, Hyderabad, India was used. Phosphate buffer, methanol, potassium dihydrogen orthophosphate, and orthophosphoric acid were from Rankem Chemicals, Haryana, India. Denver Electronic balance, BVK Enterprise pH Meter and Ultrasonicator, Thermo Scientific Hot air oven and Refrigerator, Millipore BM2EA9672R, T60 UV-Visible spectroscopy, Waters 2695 HPLC with Empower 2 software, Autoinjector, and PDA detector were used.

METHODS

Selection of detection wavelength

An ideal wavelength was used to get a good response for the analyte. Initially, daunorubicin and cytarabine were individually dissolved in diluent to scan the spectra on UV-visible spectrophotometer in the range of 200 nm–400 nm against diluent as blank. From the results, simultaneous estimation of three spectra's showed stable and maximum absorbance at 240 nm.

Preparation of solutions

Preparation of buffer: 0.01N Potassium dihydrogen orthophosphate (KH₂PO₄)

1.36 g of Potassium dihydrogen orthophosphate was added to 900 ml milli-Q water in a 1000 ml volumetric flask and sonicated. The final volume is made up with water. 1 ml of triethylamine was added and pH was adjusted to 3.8 with dil. orthophosphoric acid solution.

Preparation of diluent

Preparation of diluent: Mixture of 0.01N KH₂PO₄ (Mobile phase A) and methanol (Mobile phase B) in the ratio of 50:50 v/v.

Preparation of standard stock solutions

11 mg of daunorubicin and 25 mg of cytarabine were transferred to 25 ml volumetric flasks individually and 3/4th of diluents were added to the flasks and sonicated for 10 min. Volumes were made up with diluent and labeled as standard stock solution. (440 µg/ml of daunorubicin and 1000 µg/ml cytarabine).

Preparation of standard working solution

1 ml from each stock solution was pipetted and taken into a 10 ml volumetric flask and made up with diluent (44 µg/ml of daunorubicin and 100 µg/ml of cytarabine).

Preparation of sample stock solution

1 vial equivalent to 44 mg daunorubicin and 100mg cytarabine was transferred into a 100 ml volumetric flask. 50 ml of diluent was added and sonicated for 25 min. Further, the volume was made up with diluent and filtered with HPLC filters (440 µg/ml of daunorubicin and 1000 µg/ml of cytarabine).

Preparation of sample working solution

1 ml of filtered sample stock solution was transferred to 10 ml volumetric flask and made up with diluent (44 µg/ml of daunorubicin and 100 µg/ml of cytarabine).

Optimization of chromatographic conditions

HPLC method was developed and standardized for the analysis of daunorubicin and cytarabine. In the optimization process, various systems of stationary phase and mobile phase with several combinations were tested. Finally, acceptable retention times, good resolution, and theoretical plates were observed with Agilent C₁₈ (4.6×250 mm, 5 µm) column and 0.01N KH₂PO₄ and methanol (50:50) at pH 3.8 at a flow rate of 1.0 ml/min. Optimized chromatographic conditions are mentioned in Table 1. and the optimized chromatogram is shown in Fig. 2. Validation and stability studies of the optimized method were performed according to the ICH guidelines.

Method validation

Validation was performed as per the ICH Q2B (R2) guidelines [11-13]. The method was validated for the parameters such as system suitability, specificity, linearity, precision (system precision and repeatability), accuracy, limit of detection, limit of quantification, robustness, and assay. Stability studies such as acid degradation, base degradation, oxidative degradation, thermal degradation, photostability degradation, and aqueous degradation were carried out as per ICH guidelines [14,15].

Table 1: Optimized chromatographic conditions

Serial number	Parameter	Condition
1	Mobile phase	50% 0.01N KH ₂ PO ₄ : 50% methanol
2	Diluent	50% 0.01N KH ₂ PO ₄ : 50% methanol
3	Column	Agilent C ₁₈ (4.6 mm×250 mm, 5 µm)
4	Wavelength	240 nm
5	Column temperature	30°C
6	Injection volume	10 µl
7	Flow rate	1.0 ml/min
8	Run time	5 min
9	Retention time	2.433 min (Daunorubicin) 3.045 min (Cytarabine)

System suitability

It is performed to verify that the analytical system is working properly and can give accurate and precise results. Standard solution of daunorubicin (44 ppm) and Cytarabine (100 ppm) was injected 6 times and the parameters such as resolution, peak tailing, and USP plate count were determined. The chromatogram was presented in Fig. 3 and results of system suitability were shown in Table 2. According to the ICH guidelines, plate count should be more than 2000, tailing factor should be <2 and resolution must be more than 2. All the system suitability parameters were passed and were within the limits.

Specificity

The specificity of the method is performed by separately injecting the blank, placebo, and sample solutions. The interference observed (if any) at the retention times of each analyte in all the chromatograms is evaluated. Chromatograms are shown in Figs. 4-6. Retention times of daunorubicin and cytarabine were 2.433 min and 3.045 min, respectively. The method is specific as no interfering peaks were observed in blank and placebo at retention times of the drugs.

Linearity

Standard solutions of 25%, 50%, 75%, 100%, 125%, and 150% concentrations were prepared by taking 0.25, 0.5, 0.75, 1.0, 1.25,

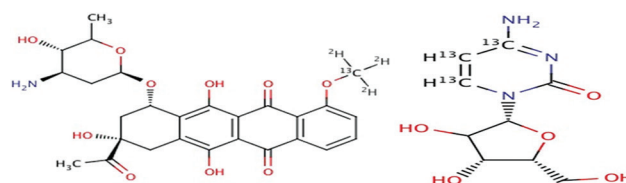
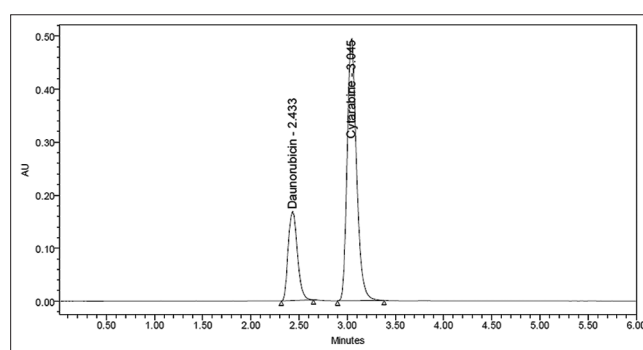


Fig. 1: (a) Structure of daunorubicin [(8S,10S)-8-acetyl-10-[[[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy]-6,8,11-trihydroxy-1-methoxy-5,7,8,9,10,12-hexahydrotetracene-5,12-dione] [11]; b) Structure of cytarabine [4-amino-1-[(2R,3S,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2-dihydropyrimidin-2-one] [12]

**Fig. 2: Optimized chromatogram****Table 2: System suitability parameters for daunorubicin and cytarabine**

Serial number	Injection						
	Daunorubicin			Cytarabine			
	Rt (min)	USP plate count	Tailing	Rt (min)	USP plate count	Tailing	Resolution
1	2.410	3220	1.20	3.013	5269	1.25	3.6
2	2.423	3426	1.23	3.030	5456	1.25	3.6
3	2.424	3400	1.22	3.031	5393	1.25	3.6
4	2.425	3338	1.22	3.031	5408	1.25	3.6
5	2.425	3364	1.20	3.031	5432	1.25	3.6
6	2.427	3311	1.19	3.032	5443	1.25	3.6

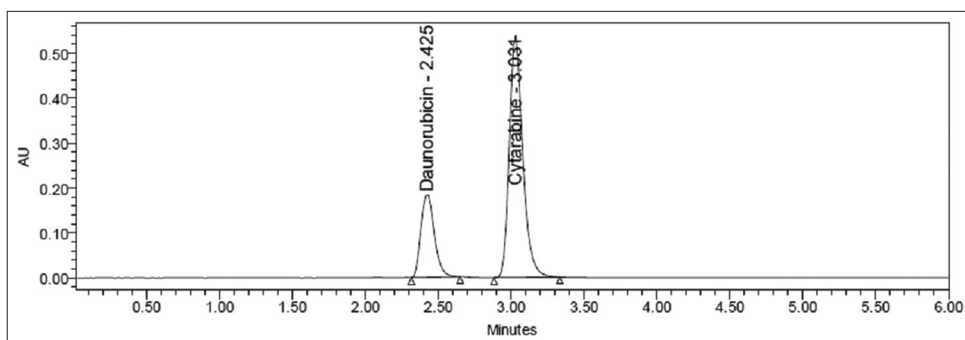


Fig. 3: Chromatogram for system suitability

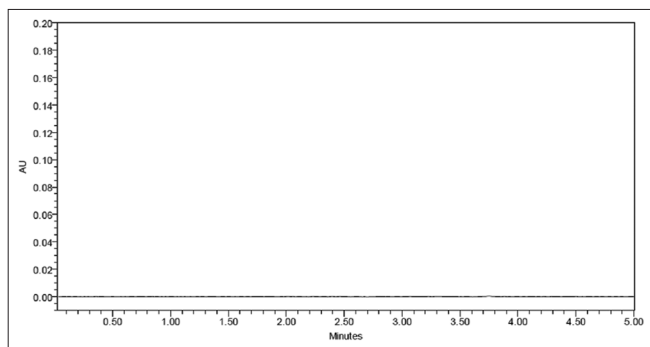


Fig. 4: Chromatogram of blank

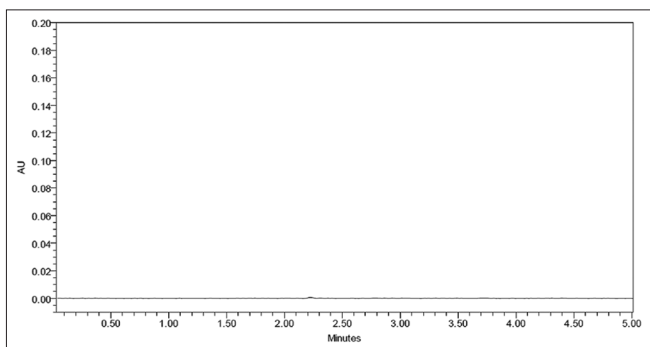


Fig. 5: Chromatogram of placebo

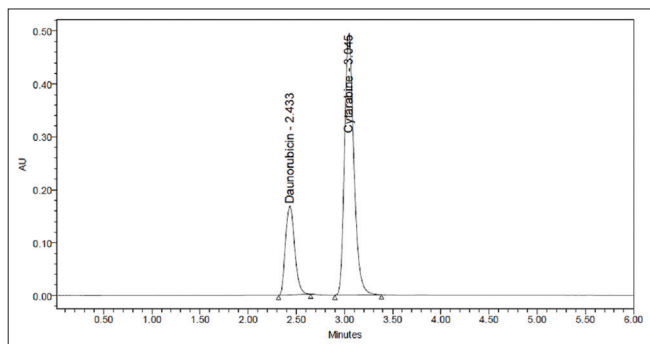


Fig. 6: Typical chromatogram

and 1.5 ml each from two standard stock solutions and made up to 10 ml. Six linear concentrations of daunorubicin (11–66 µg/ml) and cytarabine (25–150 µg/ml) were injected in a duplicate manner. Peak areas were recorded for each injected concentration and the calibration curves – concentration versus peak area were constructed Figs. 7 and 8. The results are given in Table 3. Linearity equations

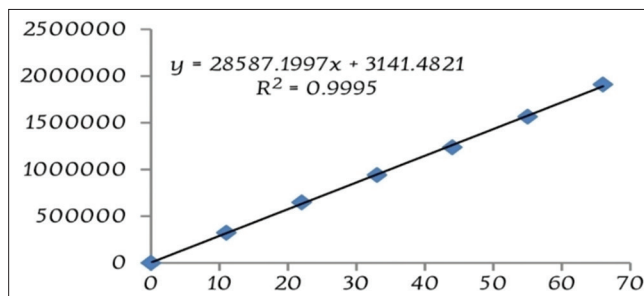


Fig. 7: Calibration curve of daunorubicin

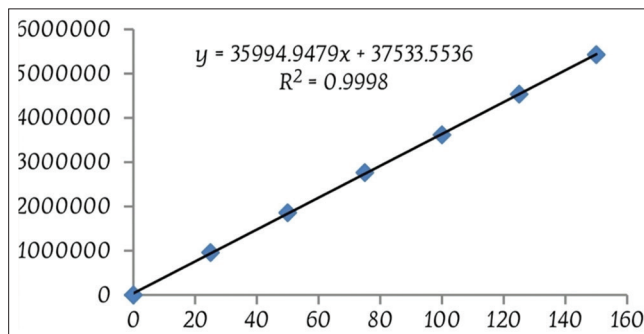


Fig. 8: Calibration curve of cytarabine

Table 3: Results for linearity of daunorubicin and cytarabine

Daunorubicin		Cytarabine	
Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
0	0	0	0
11	322962	25	958464
22	648201	50	1857576
33	941175	75	2763833
44	1237618	100	3616994
55	1565668	125	4535677
66	1910010	150	5427540

obtained for daunorubicin and cytarabine were $y=28587x+3141$ and $y=35995x+37534$. The correlation coefficient obtained was 0.999 for both drugs.

Precision

System precision

System precision was determined by injecting 15 µl standard working solution 6 times and the chromatograms were recorded. Average area, standard deviation, and %RSD were calculated for the two drugs and

Table 4: Results for system precision and repeatability of daunorubicin and cytarabine

Serial number	System precision		Repeatability	
	Area of daunorubicin	Area of cytarabine	Area of daunorubicin	Area of cytarabine
1	1276435	3551797	1268366	3609348
2	1269906	3539751	1281255	3581141
3	1264947	3524435	1271755	3599632
4	1276927	3561195	1266393	3531947
5	1271333	3529081	1270972	3578407
6	1252996	3530147	1271099	3548987
Mean	1268757	3539401	1271640	3574910
SD	8906.0	14456.0	5127.4	29550.1
RSD (%)	0.7	0.4	0.4	0.8

SD: Standard deviation

results are shown in Table 4. The %RSD obtained was 0.7% and 0.4%, respectively, for daunorubicin and cytarabine. As the limit of precision was <2, the method was precise.

Repeatability

Repeatability (method precision) was determined by multiple sampling from a sample stock solution and six working sample solutions of the same concentrations were prepared. 15 µl injection from each working sample solution was given and their peak areas are mentioned in Table 4. Average area, standard deviation, and % RSD were calculated for the two drugs and obtained as 0.4% and 0.8%, respectively, for daunorubicin and cytarabine. As the limit of precision was <2, the method was repeatable.

Accuracy

Three levels (50%, 100%, and 150%) of accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy. The results are shown in Table 5 and 6. The mean % recovery was obtained as 99.96% and 100.04% for daunorubicin and cytarabine, respectively.

Limit of detection and limit of quantification

LOD and LOQ were calculated based on signal to noise ratio. The results are shown in Table 7.

Robustness

The method was performed in duplicate under varied conditions of flow rate (± 0.1 ml/min), mobile phase compositions (± 5.0 ml of both organic components), and column temperature ($\pm 5^\circ\text{C}$) and results are given in Table 8. System suitability parameters were not affected and %RSD was within the limit. Hence, the method was considered to be robust.

Assay

Assay was performed with Vyxeous, bearing the label claim cytarabine 100mg and daunorubicin 44 mg. 20 µl of the standard and sample working solutions were injected into chromatographic system and areas for daunorubicin and cytarabine were measured and results are shown in Table 9. The average % assay for daunorubicin and cytarabine was found to be 99.83% and 100.60%, respectively.

Stability studies**Acid degradation studies**

1 ml 2N hydrochloric acid was added to 1 ml stock solution of daunorubicin and cytarabine, refluxed for 30 min at 60°C. Resultant solution was diluted to obtain 44 µg/ml and 100 µg/ml solution and 10 µl solution was injected into the system to assess the stability of the sample. The results are given in Table 10.

Table 5: Accuracy results for daunorubicin

Level (%)	Amount spiked (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	Mean recovery (%)
50	22	22.40	101.82	99.96
	22	22.16	100.73	
	22	21.80	99.09	
100	44	43.66	99.23	99.66
	44	43.85	99.66	
	44	43.81	99.57	
150	66	66.27	100.41	99.70
	66	65.80	99.70	
	66	65.63	99.44	

Table 6: Accuracy results for cytarabine

Level (%)	Amount spiked (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	Mean recovery (%)
50	50	49.76	99.52	100.04
	50	49.71	99.42	
	50	50.06	100.12	
100	100	99.80	99.80	99.80
	100	100.51	100.51	
	100	99.96	99.96	
150	150	150.02	100.01	100.01
	150	150.48	100.32	
	150	151.05	100.70	

Table 7: Results for limit of detection and limit of quantitation of daunorubicin and cytarabine

Molecule	LOD (µg/ml)	LOQ (µg/ml)
Daunorubicin	0.08	0.24
Cytarabine	0.94	2.86

LOD: Limit of detection, LOQ: Limit of quantitation

Table 8: Results for robustness of daunorubicin and cytarabine

Serial number	Condition	RSD of Daunorubicin (%)	RSD of Cytarabine (%)
1	Flow rate (-) 0.90ml/min	1.4	1.2
2	Flow rate (+) 1.1ml/min	0.4	0.3
3	Mobile phase (-) 55B: 45A	1.1	0.6
4	Mobile phase (+) 45B: 55A	0.9	0.6
5	Temperature (-) 25°C	0.9	0.3
6	Temperature (+) 35°C	0.2	1.1

Table 9: Results for assay of daunorubicin and cytarabine

Serial number	Daunorubicin			Cytarabine		
	Standard area	Sample area	Assay (%)	Standard area	Sample area	Assay (%)
1	1276435	1268366	99.4	3551797	3609348	101.6
2	1269906	1281255	100.9	3539751	3581141	101.2
3	1264947	1271755	100.5	3524435	3599632	102.1
4	1276927	1266393	99.2	3561195	3531947	99.2
5	1271333	1270972	100.0	3529081	3578407	101.4
6	1252996	1271099	101.4	3530147	3548987	100.5
Average	1268757	1271640	100.2	3539401	3574910	101
SD	8906.0	5127.4	0.90	14456.0	29550.1	1.0
RSD (%)	0.7	0.4	0.9	0.4	0.8	1.0

SD: Standard deviation

Table 10: Degradation data of daunorubicin and cytarabine

S.NO	Degradation condition	Daunorubicin			Cytarabine		
		Area	% Recover	% Drug degraded	Area	% Recover	% Drug degraded
1	Acid	1188333	93.29	6.71	3201132	90.08	9.92
2	Alkali	1206724	94.73	5.27	3373199	94.92	5.08
3	Oxidation	1230423	96.59	3.41	3249490	91.44	8.56
4	Thermal	1246892	97.88	2.12	3388688	95.36	4.64
5	UV	1253103	98.37	1.63	3443155	96.89	3.11
6	Water	1262922	98.37	1.63	3535415	99.49	0.51

Alkali degradation studies

1 ml 2N NaOH was added to 1 ml stock solution of daunorubicin and cytarabine, and refluxed for 30 min at 60°C. The resultant solution was diluted to obtain 44 µg/ml and 100 µg/ml solution and 10 µl solution was injected into the system to assess the stability of the sample. The results are given in Table 9.

Oxidative degradation studies

1 ml 20% H₂O₂ was added to 1 ml of stock solution of daunorubicin and cytarabine. The solutions were kept for 30 min at 60°C. The resultant solution was diluted to obtain 44 µg/ml and 100 µg/ml solution and 10 µl was injected into the system to assess the stability of sample. The results are given in Table 9.

Thermal degradation studies

The standard drug solution was placed in an oven at 105°C for 1 h to study dry heat degradation. The resultant solution was diluted to 44 µg/ml and 100 µg/ml solution and 10 µl was injected into the system to assess the stability of the sample. The results are given in Table 9.

Photostability studies

The photochemical stability of the drug was studied by exposing 440 µg/ml daunorubicin and 1000 µg/ml cytarabine solution to UV light by keeping the beaker in UV Chamber for 1 day. The resultant solution was diluted to obtain 44 µg/ml and 100 µg/ml solution and 10 µl was injected into the system to assess the stability of the sample. The results are given in Table 9.

Aqueous degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 1 h at 60°C [16-19]. The resultant solution was diluted to 44 µg/ml and 100 µg/ml and 10 µl was injected into the system to assess the stability of the sample. The results are given in Table 9.

CONCLUSION

A simple, accurate, and precise method was developed for the simultaneous estimation of the daunorubicin and cytarabine in tablet dosage form. Retention times for daunorubicin and cytarabine were found to be 2.433 min and 3.045 min. %RSD of the daunorubicin and cytarabine were found to be 0.7 and 0.4, respectively. %Recovery was obtained as 99.96% and 100.60% for daunorubicin and cytarabine, respectively. LOD

and LOQ values obtained from regression equations of daunorubicin and cytarabine were 0.08 µg/ml, 0.24 µg/ml and 0.94 µg/ml, 2.86 µg/ml, respectively. The regression equation for daunorubicin is $y=28587x+3141$ and $y=35995x+37534$ for cytarabine. Compared to other methods, the retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular quality control tests in industries.

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AUTHORS CONTRIBUTION

Sai Veneela performed the work and Ashritha helped in drafting and editing the journal. Rajitha is the mentor.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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None.

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