

VALIDATION OF A BIOANALYTICAL REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF NYSTATIN IN AN ANIMAL MODEL AFTER INTRANASAL *IN SITU* GEL ADMINISTRATION

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ABSTRACT

Objective: The aim of the study was to develop and validate a bioanalytical reverse-phase high-performance liquid chromatographic (HPLC) method for the estimation of nystatin in rat plasma after intranasal administration.

Methods: The reversed-phase HPLC system was equipped with a Luna C18 column, the mobile system comprised of methanol, water, and dimethylformamide (55:30:15) and the flow rate was set at 0.9 ml/min.

Results: The elution time for nystatin was 4.096 ± 0.025 min. The calibration curves constructed in rat plasma were linear from 0.25 to 50 $\mu\text{g/ml}$. The lower limit of quantification (LOQ) was found to be 0.25 $\mu\text{g/ml}$. The standards for accuracy and precision of the intra- and inter-day variation studies were in the acceptable ranges as per the FDA guidelines.

Conclusion: The LOQ value determined by the proposed method was noted to be satisfactory for inspecting the plasma pharmacokinetics of nystatin in rats' post-administration of a nasal *in situ* gelling liquid crystalline precursor formulation in an *in vivo* study.

Keywords: Nystatin, Rat plasma, Intranasal *in situ* gel, Reverse-phase high-performance liquid chromatographic, Validation.

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INTRODUCTION

The polyene class of antifungals such as nystatin, natamycin, and amphotericin B has a wide action against broad spectrum of fungi species such as *Candida*, *Cryptococcus*, *Histoplasma*, *Blastomyces*, and *Aspergillus* [1,2]. Initial efforts in accomplishing free drug administration have led to dose-limiting toxicity. It has been reported that nystatin can be loaded into liposomes and administered intravenously to reduce toxicity while retaining its antifungal activity [3-6]. At present, there are no drug therapies of nystatin that can be used to treat non-invasive nasal aspergillosis, except for nasal irrigation [7,8]. Moreover, using permeation enhancers, the intranasal delivery of nystatin can perhaps be used to achieve drug concentration in systemic circulation, thus can be applied in treatment of systemic fungal infections [9]. The mucociliary clearance plays an important role in depletion of the drug; therefore, mucoadhesive formulation that enhances residence time and control drug release can be deployed to overcome these inadequacies [10]. Biopharmaceutical performance of these newer formulations has to be assessed and necessitates the development of satisfactory analytical methodologies. In comparison to the previous methods for the detection of nystatin in plasma and tissues, our technique utilizes lower flow rate, shorter retention time, and an uncomplicated mobile phase composition which is trouble free to prepare and is neutral thus avoids instability of nystatin which is observed at low pH [11-14]. Moreover, the developed method was validated in accordance with the International Council on Harmonization. Finally, to estimate the intranasal *in vivo* performance of the *in situ* gelling liquid crystalline precursor system has led to the development of a suitable high-performance liquid chromatographic (HPLC) an analytical method that is accounted for here.

METHODS

Reagents and chemicals

Nystatin was obtained as a kind gift sample from Glenmark Limited (Mumbai, India). Plasma was separated from whole blood collected

from healthy male Wistar rats. HPLC grade solvents were procured from Merck (India) such as methanol (MeOH), acetonitrile (AcN), and dimethylformamide (DMF). Deionized was used in all experiments.

Chromatographic system

HPLC device (Waters HPLC, Alliance) with photo array detector (Waters 2996) set at a wavelength of 305 nm was used. The system was equipped with a Waters 1525 pump, an autosampler Waters 717 plus, and a column heater Waters 1500 series. The analytical column fixed in the device was a reversed-phase Luna C18 (Phenomenex) with an internal diameter of 150 mm \times 4.6 mm and with a 5 μm particle size. The column oven was kept at 25°C and confined by a Security Guard precolumn. The mobile phase comprised MeOH:H₂O:DMF (55:30:15, v/v/v). Elution was executed isocratically (25°C) at a well maintained flow rate of 0.9 ml/min and the run time was fixed for 10 min. The mobile phase was degassed by vacuum ahead of use. A 0.45 μm (Millipore Durapore) filter membrane was employed for the filtration of mobile the phase.

Preparation of standard solutions

Bulk nystatin was dissolved in MeOH by the aid of ultrasonication to obtain a standard stock solution of 500 $\mu\text{g/ml}$. Further dilution was made to the standard stock solution to prepare working standards of 50 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, and 0.25 $\mu\text{g/ml}$. Thereafter, separate stock solutions were made for standard curves and quality control (QC) samples. Six-point calibration curves with lower range 0.25–1.5 $\mu\text{g/ml}$ and higher range 1.5–50 $\mu\text{g/ml}$ were used for quantification of the drug in plasma. The calibration standards were prepared by adding stock solution 10–50 μl and adding plasma to make up the total volume to 1000 μL .

Sample preparation

Nystatin was extracted from plasma by adding HPLC grade MeOH-AcN (1:1) in the ratio of 1:2 v/v and incubating at 2–8°C (30 min) in polypropylene tubes. Thereafter, the sample was subjected to a two-step centrifugation cycle. The first cycle was applied for 10 min at 2000

g, the supernatant was separated and added to a new tube followed by the application of the second cycle for 4 min at 10,000 g. A sample of 400 µl of supernatant was filtered through a 0.22 µm Durapore filter and further centrifuged for 4 min at 4000 g. A 20 µl of aliquot was injected into the analytical column.

Validation parameters

Linearity

Calibration curves were constructed in six-point replicate sets and analyzed, repetitively, for 2 consecutive days. The curves were generated by linear regression analysis of the summing up of the peak areas of the two main peaks of nystatin versus concentrations. The determination coefficients (r^2), standard deviations (SD), and coefficient of variance (%CV) were calculated on each of 2 days. The linearity was also evaluated by plotting the interposed concentrations against their spiked concentrations.

Accuracy and precision

The proposed method's accuracy and precision were assessed by performing six replicate analyses of QC samples at intraday and interday. The concentrations were selected at three levels of low and high range the calibration curves, that is, lower QC (LQC), middle QC (MQC), and higher QC (HQC). The % accuracy of the system was calculated, whereas the precision was attributed to the %CV [15-19].

Lower limit of quantification (LOQ)

LOQ is described as the lowest standard concentration in an analytical study that can be computed with a deviation of the definite concentration and a coefficient of variation of precision <20%. Six replicate sets of samples at the lowest concentration were prepared and assayed to determine %CV and percentage accuracy [15,16].

Specificity

Blank plasma, standard drug solution, and sample formulation spiked with the known concentration of drug were injected in triplicate into HPLC system to determine method specificity. The chromatograms were observed for any interference at the retention time of the analyte peak. Percentage assay difference between mean of spiked sample results and mean of the method precision was calculated [15,16].

Recovery

To scrutinize the extraction competence of the proposed method, a set of six samples at three QC levels of the lower and higher range calibration curves, that is, LQC, MQC, and HQC were prepared by spiking nystatin in plasma and further processed (pre-extraction). Likewise, another set of plasma samples were firstly prepared and thereafter spiked, that is, post-extraction at the three QC levels of the lower and higher range calibration curve. The effectiveness of extraction for the analyte was calculated by the ratios of the peak areas of pre-extraction samples to post-extraction samples [15,16].

Stability

The stability of nystatin was tested for both processes and storage stability. For the determination of process stability, QC samples at the two concentration levels, that is, lower range (1.25 and 0.5 µg/ml) and higher range (20 and 5 µg/ml) were studied. The samples were run immediately after preparation and yet again the same samples were stored at 2–8°C, later analyzed after a time period of 12 h and 24 h. Similarly, storage stability was also estimated at the above-mentioned concentration, the samples were stored below –20°C, and the testing was performed at three timelines (baseline, 1 month, and 3 months). Drug stability was also determined after freeze and thaw cycles.

Application – pharmacokinetic studies

Male Wistar rats (250–300 g) were procured from Wockhardt Research Centre (Aurangabad, India). All the rats were inhabited in a light controlled room (22±2°C and RH 55±5%). The experimental protocol (CPCSEA/IAEC/P'ceutics-30/2016-17/125) concerning

the animal study was approved by the IAE committee of Y.B. Chavan College of Pharmacy, Aurangabad. The dose of NYS administered was 8 mg/kg as a single dose. Nasal administration was performed with a 20 µl micropipette to instil 20 µl of the *in situ* gelling fluid liquid crystalline precursor into each nostril at a time until a total of 60 µl had been instilled [20]. This spaced instillation was intended to minimize the overflow into the gastrointestinal tract. Blood samples of 0.5 ml each were withdrawn into 1.5 ml heparinized PE tubes from the retro-orbital plexus at 15, 30, 45, 60, 90, 120, 150, 180, 240, and 360 min immediately after dosing of the formulation. The plasma was separated from the whole blood sample by centrifugation at 13,000 rpm for 10 min, thereafter stored at below –20°C until the samples were analyzed.

RESULTS AND DISCUSSION

Reverse-phase (RP)-HPLC analysis

The chromatograms of blank rat plasma and plasma spiked with bulk nystatin 50 µg/ml are presented in Fig. 1.

It is well documented that nystatin is a lyophobic drug and hence its solubility at room temperature in an aqueous solvent is practically insoluble while is soluble in organic solvents such as MeOH, ethanol, AcN, and DMF [21]. It is reported elsewhere that the solubility of nystatin in polar solvents can be substantially increased in the presence of 10–20% water [21,22]. In the present study, a mobile phase system comprising MeOH, water, and DMF was prepared in volume ratio 55:30:15. It has been well established elsewhere the same mobile phase system was applied in the liquid chromatographic determination of bulk nystatin in various pharmaceutical preparations such as creams, ointments, oral suspensions, and topical powders [12]. The above-described system although studied for pharmaceutical formulations was not validated and applied for the bioanalytical analysis of nystatin in plasma and in varied body fluids such as saliva and tissues. The retention time for the major and minor nystatin peaks in the pharmaceutical formulation by the mentioned mobile phase in earlier studies was documented as 11.7 min and 17.5 min, respectively [12]. In another study, wherein the mobile phase system composed of 10 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid, 30% MeOH, and 30% AcN was applied in the bioanalytical analysis of nystatin in rabbit plasma; the retention time for the major and minor peaks were found to elute at 7.5–8.5 and 9.5–10.5 min, respectively [23].

The application of the mobile phase comprising MeOH, water, and DMF (55:30:15) leads to a reduction in tailing factor and was within the acceptable limit (United States Pharmacopoeia (USP) tailing factor <2) with high-quality resolution and improved peak symmetry with lower retention time. The flow rate was studied at 0.3, 0.6, 0.9, and 1.2 ml/min. Lower flow rates resulted in longer retention time and at higher flow rate, peak poor resolution was seen. It was noted that at a flow rate of 0.9 ml/min, the retention time for the major and minor peak was 4.096 min and 4.470 min, respectively. The result for system suitability parameters obtained is highlighted in Table 1.

The minor peak of nystatin is assigned to its isomer formed in biological fluids and was observed only at concentrations greater than 5 µg/mL. Further, the quantification was based on sum of the peak area concentration of both the peaks.

Validation parameters

Linearity and range

The data pertaining to the calibration curve are presented in Tables 2 and 3. The curves were plotted in two plots from a low range

Table 1: Data for system suitability parameters

Parameter	Observations
Retention time	4.096±0.025 min
Theoretical plates	4627±76.57
Peak symmetry	1.10±0.015

*n is number of replicates, n=6

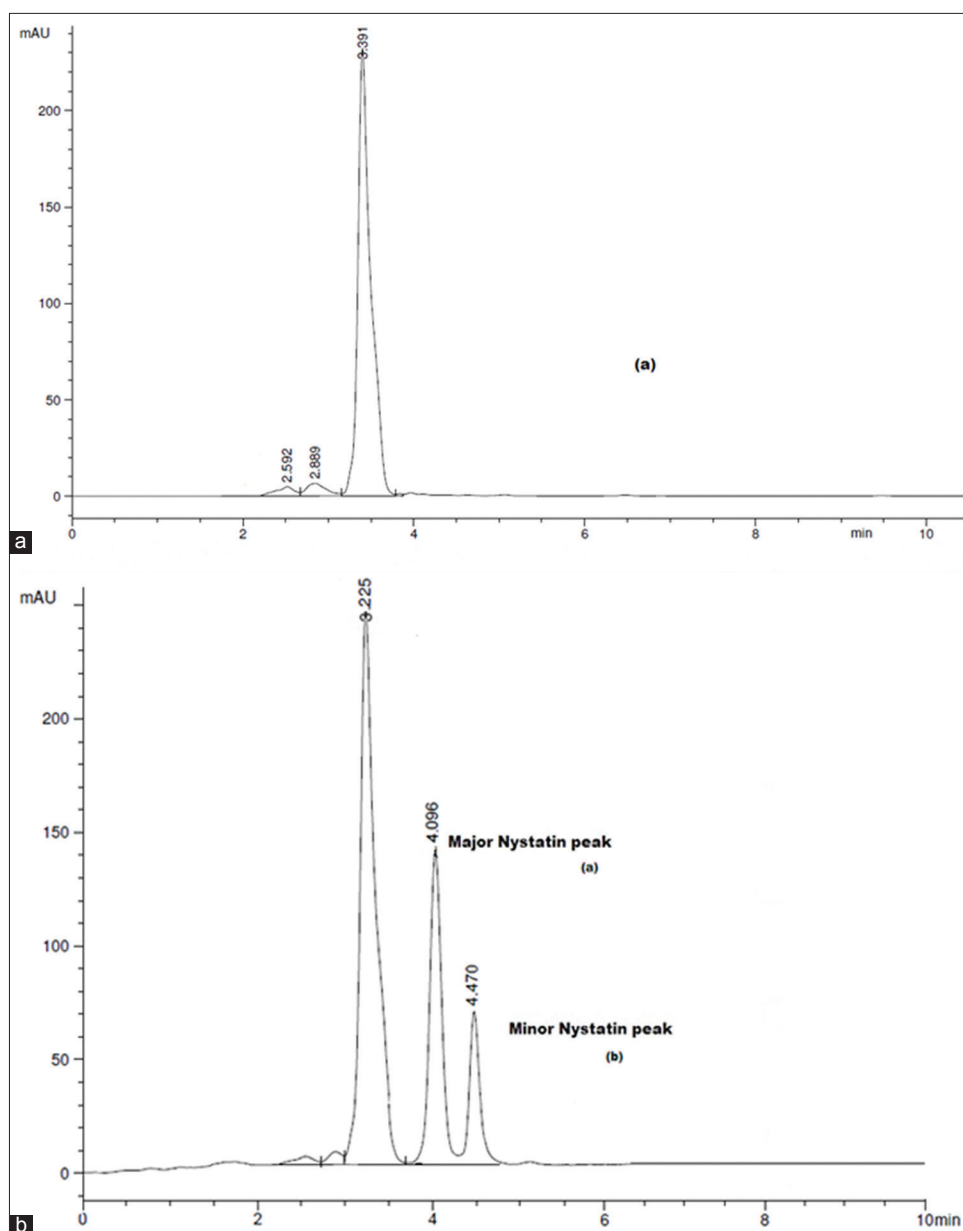


Fig. 1: High-performance liquid chromatographic chromatogram of (a) blank plasma at 3.39 min (b) extracted rat plasma spiked with bulk nystatin showing major peak at 4.096 min and minor peak at 4.470 min

Table 2: Calibration curve specifications for nystatin in rat plasma

Day	Curve	Slope	Intercept	r^2
1	1a	15101	693.4	0.996
2	2a	15378	340.2	0.999
1	1b	15678	462.1	0.999
2	2b	15478	773.9	0.998

*1a and 1b lower concentration range calibration curve at day 1 and day 2, respectively. 1a and 1b higher concentration range calibration curve at day 1 and day 2, respectively

and high range curves since the exact concentration range of drug in plasma was not clear before conducting pharmacokinetic studies. Moreover, a single calibration across a wider range could hinder the accuracy and precision of the extreme values in the range. The calibration curve exhibited linearity with $r^2 \geq 0.995$ over the range of estimation, that is, 0.25–50 $\mu\text{g/ml}$.

Accuracy and precision

A methods precision is expressed in observation to the %CV of replicates and for it to be in an acceptable range, all concentration values should be within $\pm 15\%$. The precision of the method was assessed for intraday as well for interday repeatabilities and the data are presented in Table 4. For both studies, %CV was found to be lower than 5% suggestive of satisfactory repeatability of the proposed method. The accuracy of the proposed method was determined by the nearness of the difference between the mean of the calculated values from the factual value. The values were considered to be acceptable if the actual value at all concentrations was within $\pm 15\%$. The accuracy standard in intra- and inter-day precision studies at lower and higher concentration range was within the acceptable confines.

Lower LOQ

LOQ was defined as the lowest amount of nystatin that could be detected in the plasma at a precision of $\leq 20\%$ and the accuracy, that

Table 3: Calibration curve data representing concentration of nystatin in rat plasma

Calibration curve	Spiked concentration ($\mu\text{g/ml}$)	Mean calculated concentration ($\mu\text{g/ml}$)	SD	CV (%)	Accuracy (%)
Higher range	50	50.47	0.692	1.371	105.75
	40	40.58	0.879	2.166	101.59
	32.5	32.685	0.776	2.374	98.026
	21.85	21.83	0.836	3.827	98.281
	11.5	11.48	0.462	4.028	94.130
	5.85	5.77	0.282	4.902	96.265
	1.48	1.45	0.105	7.228	100.888
Lower range	0.25	0.269	0.017	6.230	97.968
	0.48	0.487	0.059	12.156	98.638
	0.76	0.745	0.035	4.766	99.855
	0.97	0.953	0.039	4.112	99.922
	1.25	1.181	0.053	4.448	100.569
	1.56	1.503	0.048	3.241	101.466
	1.8	1.816	0.062	3.444	100.953

*SD: Standard deviation, n=12, CV: Coefficient of variance

Table 4: Intraday and interday precision data for nystatin in rat plasma

Calibration curve	Spiked level ($\mu\text{g/ml}$)	% Accuracy		% CV	
		Intraday	Interday	Intraday	Interday
Lower range	LQC (0.25)	92.18	91.64	4.56	2.94
	MQC (1)	94.203	97.147	2.721	1.702
	HQC (1.75)	89.266	92.462	3.682	3.834
Higher range	LQC (5)	94.729	95.211	2.435	2.382
	MQC (20)	95.088	96.173	2.664	2.422
	HQC (50)	96.016	95.920	4.182	4.041

*n is number of replicates, n=6, CV: Coefficient of variance. LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control

is, the difference between the mean of the calculated values from the factual value was within $\pm 15\%$. Lowest standard drug concentration was prepared in six replicates (0.25 $\mu\text{g/ml}$) and was analyzed to determine LOQ. At the estimated LOQ value, the %CV was 9.372 with an accuracy of 105.4%.

Specificity

To substantiate whether bulk nystatin could be employed as a reference standard to analyze nystatin loaded *in situ* gelling formulation, normal rat plasma was spiked with *in situ* gelling formulation at three different concentrations 5.2, 10.4, and 20.8 $\mu\text{g/ml}$. Plasma samples were extracted, assayed using a high range calibration curve constructed using bulk nystatin. The accuracies obtained at these concentrations were found to be 96.13%, 98.81%, and 97.34%, respectively. The precision as estimated by the coefficient of variation of the mean was noted to be 2.545%, 1.444%, and 3.350%, respectively. No interference was seen with rat plasma components. Thus, the method was found to be specific for estimation of nystatin from the prepared *in situ* gelling formulation as the %CV was <5%.

Recovery

The recovery data for nystatin in rat plasma is presented in Table 5. The higher values of recovery of nystatin assure that almost complete extraction of the drug was achieved and an insignificant amount of drug remained in the plasma after plasma protein extraction step. Lower values of %CV point out that the developed method demonstrated a higher scales of extraction efficiency.

Stability

In general, the analysis of the biological samples for drug content is not undertaken instantaneously after sample collection and preparation. As a result, it is imperative to determine if the drug is constant throughout the study time. Process, as well as storage stability, was determined by extracting four sets of plasma control of which two were of the lower range (1.25 and 0.5 $\mu\text{g/ml}$) and the

Table 5: Recovery data for nystatin in rat plasma

Calibration curve	Spike level $\mu\text{g/ml}$	% recovery	CV (%)
Lower range	LQC (0.25)	92.319	5.42
	MQC (1)	91.038	3.15
	HQC (1.75)	91.295	3.047
Higher range	LQC (5)	94.84	2.348
	MQC (20)	95.101	2.672
	HQC (50)	96.019	4.193

*n is the number of replicates, n=6, CV: Coefficient of variance. LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control

other two of the higher range (20 and 5 $\mu\text{g/ml}$). The samples were run immediately after preparation and yet again the same samples were stored at 2–8°C later analyzed after a time period of 12 h and 24 h, the results are depicted in Table 6. The percentage relative SD was within 4%. The data for storage stability are also presented in Table 6. The plasma samples spiked with nystatin were stored at a temperature below -20°C . The %CV for all the sample set range was within 6%. Thus, nystatin was found to be stable in plasma when stored below -20°C for at least 3 months. Furthermore, the stability of the samples was also tested post three freeze-thaw cycles. The results indicated that thawing and refreezing had the slightest outcome on the precision and accuracy of the findings (%CV was within 6%).

Application – pharmacokinetic studies

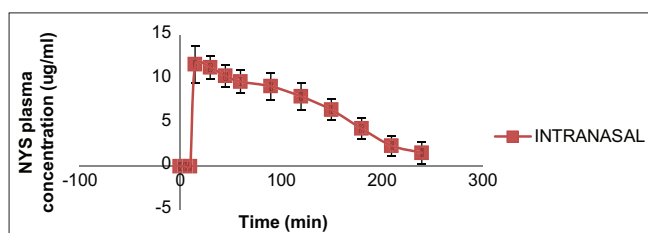
Plasma concentration versus time profile curve for the nasally administered *in situ* gelling liquid crystalline precursor formulation of nystatin in Wistar rats is presented in Fig. 2. A single dose of 8 mg/kg was administered in rats; the quantification of nystatin was performed based on the above-validated method. The maximum concentration in plasma (C_{max}) and the time required to achieve maximum concentration in plasma (T_{max}) was found to be 11.37 $\mu\text{g/ml}$ and 30 min.

Table 6: Process and storage stability data for nystatin in rat plasma

Process stability				
Range	Spiked concentration (µg/ml)	Obtained concentration (µg/ml) (mean±SD)		
		Baseline	12 h	24 h
Higher	20	19.10±0.35	18.86±0.09	18.98±0.2
	5	5.054±0.049	5.136±0.072	5.08±0.117
Lower	1.5	1.534±0.032	1.528±0.021	1.509±0.031
	0.5	0.4996±0.011	0.497±0.009	0.496±0.016

Storage stability						
Range	Concentration (µg/ml)	Baseline	1 month		3 months	
			Higher	20	19.11±0.399	18.87±0.51
	5	4.98±0.139	4.97±0.183	4.966±0.185	4.966±0.185	
Lower	1.5	1.514±0.018	1.508±0.022	1.499±0.026	1.499±0.026	
	0.5	0.538±0.038	0.521±0.021	0.518±0.03	0.518±0.03	

*SD: Standard deviation, n=3

**Fig. 2: Plasma concentration time profile of nystatin in plasma post-intranasal administration**

CONCLUSION

In the present study, a rapid and reproducible bioanalytical RP-HPLC method was developed and validated for the assay of nystatin in the plasma of an animal model. The ease of preparation of the mobile phase, the good peak symmetry, and resolution with the added benefit of the comparatively low retention time of the drug facilitate the analysis of a large number of samples over a short span of time. Moreover, the proposed method documented here has been effectively applied to estimate the biopharmaceutical performance of the novel nasal *in situ* gelling liquid crystalline precursor formulation.

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AUTHORS' CONTRIBUTIONS

Concept, design, resources, interpretation, and critical reviews – Kazi Marzuka and Dehghan Mohamed Hassan; supervision – Dehghan Mohamed Hassan; and materials, literature search, data collection and/or processing, and analysis and writing – Kazi Marzuka.

CONFLICTS OF INTEREST

“The authors declared no conflicts of interest” in the manuscript.

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