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Development and validation of eco-friendly micellar-HPLC and HPTLC-densitometry methods for the simultaneous determination of paritaprevir, ritonavir and ombitasvir in pharmaceutical dosage forms

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Abstract

Ombitasvir, ritonavir and paritaprevir are three recently discovered directly acting antiviral drugs (DAADs) used in combined single dose tablet dosage form for treatment of hepatitis-C viral infections (HCV). The methods of analysis followed by quality control and research laboratories are required to be economic and fast; however, these methods can also produce huge amounts of chemical waste. In this study two fast, economic and green HPLC and HPTLC methods were validated for the simultaneous determination of the three drugs. For HPLC, isocratic elution used a mixture of micellar aqueous mobile phase consisting of (0.15 M sodium lauryl sulfate and 0.01 M sodium dihydrogen phosphate, pH 6.2) and ethanol (56:44). Elution was done on RP-C18 Kinetix® column (5 μ m, 150 mm $\times$ 4.6 mm ID) at flow 1 mL min⁻¹ and 254 nm UV-detector. HPTLC

separations were performed on Merck® (20 cm × 10 cm) aluminum HPTLC plates coated with silica gel 60F254 using a mobile phase, Methylene chloride: methanol: ethyl acetate: ammonia (25%), (5:1:3:1, v/v/v/v) respectively. The calibration curves were linear across ranges of 3–100 µg mL⁻¹ and 0.1–2 µg/spot for both HPLC and HPTLC methods, respectively. The two methods were applied successfully for the determination of the three drugs under study in their combined tablets dosage forms.

Keywords: Analytical chemistry, Pharmaceutical chemistry

1. Introduction

More than 170 million people worldwide are infected with hepatitis-C virus (HCV). The leap forward in HCV treatment wasn't until the discovery of directly acting anti-viral drugs (DAADs) few years ago. According to the American Association for the Study of Liver Diseases and Infectious Diseases Society of America (AASLD-IDSA), the estimated average cost of regimens using DAADs ranges from 26,400 to 94,500USD per patient [1]. For example, the lowest identified price for treatment course for sofosbuvir alone in a developing country was \$ 900 in Egypt [1]. This can demonstrate the massive production size of HCV regimens all over the world which will require development of simple, fast and economic methods of analysis for determination of HCV drugs in pharmaceutical quality control (QC) and research laboratories.

Technivie® (paritaprevir, ritonavir plus ombitasvir) was approved by FDA in 2015, as single dose combination therapy [2]. Ombitasvir (OMB) is a hepatitis C virus NS5A inhibitor, paritaprevir (PAR) is a hepatitis C virus NS3/4A protease inhibitor, while ritonavir (RIT) is a CYP3A inhibitor. This combination showed activity against HCV genotype-4 with sustained virologic response of 100% [3]. HCV genotype-4 has been considered the most difficult-to-treat genotype which accounts for more than 90% of the HCV infections in Egypt alone (about 14.1% of Egyptian population) [4, 5].

Literature review reveals few LC methods for the simultaneous determination of PAR, RIT and OMB. To our knowledge, only four research methods were reported using HPLC. Three methods used acetonitrile (ACN) as organic modifier in proportion higher than 60% in the isocratic mobile phase [6, 7, 8] and one method used a 40% combination of methanol and ACN as organic modifier [9]. Two UHPLC methods were reported using MS/MS detection [10, 11].

To our knowledge, no method was reported for the simultaneous determination of the drugs under study using HPTLC.

The purpose of this research reported here was to develop a new HPTLC and green eco-friendly micellar-HPLC methods as economic and green alternatives, but yet, highly efficient methods for separation and quantification of the three DAADs. This research also compares the results and also draws conclusions on the advantages and disadvantages between HPTLC and HPLC methods for the qualitative and quantitative analysis of the three drugs.

2. Materials and methods

2.1. Materials

Sodium lauryl sulfate (SLS), Sodium hydroxide, and sodium dihydrogen phosphate dihydrate analytical grades were purchased from Merck, Germany. Ethanol (EtOH), methanol (MeOH) and ethyl acetate (EtAc) were (HPLC grades) and were purchased from J.T.Baker, Netherlands. Methylene chloride and ammonia solution (25%) were analytical grades and were purchased from Elnasr Chemicals, Egypt.

Copovidone, talc powder, sodium stearyl fumarate, sorbitan monolaurate (span-20) and propylene glycol monolaurate were pharmaceutical grades produced by Sigma-Aldrich, Germany and were kindly supplied and certified from EIPICo, Egypt.

PAR, RIT and OMB (analytical grades) were kindly supplied and certified by EIPICo., Egypt (structures shown in Fig. 1). De-ionized water was produced in-house by Millipore water purification system.

Qurevo® pharmaceutical tablet dosage form, containing (12.5 mg, 75 mg and 50 mg of OMB, PAR and RIT, respectively) was purchased from Egyptian market, (Batch number 1051458; produced by abbvie).

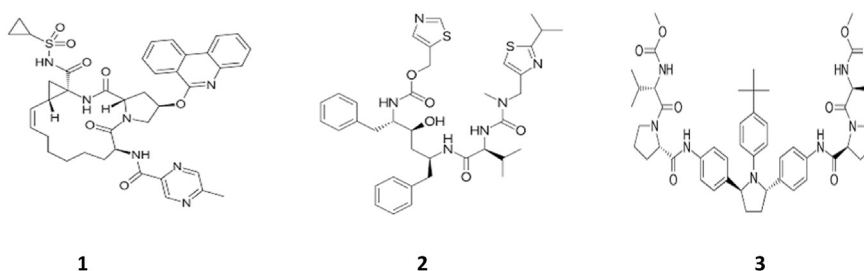


Fig. 1. Chemical structure of drugs under study (1. PAR, 2. RIT, 3. OMB).

2.2. Instrumentation and columns

Young line HPLC system model 9100 (Korea), equipped with YL9101 vacuum degasser, 20 μ L sample loop injector, Quaternary Pump (model YL9110), and YL9120 UV/Vis detector.

The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of semi-automatic sample applicator system using 100 μ L syringe, connected to a nitrogen tank and equipped with CAMAG-TLC scanner 3 which was operated by winCATS Software. Bandwidth was 4 mm and application rate was 15 μ L/sec. Densitometric scanning was done using a CAMAG TLC scanner 3 with Slit dimension was 3 $\times$ 0.45 mm and scanning speed was 20 mm/sec.

pH meter model AD111 (Adwa, Hungary) was used for adjustment of pH-values of the aqueous part of the mobile phase.

HPLC core-shell column Kinetix[®] 5 μ m-C18 (150 $\times$ 4.6 mm) was purchased from Phenomenex, USA. HPTLC aluminum silica gel 60F254 pre-coated plates (20 $\times$ 20 cm) were purchased from Merck, Germany.

2.3. Chromatographic conditions and procedure

For HPLC method, mobile phase A consisted of 0.15 M SLS, 0.01 M of sodium dihydrogen phosphate dihydrate and pH was adjusted to 6.2 using 1 M sodium hydroxide solution. Mobile phase B was HPLC grade EtOH. All analyses were done by isocratic elution (56:44, v/v) mobile phase A: B, respectively. Flow rate was set at 1 mL/min, sampler injection volume 20 μ L and column compartment was kept at 30 $^{\circ}$ C. UV-detector was set at 254 nm.

For HPTLC method, the best separation of the studied with symmetric, sharp, non-tailed peaks was attained using mobile phase consisting of methylene chloride: methanol: ethyl acetate: ammonia (25%) (5:1:3:1, v/v/v/v). HPTLC plates were divided into smaller plates of 20 $\times$ 10 cm diameter immediately before use. It was found that activation of the HPTLC plates at 60 $^{\circ}$ C for 10 min immediately before sample application improved both separation and peaks symmetry. Samples were applied as bands of 3 mm width and at 5 mm intervals under a nitrogen stream. Chromatographic elution to a distance of 9 cm in tightly closed and saturated TLC jar at room temperature. The elution time was about 6 min. The HPTLC plates were well dried after elution in a current of hot air then the plates were scanned at 243 nm at absorbance mode using the deuterium lamp as a radiation source.

2.4. Stock solutions, calibration standards and quality control samples

Stock standard solution (10 mL) of OMB, PAR and RIT was prepared in solvent mixture (EtOH: water, 1:1) at concentrations $100 \mu\text{g mL}^{-1}$ and stored in refrigerator. The working solutions then were prepared by dilution of the stock solution with the solvent mixture.

For HPLC method, linearity was tested using working calibration standard solutions prepared at concentrations of 3, 10, 20, 50, 75 and $100 \mu\text{g/mL}$. For accuracy, the quality control samples were prepared at five different concentrations which were 3, 10, 25, 50 and $75 \mu\text{g/mL}$ by spiking in freshly prepared placebo solution. Inter and intra-days precisions were assessed using three different concentrations (10, 25, and $50 \mu\text{g/mL}$) at three different days.

Placebo solution was prepared by dispersing 1 gram of Copovidone, talc powder, span-20, propylene glycol monolaurate and sodium stearyl fumarate per 100 mL water which are the excipients found in Qurevo[®] tablets.

For HPTLC method, Working solutions were prepared by dilution of stock solutions to concentrations 1, 2.5, 5, 10, 15 and $20 \mu\text{g/mL}$ in the solvent mixture. Linearity was tested on the TLC plates at concentrations of 100, 250, 500, 1000, 1500 and 2000 ng/spot. For accuracy, the quality control samples were prepared at five different concentrations which were 1, 2.5, 5, 10 and $15 \mu\text{g/mL}$ by spiking in freshly prepared placebo solution and accuracy was tested by spotting (10 μL) of each of these samples at concentrations 100, 250, 500, 1000 and 1500 ng/spot. Inter and intra-days precisions were assessed using three different concentrations (1, 2.5, and $5 \mu\text{g/mL}$) at three different days by spotting (10 μL) of each of these samples at concentrations 100, 250 and 500 ng/spot.

All stock solutions, calibration standards and quality control samples were stored in a refrigerator at $2-8^\circ\text{C}$.

2.5. Preparation of dosage forms for analysis

Ten tablets of Qurevo[®] were weighed, finely powdered and mixed thoroughly then stock solution was prepared by dissolving the average weight of one tablet in 100 mL solvent mixture. Stock solution was sonicated for five minutes then filtered. The working solution was prepared by diluting 1 mL of the filtrate to 10 mL using the solvent mixture.

For HPLC method, 20 μL of this working solution was injected directly on the chromatographic column, while for HPTLC method only 10 μL was spotted on TLC plates.

2.6. Method validation

Method validation was performed for the quantitative determination of the three anti-viral drugs under study to evaluate linearity, accuracy, specificity, precision, reproducibility and robustness of the proposed methods. Validation was carried out according to ICH guidelines [12].

3. Results and discussion

3.1. Method validation

3.1.1. Selectivity

Good separation of the three DAADs was achieved using HPLC and HPTLC methods with clear resolution between their peaks. The chromatographic parameters and performance obtained are listed in (Table 1).

Chromatograms in Figs. 2 and 3 show the separation of the three DAADs in Qurevo® tablet by the proposed HPTLC and HPLC methods, respectively. As shown, no interference from the excipients in dosage forms was observed.

3.1.2. Calibration curve and linearity

Six concentrations of PAR, RIT and OMB within the specified range were injected in triplicates. The calibration curves were constructed by plotting the peak area corresponding to each drug against its concentration. Regression data showed very good linearity within the specified range (Table 2).

The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the injected quantity giving S/N of 3 and 10 (in terms of peak area), respectively. LOD and LOQ for the proposed methods are shown in (Table 2). The results indicate that both methods are sensitive for minute concentrations of the three DAADs under study.

Table 1. Chromatographic parameters and system suitability of the proposed methods.

Drug	HPLC method			HPTLC method
	Retention time (min)	Selectivity (α)	Resolution (R_s)	Retention factor (R_f)
PAR	2.3 $\pm$ 0.1	—	—	0.2 $\pm$ 0.1
RIT	4.5 $\pm$ 0.1	2.3	8.5	0.6 $\pm$ 0.1
OMB	5.5 $\pm$ 0.1	1.3	2.6	0.7 $\pm$ 0.1

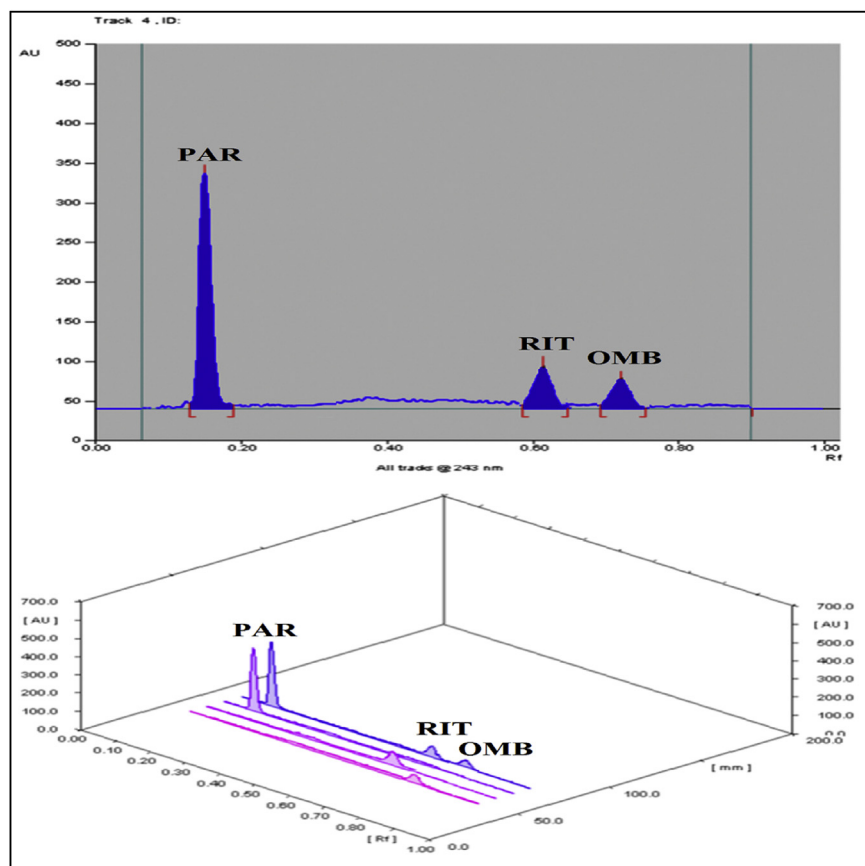


Fig. 2. Atypical 2D and 3D (HPTLC) chromatograms of Qurevo® tablets.

3.1.3. Accuracy and precision

Accuracies of the proposed methods were established across the specified ranges by analysis of the five mentioned quality control samples at low, medium and high concentration levels. The results (Table 2) showed trueness of the analytical procedures.

Inter-day and intra-day assay precisions were determined using three QC-samples injected in triplicates in the same day and on three different days, respectively. Inter-day and intra-day precision results are listed (Table 2) showing high precision results.

3.1.4. Robustness

The proposed methods were tested for minor variations in the validated chromatographic conditions. In HPLC method, the percentage of organic modifier (EtOH) in mobile phase was changed between (42, 44 and 46%) and pH of the aqueous

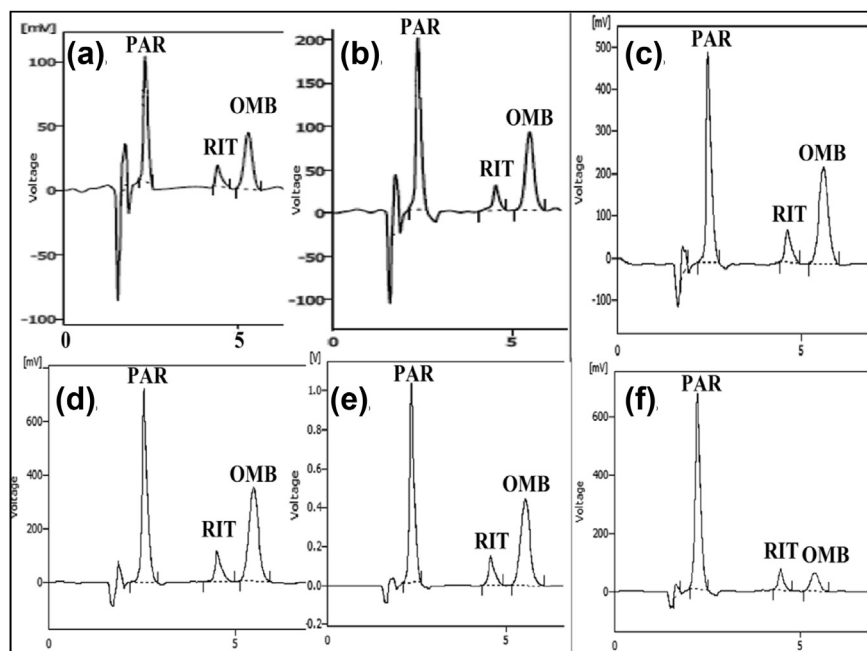


Fig. 3. HPLC chromatograms of PAR, RIT and OMB. *Laboratory prepared mixtures at concentrations (a) 10 µg/mL mixture, (b) 20 µg/mL mixture, (c) 50 µg/mL mixture, (d) 75 µg/mL mixture, (e) 100 µg/mL mixture, and (f) Qurevo® tablet.

Table 2. Linearity and accuracy data of the proposed methods.

Parameter	HPLC proposed method			HPTLC proposed method		
	PAR	RIT	OMB	PAR	RIT	OMB
Range	3–100 µg/mL	3–100 µg/mL	3–100 µg/mL	100–2000 ng/spot	100–2000 ng/spot	100–2000 ng/spot
Linearity equation	$Y = 100.1X - 152.4$	$Y = 15.2X + 62.7$	$Y = 81.7X - 43.9$	$Y = 3.7X + 1176.4$	$Y = 4.8X + 1334.4$	$Y = 7.4X + 809.6$
Regression coefficient (r^2)	0.999	0.999	0.999	0.998	0.999	0.998
LOD*	0.7 µg/mL	0.9 µg/mL	0.8 µg/mL	26.0 ng/spot	16.3 ng/spot	23.3 ng/spot
LOQ*	2.2 µg/mL	2.6 µg/mL	2.4 µg/mL	78.0 ng/spot	48.9 ng/spot	69.8 ng/spot
Accuracy** ($n=5$) (%Recovery $\pm$ RSD)	100.8 ± 1.1	100.2 ± 1.8	99.5 ± 1.7	99.9 ± 0.1	99.8 ± 0.2	99.8 ± 0.2
Intra-day precisions ($n = 3 \times 3$)***	100.1 ± 0.1	99.8 ± 0.2	99.9 ± 0.1	100.0 ± 0.3	99.9 ± 0.2	99.8 ± 0.2
Intra-day precisions ($n = 3 \times 3$) ***	99.9 ± 0.2	100.1 ± 0.2	99.8 ± 0.3	99.8 ± 0.2	100.1 ± 0.1	99.7 ± 0.4

* LOD (Limit of detection), LOQ (Limit of quantification).

** RSD (relative standard deviation).

*** Three different samples injected three different times.

Table 3. Robustness of the proposed methods for simultaneous determination of PAR, RIT and OMB.

HPLC method				
Parameter	Sample concentraion	PAR*	RIT*	OMB*
Effect of pH	QC sample (25 µg/mL)	99.8 ± 0.4	100.0 ± 0.2	99.6 ± 0.2
Effect of organic modifier%	QC sample (25 µg/mL)	100.0 ± 0.3	99.8 ± 0.3	99.9 ± 0.2
HPTLC method				
Effect of change in mobile phase composition	QC sample1 (100 ng/spot)	99.6 ± 0.5	99.8 ± 0.6	99.4 ± 0.3
	QC sample2 (250 ng/spot)	99.7 ± 0.2	99.7 ± 0.3	99.0 ± 0.3

* Results obtained = % Recovery ± relative standard deviation.

buffer was changed between (6.0, 6.2 and 6.4). In HPTLC method, three different minor changes in the composition of the mobile phase was tested; Methylene chloride: methanol: ethyl acetate: ammonia (25%) (4.5:1:3.5:1, v/v/v/v), (5:1:3:1, v/v/v/v) and (5.5:1:2.5:1, v/v/v/v), respectively. Results (Table 3) showed that minor variations did not produce significant effect on separation efficiency in terms of number of theoretical plates (N) or recovery%.

Table 4. Application of the proposed methods in determination of Qurevo® tablet dosage form and comparison with reported methods.

HPLC method				
Dosage form Qurevo® tablet	%Recovery* ± SD		t-value**	F-value**
	Proposed method	Reported [6]		
75 mg of PAR/tablet	99.8 ± 0.4	99.1 ± 0.5	2.43	2.38
50 mg RIT/tablet	99.7 ± 0.6	99.2 ± 0.6	1.25	1.01
12.5 mg OMB/tablet	100.0 ± 0.4	99.4 ± 0.4	2.41	1.21
HPTLC method				
	%Recovery* ± SD		t-value**	F-value**
	Proposed method	Reported [7]		
75 mg of PAR/tablet	99.7 ± 0.7	99.0 ± 0.6	1.9	1.2
50 mg RIT/tablet	99.9 ± 0.5	99.1 ± 0.6	2.3	1.9
12.5 mg OMB/tablet	99.6 ± 0.3	99.1 ± 0.6	1.7	2.9

* The values obtained are the mean of five determinations (n = 5).

** The tabulated t- and F-values at 95% confidence limit are 2.78 and 6.39, respectively.

3.1.5. Analytical application

The proposed methods were applied for the simultaneous determination of the three DAADs in their marketed tablet dosage form, Qurevo®. Results obtained (Table 4) were compared to results obtained from a comparison method. Student *t*-test and F-values were used for this comparison and results showed that the methods were comparable and accurate.

3.1.6. Analytical solutions stability

In order to avoid any unexpected changes in the stock solutions during the analysis due to the delay in the analysis time, we must have detailed information about the stability of the prepared drugs stock solutions. It was found that ethanolic solutions of and PAR, RIT, and OMB were stable at least 72 hours at room temperature when protected from light, and for 10 days when stored refrigerated at 4-8 °C as it showed no chromatographic or absorbance changes.

3.2. Evaluation of the proposed analytical procedures

When we compare the proposed HPLC method to other reported methods; UHPLC methods [10, 11] have lower solvents and energy consumptions than the conventional HPLC methodologies. However, UHPLC methods have higher maintenance costs due to the shorter column life-time and requirement of special instrumentation especially those coupled with MS-MS detectors [13]. That's why UHPLC use in economic establishments like pharmaceutical quality control laboratories is limited and not widespread.

According to Welch [14], each conventional LC instrument equipped with a conventional column can generate about 0.5 L of solvent wastes per day. That's why ongoing greener in chromatography we have to develop new methodologies that minimize solvents consumption and replace the ecologically dangerous ones [15]. ACN is a hazardous solvent listed in the US Environmental protection Agency of toxic chemicals, January-2019 [16], however, EtOH is a biodegradable and cheap solvent with low volatility characteristics which renders the mobile phase composition more stable upon longer storage [13]. All other reported HPLC methods utilize ACN and MeOH in the mobile phase which are both listed in the US Environmental protection Agency of toxic chemicals, January-2019 [16] which was replaced in the proposed method by the safer solvent; EtOH,. Also the proposed method lowers the percentage consumed of organic solvent in the mobile phase when compared to the other reported HPLC methods without increasing the analysis time.

The proposed HPTLC method has several advantages. Firstly, the same mobile phase could be stored and used several times for several elutions (i.e. Recycled). Secondly, the simultaneous processing of sample and standard under the exact same

conditions gave rise to improved analytical precisions and accuracies. Additionally, the low cost of HPTLC method encourages its use as an analytical tool.

4. Conclusion

In this study, two new green methods were validated for the simultaneous quantification of PAR, RIT and OMB in bulk powders and applied successfully to pharmaceutical dosage forms. The high accuracies and precisions of the assays obtained, taken together with the low solvent consumption and replacing hazardous solvents by greener ones made these methods eligible for use in different research and pharmaceutical quality control laboratories for the determination of these drugs. Moreover; the proposed methods were compared to the other reported methodologies for assessing their greenness and found more favorable for greener analysis.

Declarations

Author contribution statement

Adel Ehab Ibrahim: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Roshdy E. Saraya: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Magda Elhenawee and Hanaa Saleh: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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