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Original Research Article

A fast and validated chromatographic method for simultaneous determination of deferoxamine and D-penicillamine via chelate formation with metal ions in bulk and dosage forms

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Abstract

Purpose: *To develop a chromatographic method for the determination of deferoxamine (DFX) and D-penicillamine (D-PEN) by improving ultra violet (UV)-absorption via complex formation with Fe^{2+} and Cu^{2+} metal ions.*
Methods: *Chromatographic analysis was performed by Waters RP-HPLC system using a Symmetry® C (18) column with a mobile phase comprising 0.1 % formic acid and methanol (95:5 v/v). For complexation process, drug and metal ion solution were mixed in a ratio of 1:5 and the resulting complex directly analyzed. Validation and system suitability parameters (including chromatographic parameters) were determined.*
Results: *DFX-Fe^{2+} and D-PEN-Cu^{2+} complexes showed good UV absorption at 260 nm and were easily determined by the newly developed HPLC method. The developed method showed linearity over the concentration range of 8 - 96 µgmL^{-1} (R^2 > 0.999 for DFX and > 0.99 for D-PEN). Precision and accuracy were also within acceptable limits (100.0 ± 2.0 %).*
Conclusion: *The developed method is robust and validated, and satisfies all the system suitability requirements as per ICH guidelines. DFX injection and D-PEN capsule dosage forms can be successfully analysed with the proposed method. The method is simple, fast and cost-effective for the analysis of D-PEN and DFX individually, or simultaneously in bulk drugs as well as in capsule and parenteral formulations, using UV-detector.*

Keywords: *Deferoxamine, D-penicillamine, Chelate formation, Metal ions, HPLC, Dosage forms*

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INTRODUCTION

Studies on the development of chelating agents have been growing steadily due to their wide range of applications in the field of nutritional supplements and pharmaceuticals [1-3]. Chelation therapy has been introduced for removing toxic metal ions such as mercury, arsenic and lead, and also for correct the biological levels of the essential metals and

minimize the harmful effects due to their elevated concentrations. Deferoxamine (DFX; desferrioxamine) and *D*-penicillamine (D-PEN) are well known chelating drugs, used to treat iron and copper excesses inside the body. They have a tendency to form chelates with various divalent and trivalent metal ions [3-9].

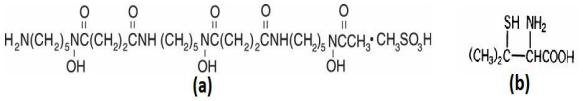

Figure 1: Chemical structures of: a) deferoxamine mesylate, b) *D*-penicillamine

The chemical structures of DFX and D-PEN lack the presence of conjugation which is essential for UV light absorption above 200 nm region. Therefore, common detectors based on UV-Vis absorption cannot be used for detection of these drugs. On the other hand, fluorescence and mass detection are more specialized techniques, although they increase the cost of analysis. The UV absorption of chelating drugs lacking conjugation can be improved by means of charge transfer, when the analytes bind with metal ions such as Fe^{3+}, Fe^{2+} and Cu^{2+} [10-12]. Chelating drug-metal ion complex formation and their stability could be estimated based on hard and soft acid and base concept (HSAB). In general, hard metal ions prefer to bind with hard ligands while soft metal ions prefer to bind with soft ligands.

Previously, DFX was determined in bulk and dosage forms after complex formation with iron by chromatographic methods using fluorometric, electrochemical and mass detectors [13-15]. In addition, HPLC-based methods based on the application of post-column derivatization technique for the detection of DFX are available [16]. Similarly, various chromatographic methods for the detection of D-PEN in different traces have also been reported [17-24].

A survey of literature reveals that no HPLC-UV or UPLC-UV method has been reported for simultaneous determination of D-PEN and DFX after complexation with metal ions. However, information on the complexes of DFX and D-PEN with Cu^{+}, Cu^{2+}, Fe^{2+} and Fe^{3+} are well documented in the literature [25-28]. Therefore, the present study was undertaken to develop a rapid HPLC-UV method that can be applied for determination of mixtures of D-PEN and DFX using pre-injection complex formation with suitable metal ions such Cu^{2+} or Fe^{2+}. The method development will rely on the stability and the reproducibility of a good chromatographic peak shapes of drug-metal ion complex as well as good separation between the drug-metal ion complexes.

EXPERIMENTAL

Chemicals and reagents

Reference standards of deferoxamine mesylate, *D*-penicillamine, copper sulphate pentahydrate and ferrous sulphate heptahydrate were procured from Sigma Aldrich. Formic acid and methanol were also procured from Sigma Aldrich. In-house Milli Q water was used for preparation of solvent systems. Artamin capsules containing 150 mg D-Penicillamine (Sandoz, Austria) and Desferal® vial for injection, containing 500 mg lyophilized deferoxamine mesylate powder (Novartis, Switzerland) were purchased from local hospital pharmacy store.

Instrumentation and chromatographic conditions

HPLC investigations were conducted on a Waters Breeze 1525 instrument using breeze LC2 software and separation was performed in Symmetry® C(18) column (0.75 cm × 4.6 mm i.d., particle size 3.5 µm) under benchtop conditions. The separation was achieved by using mobile phase at 1mL/min rate (0.1% formic acid in water and methanol; 95:5 *v/v*), injection volume of 20 µL and detector wavelength of 260 nm for a run time of 7 min.

Preparation of solutions

Mobile phase

Formic acid solution (0.1 %) in water and methanol-HPLC grade were mixed to have ratio of 95:5 *v/v*, filtered through 0.45 µm nylon filter and sonicated 30 min.

Standard drug stock solutions

Standard stock solutions containing 0.005 M of deferoxamine mesylate (0.746 mg/mL) and 0.005 M of *D*-penicillamine (3.282 mg/mL) were prepared by dissolving accurately weighed reference standards separately in Milli Q water in a volumetric flask.

Metal ion standard stock solutions

Standard stock solutions containing 0.025 M of iron (II) sulphate heptahydrate (6.952 mg/mL) and 0.025 M copper (II) sulphate pentahydrate (6.244 mg/mL) were prepared by dissolving in water, and the solutions were filtered and sonicated for 10 min.

Working standard solutions

The working standard solutions for each drug were prepared separately by transferring 1 mL of the standard drug stock solution and 1 mL of the metal ion stock solution to 5 mL. The volume was made up to 5 mL with 0.1 % formic acid to obtain metal ion/drug molar concentration ratio of 5:1. This ratio showed stable and reproducible chromatographic peak shape and area (see Results and Discussion section). The mixture was sonicated for 5 min at 60°C to accelerate drug-metal ion complexation. The resulting solutions were further diluted with 0.1% formic acid to get the final drug concentrations of 20 µg/mL before introducing them to the HPLC system for method development process. Similarly, for calibration curve, the final drug standard solutions of concentrations in range of 8 - 96 µg/mL containing both D-PEN-Cu^{2+} and DFX-Fe^{2+} complexes were prepared using 0.1 % formic acid as diluent.

Sample stock solutions of formulations

Deferoxamine mesylate: The contents of five injection vials were reconstituted with HPLC water and diluted with 0.1 % formic acid to get the final concentration of 0.005 M (0.746 mg/mL).

D-penicillamine: The contents of ten capsules were taken out, and an amount corresponding to 16.41 mg of *D*-penicillamine was diluted to 5 mL with water, filtered and sonicated for 30 min.

Sample solution from dosage forms: The test solutions were prepared by adding 1 mL of each sample drug stock solution and 1 mL of each standard stock metal ion solution to 5 mL in a volumetric flask with 0.1 % formic acid. The mixture was sonicated for 5 min at 60°C. The resulting solution was diluted with 0.1% formic acid to get the final concentrations of 20, 40 and 60 µg/mL for each drug.

Placebo stock and working solutions: The placebo solution for D-penicillamine capsule formulation was prepared by taking the equivalent amounts of excipients such as lactose and magnesium stearate into a 5 mL Eppendorf tube, and making up the volume with HPLC grade water, followed with mixing well. A placebo test solution was prepared same as sample test solution.

Validation of method

All validation and system suitability parameters including chromatographic parameters, linearity, LOD, LOQ, precision, accuracy, specificity, and solution stability were performed as per ICH guidelines. [31,32].

Linearity

Linearity was evaluated using regression analysis by plotting six concentrations (8, 16, 32, 64, 80 and 96 µg/mL) against their peak areas in six replicates.

Accuracy and precision

Three quality of control samples at low, medium and high (LQC, 16; MQC, 64 and HQC, 96 µg/mL), concentrations were selected for precision and accuracy determination. The samples were analyzed at different times on the same day, and the same analyses were performed over different days for determination of intra-day and interday precision.

The recoveries and their respective % RSD were calculated. For dosage forms, test samples at 50, 100 and 150 % of target concentration (40 µg/mL) were injected in six replicates, and the % recoveries were determined.

Quantification and limit of detection

The LOD and LOQ were calculated as per the ICH and USP guidelines.

Specificity

The specificity of the proposed method was determined by examining the chromatograms for any interfering peaks from blank and placebo.

Solution stability

The test and standard solutions were evaluated for their stabilities at different storage conditions at MQC level (64 µg/mL). The solutions were kept at 20, 4 and -20 (± 2) ^{o}C for 7, 114 and 0 days respectively. They were analyzed at the end of each storage period and the results were compared with those of fresh solutions.

Robustness

To evaluate the robustness of the method, small deliberate changes were made in the ratio of mobile phase (± 5 %), detector wavelength (± 5 nm) and flow rate (± 0.1 mL/min) in the optimized chromatographic method. The effect of these small changes on the contents of D-PEN and DFX were evaluated in triplicate.

RESULTS

Validation data

The research project was undertaken to develop a HPLC method for determination of DFX and D-PEN via complex formation with polyvalent metal ions. After investigating different mobile phase compositions of aqueous and organic phases (methanol and acetonitrile), a mobile phase consisting of 0.1 % HCOOH and CH_3OH (95:5 *v/v*) was found to be optimum at a flow rate of 1 mL/min. The data were obtained at a wavelength of 260 nm, and the retention times of D-PEN-Cu^{2+} and DFX-Fe^{2+} complexes were observed at 1.47 and 5.52 min, respectively. The chromatograms obtained by using optimized chromatographic conditions are depicted in Figure 2. Furthermore, Figure 3 shows the chromatograms of selected metal ions using the developed HPLC conditions. Symmetrical peaks for both DFX-Fe^{2+} and D-PEN-Cu^{2+} complexes were obtained with excellent resolution and the tailing factors were less than 2. Overall, the system suitability parameters obtained by the finalized chromatographic condition were within acceptable limits, indicating the suitability of the system for conducting the experiment. The observed suitability parameters are summarized in Table 1.

The limit of detection and limit of quantification for D-PEN and DFX were found to be 1.180 and 3.085 µg/mL, and 6.034 and 10.286 µg/mL respectively. No interfering peaks of matrix (blank and placebo) were observed at the retention times of the drug complexes, indicating the specificity of the developed HPLC method (Figure 3). The method was linear in the concentration range of 8 - 96 µg/mL, with mean correlation coefficient (R^2) values of 0.9906 and 0.9999 for D-PEN-Cu^{2+} and DFX-Fe^{2+}, respectively, suggesting the linearity of the proposed method. The precision and accuracy results for D-PEN-$Cu^{2+}$and DFX-Fe^{2+}in the QC samples at LQC, MQC and HQC levels are shown in Table 2. The precision was expressed as % RSD values of the peak areas, and was in the range of 0.2 - 1.3 % for D-PEN-Cu^{2+}, and 1.1 - 2.0 % for DFX-Fe^{2+} in the intra-day analysis, whereas, for inter-day analysis, the precision was in the ranges of 0.2 - 0.7 % and 0.6 - 1.5 %, respectively. To demonstrate the accuracy of the method, recovery was evaluated by standard addition method, and the results for both analytes were within 98.0 - 102.0 % range. Representative chromatograms showing overlay of six replicate injections at LQC level (16 µg/mL) are depicted in Figure 4. All the solutions were found to be stable in the selected storage conditions. The solution stability was expressed as mean percent recovery calculated from the peak area under respective storage conditions. It was found to be in the range of 99.1 - 100.9 % for D-PEN-Cu^{2+}, and 99.2 - 100.3 % for and DFX-Fe^{2+}. These results suggest that the proposed method can be operated in benchtop conditions (Table 3).

Robustness study was performed to investigate the effect of ratio of mobile phase (± 5%), flow rates of 0.9 and 1.1 mL/min, and detector wavelengths of 255 and 265 nm. No remarkable differences (100 ± 2 %) were observed between the assay results obtained by employing the optimized analytical conditions and those obtained from the experiments in which small variations in chromatographic parameters were made. Thus, the method was robust for D-PEN and DFX assays for small variations in organic solvent ratio in the mobile phase, flow rate and detection wavelength. The robustness results are summarized in Table 4.

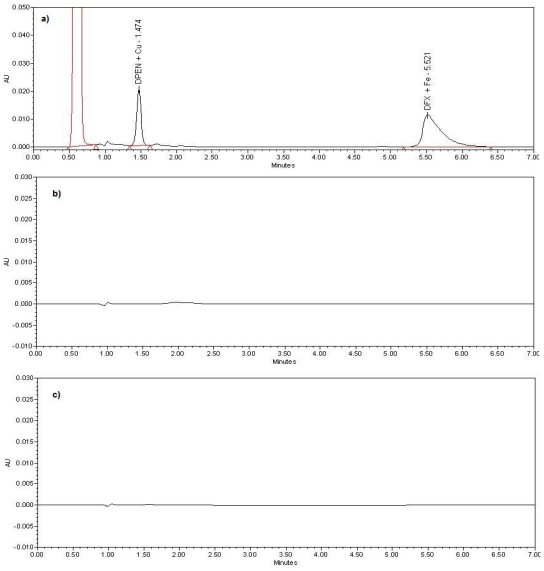

Figure 2: Representative chromatograms of a mixture of D-PEN-Cu^{2+} and DFX-Fe^{2+} complexes (a); mixture of D-PEN and DFX pure API without complex formation (b); and blank (blank: 0.1 % formic acid in water (c)

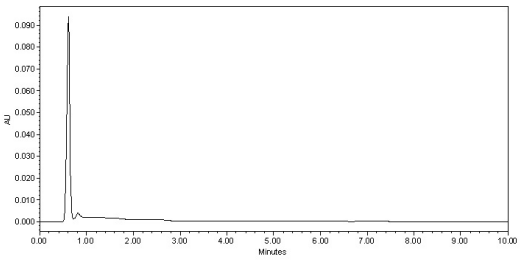

Figure 3: Representative chromatograms of mixture of iron (II) sulphate and copper (II) sulphate

Table 1: Chromatographic parameters

Parameter	D-PEN-Cu^{2+}	DFX-Fe^{2+}
Retention time (min)	1.47	5.52
Tailing factor	1.08	1.39
Resolution	7.25	11.75
Capacity factor	2.45	4.49
Theoretical plate count	2528	2624
RSD (n = 6) (20 µg/mL)	0.46	0.92

Table 2: Data on precision and accuracy

Parameter	Concentration level	D-PEN-Cu^{2+}	DFX-Fe^{2+}
Intra-day precision and accuracy[a]			
Peak area RSD (%) (average recovery, %)	LQC	1.3 (101.4)	1.2 (101.8)
	MQC	0.2 (101.8)	2.0 (99.6)
	HQC	0.4 (101.2)	1.1 (102.2)
Inter-day precision and accuracy[a]			
Peak area R.S.D. (%) (average recovery, %)	LQC	0.7 (102.15)	0.6 (100.7)
	MQC	0.2 (101.71)	0.6 (101.8)
	HQC	0.6 (99.10)	1.5 (100.5)

[a]*n* = 3; LQC, MQC, HQC - low, medium and high quality control sample concentration levels (16, 64 and 96 µg/mL, respectively)

Application of the developed method in determination of D-PEN in capsule and DFX in injection dosage forms

The validated HPLC method was used for determination of D-PEN and DFX in their capsule and injection dosage forms. The individual chromatograms for D-PEN and DFX in their respective dosage forms were compared with those recorded for placebo solution and blank chromatograms. The method was found to be specific since no peaks at the retention time of the drug complexes were observed. Accuracy studies on the respective formulations of D-PEN and DFX were carried out for sample recovery at three levels (100 ± 50 %) of the targeted concentration (40 µg/mL).

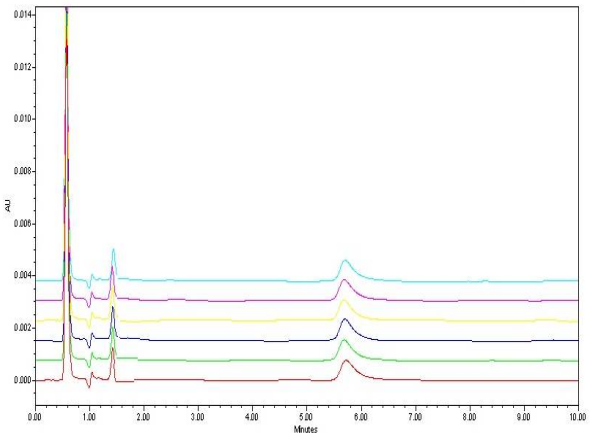

Figure 4: Representative chromatograms showing overlay of six replicate injections of standard solution containing a mixture of D-PEN-Cu^{2+} and DFX-Fe^{2+} complexes, at LQC level (16 µg/mL)

Table 3: Solution stability data

Analyte/ complex	Storage conditions	Mean recovery (%)[a]
D-PEN	20 ± 2°C; 7 days	100.9
	4°C; 14 days	100.1
	-20°C; 30 days	99.1
DFX	20 ± 2°C; 7 days	99.2
	4°C; 14 days	100.3
	-20°C; 30 days	99.9

[a]N = 6

Table 4: Data on robustness

Parameter	Content (%)	
	D-Penicillamine*	Deferoxamine*
Ratio of mobile phase (+ 5 %)	100.3	99.8
Ratio of mobile phase (- 5 %)	99.2	101.2
Flow rate (0.9 mL/min)	98.8	99.1
Flow rate (1.1 mL/min)	99.4	98.3
Wavelength (255 nm)	101.3	98.7
Wavelength (265 nm)	98.7	100.3
Without variation	99.7	99.3

**The values are the average of three determinations*

The mean percent recoveries of both analytes were within 100 ± 2 %, which indicated the aptness of the developed method for estimation of D-PEN and DFX in their capsule and injection dosage forms.

Table 5: Recovery data of D-PEN-Cu^{2+} and DFX-Fe^{2+} in capsule and injection dosage forms

Analyte	Recovery sample concentration (µg/mL)	Targeted concentration (%)	Drug recovered (µg/mL)	Recovery R.S.D. (%)[a]
D-PEN (capsule)	20	50	19.84	99.2 ± 0.6
	40	100	39.56	98.9 ± 0.2
	60	150	60.6	101.0 ± 0.7
DFX (injection)	20	50	19.78	98.9 ± 1.1
	40	100	40.75	101.9 ± 0.1
	60	150	59.76	99.6 ± 1.1

[b] $n = 6$

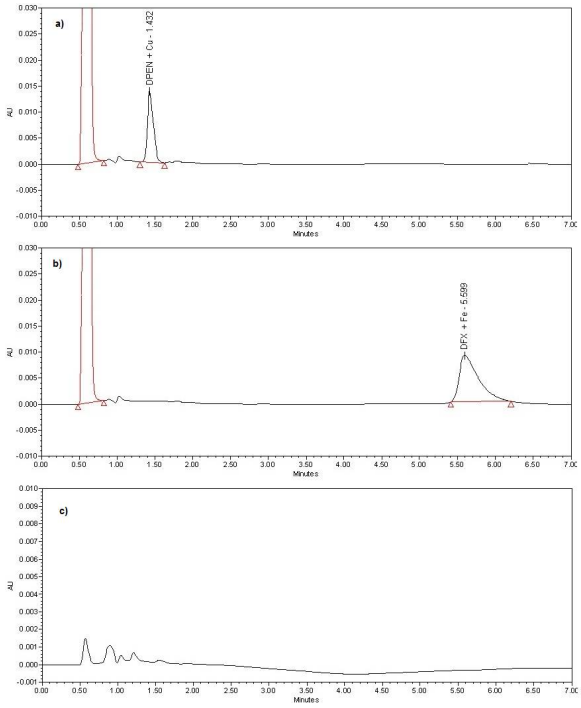

Figure 5: Representative chromatograms obtained from pharmaceutical formulations (capsule for D-PEN and injection for DFX) using the developed method a): D-PEN-Cu^{2+} complex; b): DFX-Fe^{2+} complex); c) placebo for D-penicillamine capsule

The calculated mean recovery data of D-PEN-Cu^{2+} and DFX-Fe^{2+} in D-PEN and DFX formulations are summarized in Table 5, while the representative individual chromatograms of D-PEN capsule, DFX injection and placebo are given in Figure 5.

DISCUSSION

The present research was undertaken in order to improve the UV-absorption of DFX and D-PEN by forming their complexes with different metal ions. No peaks were detected up to 7 min run time when pure DFX and D-PEN were injected into the HPLC system. This confirmed that these drugs cannot be detected directly by UV detector. Therefore, copper (II) chloride, copper (II) sulphate, iron (III) chloride and iron (II) sulphate were selected for improving the UV absorption of DFX and D-PEN by chelate formation. Different combinations of drug and metal ions were tested to find out the metal ion which forms a stable complex with drugs along with good and reproducible peak height and area. During the development of the method, no peak was detected up to 10 min of runtime when D-PEN was complexed with copper (II) chloride, iron (III) chloride as well as with iron (II) sulfate. Similar results were obtained when DFX was treated with copper (II) chloride, copper (II) sulfate and iron (III) chloride. However, when D-PEN and DFX were complexed with copper (II) sulfate and iron (II) sulfate, respectively, two distinctly separated peaks at retention times of 1.47 and 5.52 min belonging to D-PEN-Cu^{2+} and DFX-Fe^{2+}complexes were observed.

After the selection, concentrations of metal ions were optimized with respect to DFX and D-PEN concentrations in order to obtain stable complexes. Different drug/metal ion molar concentration ratios of DFX and D-PEN (1:1, 1:2, 1:5, 1:10 and 1:20) and their suitable metal ions (Fe^{2+} and Cu^{2+}) were investigated. At lower metal ion concentrations (1:1 and 1:2), lower peak areas were observed, whereas, at higher metal ion concentrations (1:10 and 1:20), remarkably broad peaks were obtained with a lot of noise. However, at 1:5 concentration ratio, symmetric and well separated peaks corresponding to DFX-Fe^{2+} and D-PEN-Cu^{2+} complexes were achieved. As a result, five folds higher metal ion concentration than drug was found to be most optimum for quick formation of stable drug-metal ion complexes with acceptable peak shape.

In HPLC technique, the interaction between chelating drugs and metal ions can be achieved by using two approaches. In the first approach (in-column), chelating agents and metal ion solutions are premixed to allow for complex formation and then the resulting solutions are injected into the system for analysis, whereas, in the second approach (out-column), metal ion solutions are mixed with the mobile phase and

the interaction between analyte/drug and metal ion takes place inside the HPLC column during the chromatographic run. In the present analysis, the first approach was successfully applied, wherein the solutions of DFX and D-PEN were mixed with Fe^{2+} and Cu^{2+} solutions, respectively, during the preparation of the sample/working solutions. The formed DFX-Fe^{2+} and D-PEN-Cu^{2+} complexes were then analyzed by injection into the HPLC system.

The second approach of complex formation was also studied during the development of the method, where the metal ion solutions were mixed with mobile phase (aqueous phase, *i.e*., 0.1 % formic acid), and noisy chromatograms with multiple small peaks were obtained. Furthermore, the baseline noise was high and the acceptable system suitability parameters were not achieved.

The pH of the solution had significant influence on complexation process. Another solution which offered acceptable separation of analytes was ammonium formate buffer at pH 3.5. However, in this case, additional peaks were observed. Moreover, broad peaks of both complexes were seen, which might be due to the interference of ammonium ion with the drugs, resulting in adverse effects on the stability of the complexes. Hence, 0.1% formic acid was found to be most suitable to be used as the aqueous phase.

CONCLUSION

The chelating property of deferoxamine and *D*-penicillamine has been examined, and a suitable chromatographic technique has been developed for quantification of these drugs in their complexed forms with Fe^{2+} and Cu^{2+}, respectively. The key improvement associated with the present method is that it provides fast and accurate analysis of D-PEN and DFX in bulk drugs as well as in capsule and injection formulations. The proposed method also offers a fast and easy complexation process that can be utilized for routine quality control analysis of these agents.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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