Dissolution-Controlled Salt of Pramipexole for Parenteral Administration: In Vitro Assessment and Mathematical Modeling

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ABSTRACT
Parenteral suspensions of poorly water-soluble salts for intramuscular administration retain therapeutic drug concentration over a long duration. In vitro drug dissolution testing is a prerequisite to assess batch-to-batch variability as well as to assure appropriate drug release during formulation development. The purpose of the present work was to compare the drug release kinetics of two salts of pramipexole in powdered and suspension forms. The two salts employed for the dissolution study were commercially available soluble salt-pramipexole dihydrochloride monohydrate (PRP HCl) and in-house synthesized poorly water-soluble salt, pramipexole pamoic acid salt (PRP PAM). Modified USP apparatus 2 (paddle) using dialysis sac and open loop USP apparatus 4 (flow-through cell) were used for the in vitro dissolution studies. The drug release was estimated using high-performance liquid chromatography. The release kinetics were statistically analyzed using various mathematical models. Results obtained from in vitro dissolution testing showed an immediate-release profile for PRP HCl and a sustained-release profile for PRP PAM, indicating its long-acting potential. The developed method could discriminate between different particle sizes of the PRP PAM salt. The results indicated that the release profile of the PRP PAM salt using modified USP apparatus 2 with dialysis sac more closely mimicked the desired in vivo conditions of intramuscular administration as compared to open loop USP apparatus 4. The developed dissolution method can be used as a quality control tool for PRP PAM injectable suspension.

KEYWORDS: Dissolution, dialysis sac, paddle apparatus, flow-through cell apparatus, kinetic modeling, long acting parenteral suspension, USP apparatus 2, USP apparatus 4, Pramipexole

INTRODUCTION
Long-acting parenteral suspensions have attained considerable attention of scientific community in the last decade. Long-acting suspensions improve patient compliance by reducing dosing frequency and maintain plasma drug concentration for a prolonged period of time. They release the drug in the localized target area, thereby minimizing the drug exposure to non-target sites and decreasing chances of toxicity (1).

Poorly water-soluble salt formation is one of the simplest approaches for the development of parenteral depot formulations. This approach permits modifications in the physicochemical properties of the drug. It helps in development of dosage forms with improved stability and bioperformance and facilitates a different route of administration (2).Generally, poorly water-soluble salts are prepared to reduce the drug dissolution (release) rate. As a consequence, drug release can be attained over an extended period of time (3). These formulations pose a greater risk to patients due to complex release behaviour and less chance of reversal therapy; i.e., once administered parenterally, they are difficult to remove completely in case of an adverse event (4, 5). Hence, appropriate drug release estimations of poorly water-soluble salts are a prerequisite for the assessment of their biocompatibility, safety, and efficacy (6). Concerning this, an in vitro dissolution study is considered as an important tool to assure product performance.

In vitro dissolution profiling is a predictor of drug’s in vivo behaviour and a hint for establishing correlation with its bioavailability. Moreover, drug release data can help in assessing risks related to drug-drug interactions in the body, dose dumping, or unanticipated drug release (7, 8). The relationship between an in vitro property and a relevant in vivo response can be predicted by the mathematical tool known as in vitro-in vivo...
correlation (IVIVC) (9). Through the establishment of a biowaiver, IVIVC can help to reduce regulatory burden of bioequivalence studies, greatly saving time and expenses (9).

Apart from that, the in vitro dissolution study assists in product quality control by evaluation of batch-to-batch consistency at the early stages of the product development and facilitates regulatory approvals by assessment of scale-up and post-approval changes (SUPAC) (10, 11).

Despite the considerable importance of in vitro drug release testing, devising a universal in vitro method for non-oral dosage forms is quite challenging. The reasons include differences in the formulation designs, variation associated with the route of administration, diverse physicochemical properties, and complexity of the in vivo environment (12). Hence, there is a lack of a standardized, compendial in vitro method to simulate in vivo conditions for poorly water-soluble salts. Various regulatory bodies are highly interested in developing dissolution methods for parenteral suspension formulations (10).

Pramipexole (PRP) is a non-ergot dopamine agonist used for the treatment of Parkinson’s disease. It helps in the management of motor symptoms in earlier and advanced stage of Parkinson’s disease. PRP is a Biopharmaceutics Classification System (BCS) class-I drug, having a highly basic nature (13, 14). Pamoic acid is one of the pharmaceutically acceptable excipients for the preparation of poorly water-soluble salts with drugs of basic nature (15). Pramipexole pamoic acid salt (PRP PAM) – a dissolution-controlled poorly water-soluble salt of pramipexole with pamoic acid, was synthesized in-house. It was prepared by solvent-antisolvent precipitation method and was crystalline in nature.

As per the current literature, various compendial apparatuses have been used for in vitro dissolution profiling of such formulations, including the paddle (USP apparatus 2), flow-through cell (USP apparatus 4), and reciprocating disc (USP apparatus 7) (16). The noncompendial methods used for the same purpose include rotating dialysis cell, rotating basket/bottle, reverse dialysis bag, shake flask, inverted cup, single drop, and dialysis tube (17).

The present study aims to compare release kinetics of two salts of the drug pramipexole, including commercially available water-soluble salt, pramipexole dihydrochloride monohydrate (PRP HCl) and PRP PAM (in-house synthesized, poorly water-soluble salt) in powdered and suspension forms. A detailed in vitro dissolution profiling was executed using USP apparatuses 2 and 4 to evaluate product performance and assess prolonged release properties of PRP PAM. Considering the poor perfusion of intramuscular route of administration, USP apparatus 2 was modified using dialysis sac method to get a better biosimulation. The particle size of both the salts was measured to get insight into the relationship between physical attributes and release kinetics. Further, the dissolution profiles of both salts in powdered and suspension forms were compared by employing model-dependent (curve fitting) and statistical analytical methods.

**MATERIAL AND METHODS**

**Materials**

PRP HCl was procured as a gift sample from Zydus Pharmaceuticals, Ahmedabad, India. Pamoic acid was gifted by Megafine Pharma, Ltd., India. PRP PAM and PAP PAM-2 were synthesized using a solvent-antisolvent precipitation method at our laboratory with PRP and pamoic acid. Potassium dihydrogen phosphate (KH₂PO₄) was purchased from S D Fine-Chem Ltd., Mumbai, India. All reagents used for this work were of analytical grade. Syringe filters were purchased from Millipore Ltd., Bangalore, India.

**Particle Size Analysis**

Particle size distribution of powdered PRP HCl, PRP PAM, and suspension of PRP PAM was determined by laser particle size analyzer, and particle size was measured using laser diffraction (Symantec Helos BF module, Sympatec Inc., Germany).

**Solubility Study**

The saturation solubility of PRP HCl and PRP PAM was determined using shake flask method, where excess of PRP HCl and PRM PAM were introduced separately in a volumetric flask containing 5 mL of dissolution media (0.05 M phosphate buffer, pH 6.8). Then, the saturated solution of PRP HCl and suspension of PRP PAM were kept in incubator shaker at 37 °C for 24 h followed by centrifugation at 12,000 rpm for 15 mins. Subsequently, each saturated solution was filtered through 0.45-μm syringe filter and further diluted with buffer. The solubility of drug and its salts was estimated using high-performance liquid chromatography (HPLC). The solubility study was performed in triplicate, and the mean values were calculated.

**In Vitro Release Studies**

The in vitro dissolution study of PRP HCl and PRP PAM was performed for both powdered and suspension forms.
The dissolution media used for each study was 0.05 M phosphate buffer pH 6.8, kept at 37 °C ± 0.5 °C. Fractions of each samples were withdrawn at predetermined time points and, after filtration through 0.45-µm syringe filter, they were directly injected into the HPLC system. Two dissolution apparatus were used for the study: USP apparatus 2 (paddle) (DS 8000, Electro lab India, Mumbai) and USP apparatus 4 (flow-through cell) (Erweka GmbH, Germany).

First, for dissolution study of powdered form of both salts, 31.5 mg of PRP HCl and its equivalent PRP PAM were introduced in 500 mL of dissolution media maintained under sink condition with apparatus 2. The paddle speed was kept at 50 rpm and the vessel temperature was maintained at 37 °C. Second, the dissolution study was performed with a modified apparatus 2 using dialysis sac method with a paddle rotating at 25 rpm speed. The media volume of 300 mL was used to provide sink conditions. The dialysis membrane (LA 401, Himedia, Mumbai, India) used for release studies had a 12,000-14,000 Da molecular weight cut off. Accurately weighed 31.5 mg of PRP HCl and its equivalent PRP PAM were weighed and dispersed in 2 mL of buffer media in case of powder. Whereas in case of suspension, they were dispersed in 2 mL of vehicle (Sodium CMC, Tween 80, propylene glycol, and mannitol). Each resulting sample was further packed in dialysis sac and tied with paddle (Fig. 1a). Aliquots of 5 mL were withdrawn and replenished by dissolution medium.

The USP apparatus 4 consisted a semi-automated DFZ 720 flow-through cell with heater (Fig. 1b), HKP 720 piston pump, and FRL 724 sample collector (Fig. 1c). The apex of a 22.6-mm tablet cell was packed with ruby bead of 5 mm diameter and glass beads of 1 mm diameter to provide laminar flow of the dissolution medium (Fig. 1b). Accurately weighed 31.5 mg powdered sample or 2 mL suspension was kept in cell with open mode at a flow rate of 2 mL/min at 37 °C ± 0.2 °C. Aliquots of 5 mL were collected at each time point using fraction collector and, after filtration through 0.45-µm syringe filter, samples were injected into the HPLC system.

The studies were carried out in triplicate and the mean was calculated. The results were graphically represented as cumulative release (%) vs time for both powders and suspensions of PRP HCl and PRP PAM.

**Figure 1.** Dissolution apparatuses: a. paddle apparatus (USP-2) with dialysis sac method; b. semi-automated DFZ 720 flow-through cell with heater (USP-4); c. complete flow-through cell system (USP-4).
**HPLC Analysis**

All dissolution samples were analyzed using Agilent 1260 infinity HPLC system (Santa Clara, CA, USA) equipped with UV detector and linked to OpenLAB EZChrom software (version A.01.03, Agilent Technologies). HPLC method of USP was adopted for dissolution sample analysis with a few modifications in a gradient to detect pamoic acid along with pramipexole. The mobile phase A consisted potassium dihydrogen phosphate buffer along with sodium octane sulphonate monohydrate, and the pH was adjusted to 3.0 ± 0.02 using orthophosphoric acid. The mobile phase B was composed of 1:1 ratio of buffer: acetonitrile (ACN). Mobile phase A and B were used in a gradient mode [Time(min)/A:B (v/v); T0 60/40; T15 20/80; T20 20/80; T22 60/40; T27 60/40]. The injection volume was 20 μL. Mobile phases were passed through a Purospher STAR RP-18 endcapped column (250 × 4.6 mm, 5-μm particle size, Merck, Germany) at a flow of 1.5 mL/min. The UV detector was set at 264 nm.

**Kinetic Modeling of Dissolution Profiles**

Different mathematical kinetic models were applied for the statistically analysis of dissolution data. DDSolver software (Microsoft Excel add-in) was utilized for the in vitro release data treatment. The best fit models were determined by comparing coefficient of determination, $R^2$, $R^2$ adjusted, Akaike information criterion (AIC), and model selection criterion (MSC).

**RESULTS AND DISCUSSION**

**Particle Size Analysis**

The particle size of salts and suspension can influence the physical properties of the formulation associated with dissolution; hence, it becomes essential to measure their particle size and control particle size distribution (18). The average diameter values for PRP HCl were $X_{10}$: 0.87 μm, $X_{50}$: 5.17 μm, and $X_{90}$: 24.87 μm. The average diameter values for PRP PAM were $X_{10}$: 0.81 μm, $X_{50}$: 2.26 μm, and $X_{90}$: 6.56 μm, whereas the same for PRP PAM suspension were $X_{10}$: 3.12 μm, $X_{50}$: 11.69 μm, and $X_{90}$: 27.88 μm. The obtained results displayed narrow particle size distribution.

**Solubility Study**

The solubility of PRP HCl and PRP PAM was determined in dissolution media (phosphate buffer pH 6.8). The solubility of PRP HCl and PRP PAM were greater than 500 and 0.48 mg/mL, respectively. The obtained results confirmed that PRP PAM has reduced solubility ~1000 fold compared to PRP HCl (native form).

**In Vitro Release Studies**

The dissolution profiles of PRP HCl and PRP PAM were determined in both powdered and suspension form by using USP apparatuses 2 and 4 for quality control and evaluation of batch-to-batch variation.

**USP Dissolution Apparatus 2**

All Initially, powdered samples of PRP HCl and PRP PAM were introduced into 500 mL of dissolution media under sink condition. The drug was released completely within 15 min for PRP HCl whereas only 30% release was observed within same time for PRP PAM, which released completely after 10 h. The in vitro release profile showed retarded dissolution for PRP PAM compared to PRP HCl. Hence, the current results confirmed the sustained-release potential of PRP PAM salt.

PRP PAM is formulated to be injected intramuscularly, where the volume of blood is less. In this case, sink conditions are different than oral dosage forms, and the drug releases over a longer time period due to formation of depot at the site of injection. Therefore, a modified dissolution method was required to mimic the in vivo conditions (10). So, further dissolution studies were performed using a dialysis sac in USP apparatus 2 with 300 mL of dissolution media and a paddle speed of 25 rpm. The resulting dissolution profile for powders of PRP HCl and PRP PAM dispersed in buffer media were compared. (Fig. 2a). PRP HCl showed complete drug release within 2 h, whereas PRP PAM showed minimal release of only ~12% and reached 65% over the course of 12 h and 90% after 24 h. Thus, the prolonged release observed in the dissolution profile verified that PRP PAM is a dissolution-controlled salt as compared to PRP HCl.

Further, to evaluate discriminative power of the dissolution method, one batch of PRP PAM was synthesized with higher particle size (PRP PAM-2) (average diameters: $X_{10}$: 3.14 μm, $X_{50}$: 17.71 μm, and $X_{90}$: 39.27 μm). The in vitro release profile of PRP PAM was compared with PRP PAM-2. The PRP PAM-2 demonstrated 79% release within 12 h. PRP PAM-2 (having higher particle size) showed higher in vitro release as compared to PRP PAM (having lower particle size) (Fig. 2a). The contrary release behaviour of these salts was due to the large hydrophobic surface area of PRP PAM, which make the salt difficult to wet. This leads to a reduced dissolution rate for salts having smaller particle size (19, 20).

Microsuspension samples were prepared using aqueous vehicle as mentioned previously, where PRP HCl with
higher solubility made a solution instead of suspension, and PRP PAM formed a suspension. Comparative drug release profiles of PRP HCl and PRP PAM suspensions under identical conditions are represented in Figure 2b. Drug was released completely within 1 h in PRP HCl, whereas only 20% release was observed after 1 h in PRP PAM and 80% release was achieved after 24 h. Thus, the dissolution-controlled salt, PRP PAM, showed a sustained-release profile compared to its native form, PRP HCl. Unlike powdered samples of PRP PAM with various particle size, their suspensions showed no significant difference in the release pattern as there was no issue of wettability.

**USP Dissolution Apparatus 4**

Powdered PRP HCl and PRP PAM were introduced into tablet cells separately above the bed of glass beads in an open mode. In open loop system, fresh dissolution media flowed continuously, which provided infinite sink conditions for the samples (21). After 30 min, PRP HCl released almost 100%, whereas only 5.4% was released in PRP PAM. Over 8 h, only 52% release was recorded in PRP PAM, despite higher sink conditions in USP apparatus 4 compared to apparatus 2 (Fig. 3a). Hence, the experiment was discontinued. The slow dissolution of PRP PAM was due to its wettability issues. This was further confirmed by using dissolution media (pH 6.8) with 0.2% Tween 80, and 90% drug release was observed within 2 h in PRP PAM (Fig. 3b).

Suspension samples of both salts were also studied using the same experimental conditions with USP apparatus 4 (Fig. 3c). Here, 100% release was observed within 20 min in PRP HCl, and complete drug release was noted in 8 h for PRP PAM. The problem of wetting was not encountered with the suspensions.

The discriminative capacity of dissolution method using USP apparatus 4 was determined by two different
batches of PRP PAM with varying particle size, as mentioned for the USP-2 apparatus. Similar results were obtained with apparatus 2 and 4. PRP PAM-2 (having higher particle size) showed 65% drug release following 8 h, which was higher than PRP PAM (having lower particle size) (Fig. 3a).

**Kinetic Modeling of Dissolution Profiles**

The mechanism of in vitro drug release was predicted using various kinetic models, including zero-order, Higuchi, first-order, Korsmeyer–Peppas and Hixson–Crowell (22–24). Various parameters like $R^2$, $R^2_{adj}$, AIC, and MSC for the different kinetic models using USP apparatus 2 and 4 are given in Tables 1 and 2, respectively. The results of kinetic modeling were insignificant for the immediate-release salt (PRP HCl).

Based on the values of selected criteria for mathematical modeling, PRP PAM powdered and suspension samples followed different drug release kinetic models, indicating that dissolution is not controlled by single mechanism. For powdered PRP PAM, the best-fit model was first-order kinetics when estimated by USP apparatus 2, whereas with the flow-through cell, the correlation coefficients were very close for first-order and the Korsmeyer–Peppas models. Based on these results, the drug release profile was concentration-dependent and progressed with an increase in wetting of the powder. USP apparatus 4 displayed mixed dissolution profiles for the first-order and Korsmeyer–Peppas models.

In PRP PAM suspension, the drug release for USP apparatus 2 and 4 was best fit to the Korsmeyer–Peppas and first-order models, respectively. Therefore, drug release from suspension was diffusion controlled. Also, on the basis of $n$, calculated from the regression coefficient of Korsmeyer–Peppas model, the drug release obeys quasi-Fickian diffusion ($n < 0.45$), which means partial diffusion. In our case, the drug was first dissolved inside the dialysis bag and subsequently diffused inside the dissolution media (25). Therefore, both dissolution methods using USP apparatus 2 and 4 are useful for quality control purposes. Also, both have shown discriminative dissolution profiles for PRP PAM salts having different particle sizes. The sustained-release potential of PRP PAM was also confirmed by both the methods.

However, the modified USP apparatus 2 with dialysis sac better facilitated drug release for the poorly water-soluble salt system to be administered via the less perfused route.

### Table 1. Modeled Dissolution Characteristics Using USP Apparatus 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Parameter</th>
<th>Kinetic Models</th>
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<tr>
<td></td>
<td></td>
<td>Zero order</td>
<td>First order</td>
<td>Higuchi</td>
<td>Korsmeyer–Peppas</td>
<td>Hixson–Crowell</td>
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<tr>
<td>Powder</td>
<td></td>
<td>$R^2$</td>
<td>-0.0948</td>
<td>0.9900</td>
<td>-0.5910</td>
<td>0.7094</td>
<td>-0.8605</td>
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<tr>
<td>PRP HCl</td>
<td>$R^2_{adj}$</td>
<td>-0.0948</td>
<td>0.9900</td>
<td>-0.5910</td>
<td>0.6731</td>
<td>-0.8605</td>
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<tr>
<td></td>
<td>AIC</td>
<td>106.68</td>
<td>46.57</td>
<td>97.23</td>
<td>82.22</td>
<td>98.79</td>
<td></td>
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<tr>
<td></td>
<td>MSC</td>
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<td>4.40</td>
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<td>0.83</td>
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<tr>
<td>PRP PAM</td>
<td>$R^2$</td>
<td>0.8960</td>
<td>0.9945</td>
<td>0.9268</td>
<td>0.9752</td>
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<td></td>
<td>$R^2_{adj}$</td>
<td>0.8960</td>
<td>0.9945</td>
<td>0.9268</td>
<td>0.9721</td>
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<td></td>
<td>AIC</td>
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<td>39.16</td>
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<td>5.00</td>
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<td>3.29</td>
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<td>Suspension</td>
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<td>$R^2$</td>
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<td>0.9952</td>
<td>-2.3786</td>
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<tr>
<td>PRP HCl</td>
<td>$R^2_{adj}$</td>
<td>-6.0561</td>
<td>0.9952</td>
<td>-2.3786</td>
<td>0.5419</td>
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<td>PRP PAM</td>
<td>$R^2$</td>
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<td>0.8794</td>
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<td>0.9993</td>
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<tr>
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<td>0.9881</td>
<td>0.9946</td>
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<td></td>
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<td>1.89</td>
<td>4.20</td>
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</table>

PRP HCl, pramipexole dihydrochloride monohydrate; PRP PAM, pramipexole pamoic acid salt; $R^2$, coefficient of determination; $R^2_{adj}$, adjusted coefficient of determination; AIC, Akaike information criterion; MSC, model selection criterion.
The dialysis sac mimics in vivo conditions, where particles are immobilized upon intramuscular administration and surrounded by a stagnant layer. This causes slow diffusion of drug because sink conditions are not maintained (26).

In open loop mode using USP apparatus 4, continuous flow of media generated infinite sink conditions, which helped in release of the drug, but the volume of media was too large to imitate intramuscular in vivo conditions. Moreover, the open loop mode with apparatus 4 faced challenges related to wettability. Although it might be useful for poorly water-soluble drugs for mimicking in vivo conditions, apparatus 4 in open loop mode requires further modifications in our case. Hence, a dissolution study by close loop mode with apparatus 4 is desired to provide finite sink conditions and for better correlation of intramuscular drug release of poorly water-soluble salt PRP PAM, which will be explored in future work.

**CONCLUSION**

The present work compared drug release from in-house synthesized PRP PAM salt with its commercially available soluble salt, PRP HCl, in both powdered and suspension forms using modified USP apparatus 2 with a dialysis sac and open-loop USP apparatus 4. In all cases, PRP HCl and PRP PAM demonstrated immediate and prolonged release profile, respectively. PRP PAM showed a discriminative dissolution profile based on its particle size distribution for both apparatuses. In addition, the release kinetics were compared using various mathematical models. Overall, for poorly water-soluble salt of pramipexole, modified USP apparatus 2 with dialysis sac facilitated better drug release for the optimization of a long-acting microsuspension compared to open-loop USP apparatus 4. However, future studies of closed-loop USP apparatus 4 might provide a more appropriate drug release profile. The microsuspension of PRP PAM fit a Korsmeyer–Peppas model for USP type 2 and showed a diffusion-controlled release mechanism. These dissolution methods for delayed release suspensions are useful during formulation optimization and quality control.

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CONFLICT OF INTEREST
The authors disclosed no conflicts of interest related to this article.

REFERENCES