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Article

In vitro dissolution and *in vivo* response of pseudoephedrine dosage forms

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SUMMARY

The pharmacological activity of therapeutic oral drugs depends on the speed of release, location within the gastrointestinal tract, and time needed for absorption into the bloodstream. Moreover, the study of the relationship between *in vitro* dissolution and *in vivo* pharmacological response is critical, and there is an increasing interest in designing relevant *in silico* models. Pseudoephedrine HCl is a nasal decongestant with a side effect of increased heart rate. Our research looked at the relationship between pharmacokinetics of various forms of pseudoephedrine HCl and the pharmacodynamic response on *Daphnia magna*. We hypothesized that drug release kinetics *in vitro* may not correlate directly with the *in vivo* response. Our study was comprised of two-parts: i) comparing dissolutions of immediate-release, 12-hour, 24-hour, and enteric-coated pseudoephedrine formulations in gastric and intestinal environments, and ii) evaluating the effect of released pseudoephedrine HCl on heart rate. Our results revealed that maximum drug release was at 55 minutes for the immediate-release form, ~4 hours for the 12-hour release form, and ~18 hours for the 24-hour release form at the two pHs tested. The enteric-coated capsules released only at pH 7.2. There was a positive correlation between drug release and heart rate, with a 36% increase at 120 minutes. Although the drug dissolution kinetics from the immediate-release tablet reached its maximum at 55 minutes, the maximum *in vivo* response occurred at 120 minutes. Therefore, *in vitro-in vivo* correlation studies could help in the development of comprehensive *in silico* models capable of predicting pharmacological parameters for novel drugs in the future.

INTRODUCTION

Therapeutic drugs are available in all types of dosage forms. They can be injected intravenously, taken orally, or applied topically. Drugs taken orally are delivered in various forms, such as tablets, capsules, or liquids (1). Absorption of drugs from tablets and capsules, which are usually referred to as drug products, depends on how quickly the drug substance is released from the drug product and its location within the gastrointestinal system at the time of release (2). It also depends on the physiological condition of the release and how quickly the drug gets absorbed into the bloodstream (3). Therefore, the study of the relationship between *in vitro* dissolution and *in vivo* pharmacological response is critical. The branch in pharmacology which studies the movement of any drug through the body of an organism is called pharmacokinetics (4). The branch of pharmacology that studies the physicochemical effects of any drug in the body of an organism and its mechanism of action is called pharmacodynamics (5).

Pseudoephedrine HCl is a common nasal decongestant for colds and allergies used by millions of people worldwide (**Figure 1A**) (6-9). The mechanism of action of pseudoephedrine is to shrink blood vessels present within the nasal passage, which drains the mucus and fluids, and makes it easier to breathe (10). However, this effect also occurs throughout the rest of the body, causing side effects, such as increased heart rate (11). This is especially dangerous in people with certain medical conditions such as diabetes, hypertension, and other metabolic and endocrine disorders (12).

Daphnia magna is a microcrustacean and a versatile organism due to its short life cycle (**Figure 1B**) (13). It is used as a model system for pharmacology and toxicology studies because it is highly sensitive to environmental changes (14). *D. magna* has a myogenic heart that responds to therapeutic drugs in a very similar manner to the human heart. Therefore, in *D. magna,* pseudoephedrine HCl is expected to act as a pro-arrhythmic agent and show a quick pharmacodynamic effect by increasing the rhythmic function of its myogenic heart (15).

In a one-compartment model, where the body is assumed to be a single and uniform compartment, there would be a perfect overlap between the release kinetics from an immediate-release tablet and the increase in heart rate of *D. magna.* However, if the distribution of pseudoephedrine within *D. magna* followed a two-, three-, or multi-compartment model, where different organs within the body absorb, distribute and eliminate drugs at different rates, we would expect a difference in the release kinetics from the tablets and the kinetics of heart rate increase in our model organism (16). Drugs that follow a two-, three-, or multi-compartment distribution and elimination model usually move from a central compartment into peripheral compartments where they show their pharmacodynamic activity, and/or get eliminated. In these cases, the drug's effect will depend on the local concentration at the site of action and are more complicated to model.

The FDA Modernization Act 2.0, signed in 2022, allows the US Food and Drug Administration to approve new drugs for human clinical testing even in the absence of safety testing in animals, so long as the results from *in vitro*, *in silico*, or *in chemico* tests are convincing. In addition, non-

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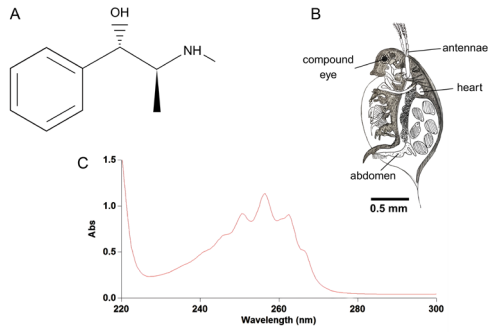

Figure 1. Background Research. A) Pseudoephedrine is a member of the class of phenylethanolamines with a molecular formula of $C_{10}H_{15}NO$ and a CAS Registry Number of 90-82-4. **B)** A pictographic representation of a female *Daphnia magna,* a small planktonic crustacean belonging to the subclass Phyllopoda. Adults usually grow to a length of 1.5-5.0 mm (27). **C)** A scan was performed between 220-300 nm using a spectrophotometer to determine the absorption maximum (Abs) of pseudoephedrine. The absorption maximum was found to be ~257 nm.

human *in vivo* tests, human cell-based assays, organ-on-chips, bioprinted organs as well as computer models are now allowed (17). However, we hypothesized that tests conducted *in vitro* or *in silico* may not translate directly *in vivo*, unless all *in vivo* parameters have been evaluated and understood. Therefore, we set out to understand what variables need to be accounted for to accurately develop a computer model of a pharmacokinetic and pharmacodynamic study of any drug *in vivo*.

We performed an *in vitro-in vivo* correlation (IVIVC) evaluation using *D. magna* and pseudoephedrine HCl to see if additional parameters other than simple drug release from tablets are involved in modulating a pharmacodynamic response to pseudoephedrine HCl. In Part I, we compared the dissolution kinetics of three different commercially available formulations of pseudoephedrine; immediate-release, 12-hour extended-release, and 24-hour extended-release pseudoephedrine formulations in solutions that mimic the gastric environment (low pH) and the intestinal environment (neutral pH) (18). In addition, we produced a novel formulation of pseudoephedrine HCl in the form of enteric-coated capsules and tested its pharmacokinetic profile in the above two gastrointestinal environments. Enteric-coated capsules are used in the oral delivery of drugs that rapidly degrade in the stomach (19). In Part II of the study, we generated the pharmacodynamic profile of drug released from immediate-release tablets at various time points by testing its effect on the heart rate of *D. magna* and correlating it with the drug release kinetics. Our study deciphered the relationship between the pharmacokinetics — the release of various forms of a model drug (pseudoephedrine HCl) — and the *in vivo* pharmacodynamic response in the model organism *D. magna*.

Our results showed that the maximum drug release timepoint to be 55 min for immediate-release formulation, between ~4 hours for 12-hour release formulation depending on the environmental pH condition, 18 hours for the 24-hour release formulation, and 35 min in the intestinal environment for the enteric coated capsules. In addition, we showed that there was a difference between the time to maximum drug release and the time for the maximum increase in heart rate in the model organism tested. As a result, we concluded that such *in vitro-in vivo* correlation studies are important and relevant because they give us the much-needed insight into building effective *in vitro* or *in silico* models in order to move away from drug testing in animals prior to initiating human clinical studies.

RESULTS

Dissolution kinetics of pseudoephedrine tablets

We compared the drug release kinetics of each of four pseudoephedrine HCl tablets (immediate-release, 12-hour extended-release, 24-hour extended-release, and enteric-coated) to see if there was a difference in release time in solutions of pH 1.5, to model the stomach environment, and neutral pH of 7.2, to model the small intestinal environment. After placing the relevant pseudoephedrine tablet in one of the buffer solutions, we recorded the absorbance (optical density, OD) at different timepoints at 257 nm. An increase in absorbance would indicate that the drug is being released from the tablet into the buffer solution.

The release of pseudoephedrine HCl from the immediate-release tablets was similar at both pH 1.5 and 7.2, indicating that the amount of drug released in both stomach and intestinal environments may be similar (**Figure 2**). Release saturation was reached around 55 minutes, and no additional drug was released until 105 minutes. The release of pseudoephedrine HCl from the 12-hour extended-release tablets was significantly lower at pH 7.2, compared to pH 1.5 ($p < 0.01$ at all timepoints except 5, 95, 215 and 305 minutes) (**Figure 3**). Release saturation was reached around 245 minutes (approximately 4 hours) at an environmental pH of 1.5 and around 305 minutes (approximately 5 hours) at an environmental pH of 7.2. The release of pseudoephedrine HCl from the 24-hour extended-release tablets was similar at both pH 1.5 and pH 7.2 (**Figure 4**). Release saturation was reached around 18 hours at both environmental pH 1.5 and environmental pH 7.2, with no statistically significant difference at any time point ($p > 0.05$). Lastly, we looked at the release of drug from enteric-coated capsules, where the enteric coating prevents the drug from being released in the acidic conditions of the stomach before reaching the intestine. Maximum drug release from these enteric coated capsules occurred within 15 minutes at pH 7.2 and saturated at approximately 35 minutes (**Figure 5**). There was no drug released in the pH 1.5 buffer, illustrating the integrity of the enteric-coated capsules. The drug release kinetics between pH 1.5 and 7.2 at all time points after 5 minutes were statistically different ($p < 0.0001$).

Effect of pseudoephedrine HCl released from immediate-release tablets on the heart rate of *D. magna*

To determine if there is a positive correlation between the amount of drug released from pseudoephedrine HCl into the environment and an increase in the heart rate of *D. magna*, we added immediate-release pseudoephedrine samples to *D. magna* in a timeline order and recorded the change in heart rate over time. Treatment with caffeine and ethanol, two known arrhythmia-causing substances were used as positive controls to validate our experimental procedure (20). The caffeine treatment at 0.3 mg/ml resulted in a negligible increase in heart rate compared to no treatment control (**Figure 7A**).

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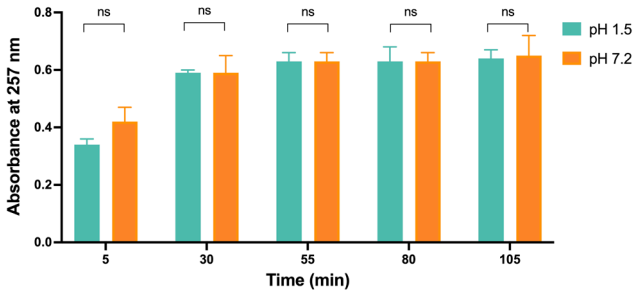

Figure 2. Release of pseudoephedrine HCl from immediate-release tablets. Pseudoephedrine HCl release for each timepoint tested with the immediate-release tablets. Release saturation was reached around 55 minutes at both pH conditions. We performed statistical analysis using a multiple unpaired t-test for drug release between pH 1.5 and 7.2 using GraphPad Prism. No statistically significant difference (ns) was found at any of the time points. Data shown as mean ± standard deviation (n=9).

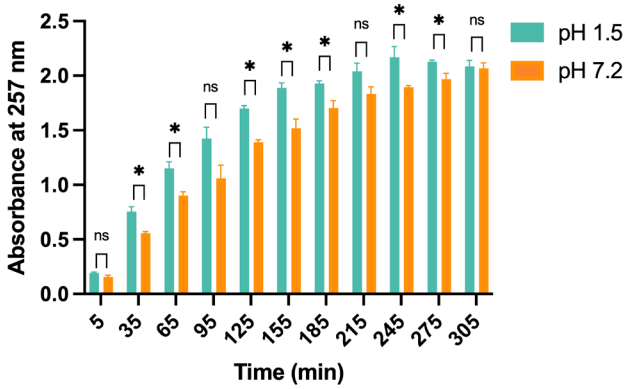

Figure 3. Release of pseudoephedrine HCl from 12-hour extended-release tablet. Pseudoephedrine HCl release for each timepoint tested with the 12-hour extended-release tablets. Release saturation was reached around 245 minutes at pH 1.5 and around 305 minutes at pH 7.2. We performed statistical analysis using a multiple unpaired t-test for drug release between pH 1.5 and 7.2 using GraphPad Prism. At 35, 65, 125, 155, 185, 245, and 275 minutes, the drug release kinetics at pH 1.5 had a statistically significant difference, *p < 0.01. Data shown as mean ± standard deviation (n=9).

Ethanol treatment at a concentration of 1% slowed heart rate by 19% compared to the no treatment control. When caffeine treatment was followed by ethanol treatment, there was an increase in heart rate of 36%.

We observed a positive correlation between the rate of drug release from the tablet and increase in the heart rate of *D. magna*. However, although the drug release from the immediate-release tablet reached its maximum at 55 minutes, the *in vivo* response continued increasing until 120 minutes (**Figure 8**). At 120 minutes, there was a 36.3% increase in the heart rate relative to time T0.

DISCUSSION

The results from the *in vitro* part of the experiment revealed differences in release kinetics between immediate and extended-release tablets, with the immediate form releasing faster than extended-release forms, as expected. However, varying the pH to mimic either stomach or intestinal environments did not largely affect the release rates within each tablet form. Immediate and extended-release tablets showed similar release kinetics in the stomach and intestine environmental conditions, indicating that pseudoephedrine HCl can be released in both stomach and intestinal environments equally. There was a direct correlation between the amount of pseudoephedrine HCl in the dose formulations (30 mg vs. 120 mg vs. 240 mg) and the absorbance at 257 nm at different time points (**Figures 2-4**). Immediate-release formulation had the least absorbance, reaching a maximum OD of 0.6, 12-hour extended-release reached a maximum OD of ~ 2.2, and 24-hour extended-release had the highest absorbance, reaching a maximum OD of ~ 4.3. Surprisingly, maximum release for both extended-release formulations was reached at ~4 hours and 18 hours, long before the expected time of 12 or 24 hours. Caffeine and ethanol, the two positive controls used in the *in vivo* part of our study, had an effect on the heart rate of *D. magna* that was consistent with literature and confirmed that our experimental setup was appropriate. Similarly, when caffeine treatment was followed by ethanol treatment, the 36% increase in heart rate was similar to a previous report, where rats fed with alcohol and caffeine induced spontaneous ventricular tachyarrhythmias in a synergistic way, potentially due to increased Ca^{2+} sparks (21). Treatment with the

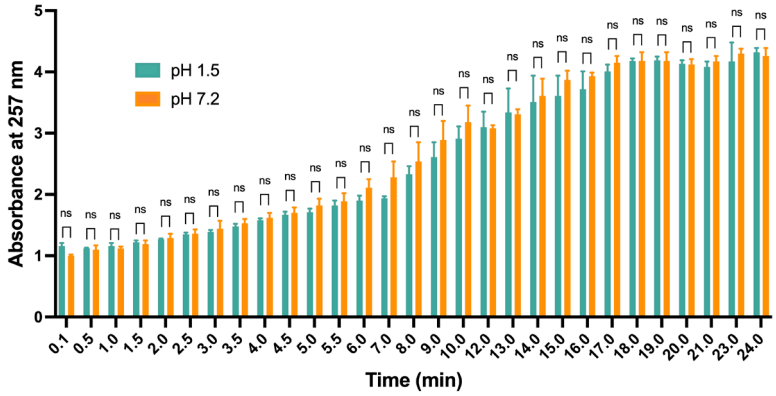

Figure 4. Release of pseudoephedrine HCl from 24-hour extended-release tablet. Pseudoephedrine HCl release for each timepoint tested with the 24-hour extended-release tablets. Release saturation was reached around hour 18 at both pH conditions. We performed Statistical analysis using a multiple unpaired t-test for drug release between pH 1.5 and 7.2 using GraphPad Prism. No statistically significant difference (ns) was found at any of the time points. Data shown as mean ± standard deviation (n=9).

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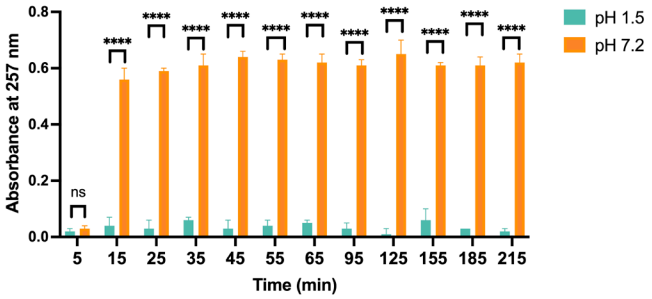

Figure 5. Release of pseudoephedrine HCl from enteric-coated capsules. Pseudoephedrine HCl release for each timepoint tested with the enteric-coated tablets. Release saturation was reached around 35 minutes at pH 7.2. Statistical analysis was performed using multiple unpaired t-tests for drug release between pH 1.5 and 7.2 using GraphPad Prism. No statistically significant difference (ns) was found at 5 minutes. After 5 minutes, the difference in drug release between pH 1.5 and 7.2 was statistically significant (****p < 0.0001) at all time points. Data shown as mean ± standard deviation (n=9).

immediate-release formulation of pseudoephedrine HCl sampled at various timepoints displayed a positive correlation between the amount of drug product released and the heart rate of *D. magna*. There was a 36.3% increase in heart rate over time. The positive correlation between the rate of pseudoephedrine HCl release and increase of heart rate is as expected for this drug because it is adrenergic and increases the irritability of heart muscle to cause the observed arrythmia (22). However, there was also a time difference between the *in vitro* immediate-release tablet release maximum, which was reached by 55 minutes, and the *in vivo* heart rate maximum, which continued climbing even at 120 minutes.

We hypothesized that if there were no additional parameters involved, there would be perfect overlap between the release kinetics from the immediate-release tablet and the increase in heart rate of *D. magna*. However, this was not the case, indicating that additional parameters were involved in the pharmacodynamic response. Since *D. magna* is a biological organism, these parameters likely include the time taken for pseudoephedrine HCl to be absorbed into the bloodstream, travel to the animal's heart, and enter the heart cells before it can affect its heart rate.

We also observed a high degree of variability of heart beats per minute within each time point. Literature shows that there are sex-dependent ageing rates in *D. magna* (23). There are significant differences between male and female *Daphnia* in several physiological markers including lifespan, growth rate, heart rate, and swimming speed. Additionally, the authors found that the heart rates of females decrease by 1.6-fold in 100-days old *Daphnia* compared to 10-days old *Daphnia.* As they age, the beats per minute of male *Daphnia* decline 4.04-fold faster compared to female *Daphnia* (23). Although similarly sized *D. magna* were picked from the culture for our study, we were unable to exactly match for age. In addition, we did not distinguish organisms based on their sex for any of our experiments. It is well known that the anti-stress machinery in female *Daphnia* differ greatly from males (24). Therefore, another factor that could contribute to higher heart rate in some animals compared to others at each time point could be environmental stress, namely placing the *D. magna* on a cavity slide rather than in its previously acclimatized environment within the culture bottle.

In conclusion, our study successfully demonstrated that *in vitro* drug dissolution studies do not always accurately predict *in vivo* response in organisms unless they account for these additional parameters. Model-organism-specific *in vitro-in vivo* correlation studies need to be conducted to aid the development of comprehensive *in silico* models capable of predicting pharmacological parameters for novel drugs in the future.

MATERIALS AND METHODS

Absorption spectra of pseudoephedrine HCl

Before the start of the study, an initial scan was performed on a spectrophotometer to evaluate the absorption spectra of pseudoephedrine HCl. To control for background absorbance of the solvent, a scan was performed with 1 mL DPBS pH 7.2 (Cytiva, Catalog number SH30028.02) and used for background subtraction. Next, one immediate-release tablet (30 mg, Johnson & Johnson Consumer Inc., Lot Number: BSC107) was crushed using a mortar and pestle and resuspended in 8 mL of DPBS pH 7.2. 1 mL of this solution was transferred to a micro quartz cuvette, and a UV-visible Spectrophotometer (Varian, Cary) was used to perform an initial scan of the absorption spectra between 250-300 nm range. The maximum absorbance was found to be at 257 nm, consistent with the literature (**Figure 1C**) (8).

To reduce the amount of sample needed for further experiments, the absorbance at 257 nm was recorded using a Nanodrop 2000 (Thermo Fisher Scientific). Briefly, 2 µl of the solution from a microcentrifuge tube was pipetted onto the lower pedestal of the Nanodrop. The arm was lowered, and absorbance was recorded at 257 nm.

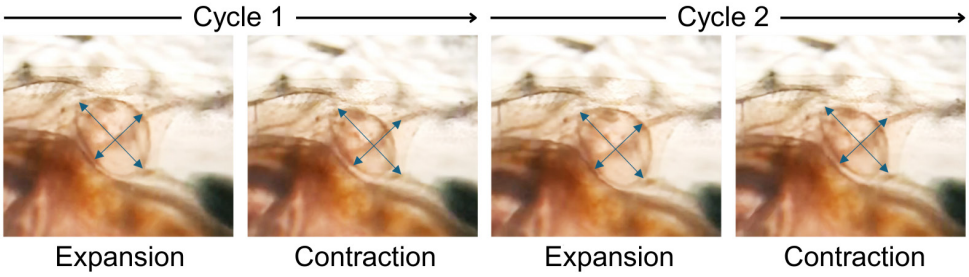

Figure 6. Images of *D. magna*'s cardiac cycle. Real time images of two successive cardiac cycles in *D. magna*, with arrows indicating expansion and contraction of the heart. We considered one expansion and one contraction to be one cycle of a heartbeat.

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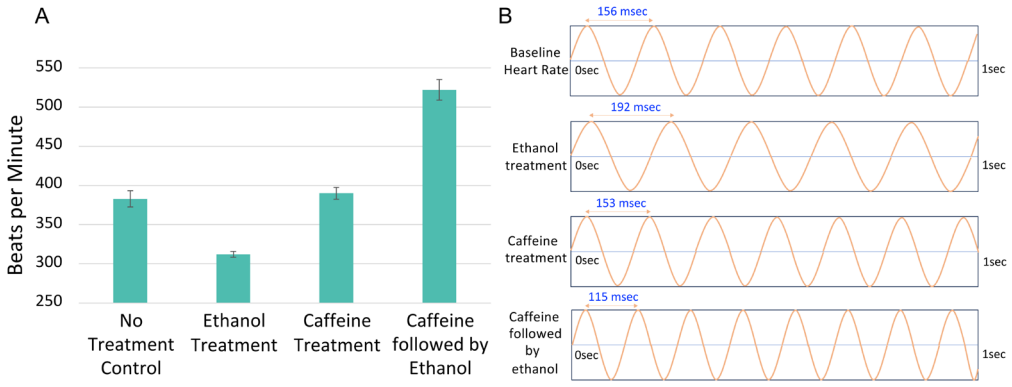

Figure 7. Heart rate of *D. magna* in response to controls. A) Heart rate of *D. magna* with various treatment controls in heartbeats per minute. A higher heart rate represents tachycardia while a lower heart rate represent bradycardia. Data shown as mean ± standard deviation (n=3). **B)** Sinusoidal graph displaying cardiac rhythm over one second for treatment controls. Number of milliseconds between two peaks and two troughs were calculated and displayed in dark blue. Tachycardia is accompanied by a decrease in time between two peaks, and bradycardia is accompanied by an increase in time between two peaks compared to the baseline heart rate.

Tablet dissolution testing

The tablets used in this study were pseudoephedrine HCl immediate-release tablets (Johnson & Johnson Consumer Inc., Lot Number: BSC107), pseudoephedrine HCl 12-hour release tablets (Johnson & Johnson Consumer Inc., Lot Number: 2LE1805), and pseudoephedrine HCl 24-hour release tablets (Johnson & Johnson Consumer Inc., Lot Number: 2005502). In addition, enteric-coated capsules were prepared according to the methods described below.

The four different forms of pseudoephedrine HCl tablets were placed in 125 ml clear PET plastic media bottles with caps. 50 mL of DPBS buffer pH 7.2 (Cytiva, Cat No: SH30028.02), or Glycine pH 1.5 (Cytiva, Cat No: BR100354) were added to the tablets and the bottles were placed on a shaker inside the incubator with shaking at 200 rpm at 37°C. At various time points, 20 μL of solution from each bottle was transferred into 6 different microcentrifuge tubes. The tubes were centrifuged for 5 minutes at 1,000 rpm to clear the cloudy liquid. The supernatants were transferred into fresh labeled tubes. 2 μL of the solution was pipetted onto the lower pedestal of the nanodrop, and the absorbance was recorded at 257 nm. The remaining solution in the tubes was stored at -20°C for *in vivo* testing.

The timepoints for the immediate-release tablets were every 25 minutes from 5 minutes to 105 minutes for Part I of the study and 0, 2.5, 5, 15, 30, 45, 60, 90, 120 minutes for Part II of the study. The time points for the 12-hour release capsules were every 30 minutes from 5 minutes to 305 minutes. The time points for the 24-hour release capsules were every 30 minutes for the first 6 hours, followed by every hour for the remainder of the experiment. The time points for the enteric-coated capsule experiment were every 10 minutes from 5 minutes until 65 minutes, followed by every 30 minutes until 215 minutes. All the experiments were repeated in triplicate.

Preparation of enteric-coated capsules

Immediate-release tablets were crushed to a powder using a mortar and pestle. Powder from one immediate-release tablet was packed into one empty enteric-coated capsule (Eudracap, Batch # F210359002).

D. magna care and handling

A healthy *D. magna* culture was purchased from Carolina Biological Supply Company, (Item Number: 142330) (25). The lid of the culture jar was unscrewed to allow for air exchange, and the jar was stored in a cool area (21-23°C) out of direct

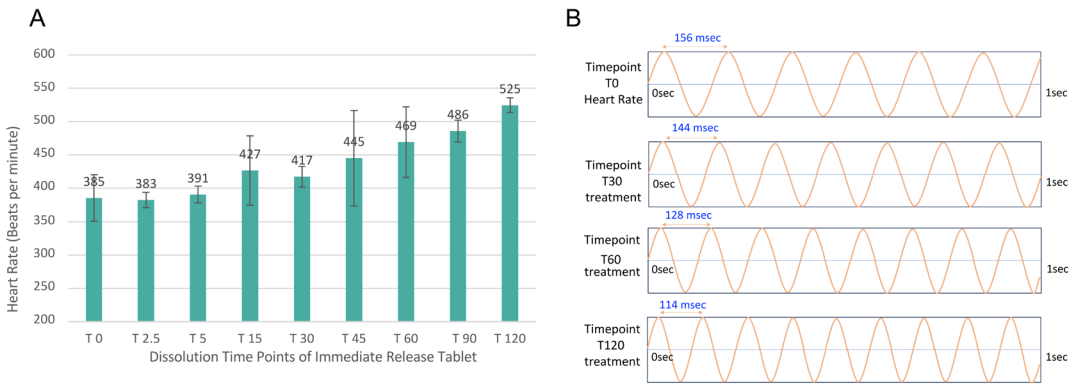

Figure 8. Heart Rate of *D. magna* in response to pseudoephedrine HCl treatment. A) *In vivo* pharmacodynamic effect of immediate-release pseudoephedrine tablets on the heart rate of *D. magna* at different timepoints. A higher heart rate represents tachycardia. Data shown as mean ± standard deviations (n=9). **B)** Sinusoidal graph displaying cardiac rhythm over one second for pseudoephedrine treatment. Number of milliseconds between two peaks and two troughs were calculated and displayed in dark blue.

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sunlight for two days to acclimatize before experimentation. Per the supplier's recommendation, since the *D. magna* was used within 3-4 days of receipt, no further feeding was needed.

Visualization and calculation of heart rate in *D. magna*

A compound microscope was set up and focused on an empty concave slide. A Pasteur pipette was used to pick up one *D. magna* and place it on the slide. A small piece of cotton wool was placed next to the *D. magna* to prevent it from moving (26). Enough DPBS buffer at pH 7.2 was added to cover the *D. magna*, and heart rate was measured by recording a video of the heart for 10 seconds. The video was imported into iMovie and slowed down by 20X. The number of expansion-contraction cycles was counted manually and multiplied by six to calculate the beats per minute. This was done three times per animal for three animals in total.

Caffeine and ethanol control experiments

A 0.3 mg/ml caffeine stock solution and a 1% ethanol solution were prepared in water separately. Enough caffeine or ethanol solution was pipetted to cover a new *D. magna*. The *D. magna's* heart rate was recorded for 10 seconds and counted afterwards. In addition, we treated *D. magna* first with caffeine for 5 minutes followed by the removal of caffeine solution and addition of ethanol solution for 5 minutes prior to recording its heart rate for 10 seconds. A total of three trials were performed per treatment.

Testing effect of immediate-release tablets on *D. magna* heart rate

The frozen immediate-release samples from the *in vitro* experiment (Timepoints in minutes: 0, 2.5, 5, 15, 30, 45, 60, 90, 120) were used for this procedure. In order to mimic the exposure of pseudoephedrine HCl in humans over 120 minutes, a single *D. magna* was used for the entirety of the timepoints. All of the samples were allowed to thaw to room temperature completely before adding to the *D. magna* sequentially. The timepoints were applied in chronological order, starting with Timepoint 0. The following steps were then completed: a) enough solution from the chosen timepoint was pipetted to cover a new *D. magna* and b) a video of the *D. magna*'s heart rate was recorded for 10 seconds to count heart rate afterwards. Steps a) and b) were repeated thrice for each timepoint. This process was then done again with a new organism, resulting in a total of three organisms.

Data analysis

Absorbance was plotted at each time point for Part I and heart rate was plotted at each time point for Part II. Statistical analysis on the difference in drug release kinetics between the two pHs was performed on GraphPad Prism 10 for macOS using multiple unpaired t-tests. For the *in vivo* part of the experiment, data was recorded in a table, and a graph was created in Microsoft Excel to display the average change and standard deviation of the nine values for any given variable. Averages and standard deviations were calculated in Microsoft Excel.

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