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Serum Buprenorphine Concentrations and Behavioral Activity in Mice After a Single Subcutaneous Injection of Simbadol, Buprenorphine SR-LAB, or Standard Buprenorphine

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Buprenorphine, an analgesic commonly used in rodent surgery, requires repeated dosing every 4 to 6 h in order to provide adequate analgesia. However, redosing requires repeated handling, which may itself cause stress. Buprenorphine SR-LAB, which reportedly maintains serum levels of buprenorphine greater than 1 ng/mL for 48 to 72 h, is commercially available. However, the viscosity of the product and small dosing volumes make accurate dosing a challenge. Simbadol is a concentrated formulation of buprenorphine hydrochloride labeled for use in cats with recommended dosing frequency of every 24 h. We measured serum concentrations over time after a single injection of this product in C57BL/6NCrl mice and compared it to standard buprenorphine (Buprenex) and Buprenorphine SR-LAB. Male and female mice were injected subcutaneously with one of the 3 buprenorphine formulations at a dose of 1 mg/kg at time 0. Groups of mice (n = 8) were euthanized at 1, 4, 8, 12, 16 h for all groups and 24 h for the Simbadol and the Buprenorphine SR-LAB. Liquid chromatography-mass spectrometry (LC-MS/MS) was used to determine concentrations of buprenorphine in each serum sample. High concentrations were observed in both Simbadol and standard buprenorphine groups one hour after injection (>50 ng/mL). These groups had similar buprenorphine concentration curves, including rates of decline. The standard buprenorphine group had mean concentrations less than 1 ng/mL by 12 h and the Simbadol group by 16 h. In contrast, the Buprenorphine SR-LAB group remained above the 1 ng/mL therapeutic threshold throughout the 24 h. In addition, clinical signs, including increased activity, that lasted for up to an hour after the injection in the Simbadol and standard buprenorphine groups. We conclude that Simbadol does not offer dosing advantages over the standard buprenorphine formulation when given at 1 mg/kg. Buprenorphine SR-LAB maintained a steady concentration of buprenorphine above 1ng/mL for at least 24 h, and as such is a superior choice for providing long-term analgesia.

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Buprenorphine hydrochloride (HCl) is a semisynthetic, partial *mu*-opioid agonist and partial k-opioid antagonist that has been shown to be an effective analgesic in laboratory animals.[5,7,11,21,22] The duration and efficacy of buprenorphine dependent on dose, route of administration, and species, and recommended dosages and dosing intervals vary substantially in the literature.[5,6,10,11,13,18] A serum concentration of 0.5 ng/mL – 1 ng/mL has previously been reported to be the analgesic threshold of buprenorphine.[15,26] For mice, doses used have ranged from 0.05 to 4 mg/kg,[6,10,11,13,16,18,21,27] depending on the procedure and the strain, with dosing intervals every 4 to 6 h. Although dosing intervals vary by the dose given, repeated injections of buprenorphine HCl are required to provide the continuous analgesic coverage necessary for animals undergoing a major survival surgery. The handling required for repeated injections may be stressful and is known to affect physiologic and behavioral parameters.[29]

Providing adequate relief of postsurgical pain while minimizing the stress of repeat injections has long been a concern to researchers and veterinary staff.[3,12,28] To meet this need, sustained-release formulations of buprenorphine are commercially available for laboratory rodent use; these reduce the number of injections required for providing continual analgesia.[4,6,12,17-20] Currently, the most widely used sustained-release product is Buprenorphine SR-LAB (Zoopharm, Windsor, CO), which can only be obtained by prescription (requiring a licensed veterinarian with a practitioner DEA license). This requirement adds to the complexity of obtaining, securing, and dispensing the product for an institution and for the veterinarian. Moreover, Buprenorphine SR-LAB is viscous, concentrated, and cannot be diluted, making accurate dosing difficult.

A potential alternative that could deliver appropriate buprenorphine dosing to rodents (particularly mice) while mitigating known viscosity issues is Simbadol. Simbadol is a commercially available high-concentration formulation of buprenorphine labeled for use in cats. It is readily available

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from veterinary distributors and can be purchased with a DEA researcher license. It is supplied at a concentration of 1.8 mg/mL of buprenorphine hydrochloride. Although highly concentrated, the solution is not viscous. A recent publication in cats compared plasma concentrations and analgesia effect after administration by various routes (IV, buccal, subcutaneous).[9] This study found that Simbadol administered subcutaneously provided sustained plasma concentrations and prolonged analgesia (≥24 h).

Other authors have evaluated the effectiveness of Simbadol in other species.[1,24] Although serum concentrations were not measured, rats dosed with either Simbadol or buprenorphine displayed analgesic effects for only one hour, and self-injurious behavior was seen in all treatment groups when dosed with 0.3 mg/kg.[1] More recently, another group measured plasma concentrations of Simbadol in Rhesus macaques.[24] They found that the mean concentrations of buprenorphine exceeded 0.1 ng/mL for 48 h after a dose of 0.24 mg/kg subcutaneously and for 72 h after a dose of 0.72 mg/kg subcutaneously.

Our goal in this study was to determine whether Simbadol would maintain serum concentrations of buprenorphine at 1 ng/mL over a 24 h period in mice. If therapeutic concentrations of buprenorphine could be sustained in the blood with a single dose of Simbadol, it could be an alternative to Buprenorphine SR-LAB, avoiding the issues of viscosity, accuracy of the administered dose due to small dosing volume, and ease of procuring and dispensing the drug. Therefore, we measured and compared the serum levels of buprenorphine in mice for 24 h after a single subcutaneous injection of 1 of 3 buprenorphine formulations: buprenorphine HCl (Buprenex – considered the base or standard formulation), Buprenorphine SR-LAB (a long-acting formulation) and Simbadol. This dose was selected because it is the recommended dose of the longer acting Buprenorphine SR-LAB. Both male and female mice were evaluated and compared.

Materials and Methods

Animals. All animal procedures used in this study were reviewed and approved by The National Institute of Environmental Health Sciences Animal Care and Use Committee. Male and female C57BL/6NCrl mice (Charles River, Raleigh, NC for the serum concentration study) or C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME for the activity study) were used at 6 to 7 wk of age. Mice were group housed 4/cage in individual ventilated caging (IVC) on a double-sided rack (Techniplast, Exton, PA). Cages contained autoclaved hardwood bedding (Sani-chips, PJ Murphy, Montville, NJ), autoclaved Enviro-dri nesting material (Shepherd Specialty Papers, Watertown, TN) and Cotton squares (Ancare, Bellmore, NY) for environmental enrichment. Mice were provided ad libitum autoclaved rodent diet (NIH31, Harlan Laboratories, Madison, WI) and deionized water treated by reverse osmosis. Housing and experiments took place in an AAALAC International accredited facility under constant environmental conditions (70 to 73 °F, relative humidity 40% to 60%, 12h:12h light- dark cycle). Mice were negative for MHV, MPV1-5, MVM, MNV, TMEV, MRV/EDIM, Sendai virus, PVM, REO3, LCMV, Ectromelia, *Pasteurella* spp., *Mycoplasma pulmonis*, *Helicobacter* spp., CARB/Filobacterium, and endo-and ectoparasites upon receipt and no agents were detected in sentinel mice during the study.

Buprenorphine formulations. Buprenex (BUP) (Par Pharmaceutical, Chestnut Ridge, NY) and Simbadol (SIM) (Zoetis, Parsippany, NJ) were diluted in sterile water to a concentration of 0.1 mg/mL prior to injection. The Buprenorphine SR-LAB (BSR) (Zoopharm, Windsor, CO) was supplied from the manufacturer at a concentration of 0.5 mg/mL and was used undiluted. Each mouse was dosed at 1 mg/kg SQ with one of the buprenorphine products using a 25-gauge × 5/8-in needle. Mice were observed closely for 1 h after injection.

Study design for serum concentration. Immediately upon receipt, the room technician randomly took mice from the shipping container and placed them into IVC cages, with 4 mice per cage. After a one-week acclimation period, cages were assigned to one of the 3 treatment groups: BUP, SIM, or BSR. Individual mice within a cage were not assigned to different cages to avoid potential fighting in the male mice, and females were not reassigned to replicate how males were handled. All mice in a given cage were dosed with the same buprenorphine product, and all mice in a cage were used at the same timepoint. Both female and male mice were used to discern any sex differences. The study using females was done 2 wk before the study that used the male mice. The treatment groups consisted of 48 mice, and sex was evaluated for the SIM and BSR treatments (8 mice for each of 6 time points – 1, 4, 8, 12, 16, 24 h after administration). Because therapeutic buprenorphine levels were not expected to occur beyond 16 h after dosing for the BUP treatment group, only 40 mice per sex were used (that is, 8 mice for each of 5 time points – 1, 4, 8, 12, 16 h after administration. Each mouse was manually restrained and injected subcutaneously at time 0 with one of the 3 buprenorphine formulations (BUP, SIM, or BSR). Mice were injected 1 to 2 min apart so that the 1- hour timepoint could be accurately timed. At 1, 4, 8, 12, 16 and 24 h after injection for the SIM and BSR groups, 8 mice per sex were euthanized with carbon dioxide in their home cages with a 30% fill rate. Blood was collected immediately by cardiocentesis, and death assured by cervical dislocation. Mice were euthanized rather than bled at each timepoint due to the volume of blood needed for duplicate analyses. Blood samples were placed in serum collection gel-lock tubes and centrifuged within 2 h. Serum (0.5 mL) was removed and stored in 2 mL cryovial tubes at −80 °C until analyzed. Two naive male mice from the same shipment were euthanized at time 0 for use in calibrating the buprenorphine assay.

Quantification of buprenorphine concentration. Serum concentrations at each time point for males and females were quantified by LC-MS/MS. Samples were run in duplicate and calculated concentrations were averaged. Serum samples (approximately 50 µL) were loaded on top of Phenomenex Phree phospholipid removal columns (Phenomenex, Torrance, CA) that had been prerinsed with 1% acetic acid in acetonitrile. Proteins were precipitated with 450 µL 1% acetic acid in acetonitrile containing 50 pg of buprenorphine-d4 heavy isotope labeled internal standard (Cerilliant, Round Rock, TX). A Cerex System 48 positive pressure manifold (Tecan, Baldwin Park, CA) equipped with compressed nitrogen gas was used to elute the samples from the stationary phase at a flow rate of approximately 0.1 mL/min. Samples were collected in glass centrifuge tubes. Solvent was removed under vacuum at 40 °C (Centrivap, Labconco, KS City, MO). Samples were reconstituted in 50 µL of 0.05% formic acid in 20% methanol and transferred to autosampler vials with limited volume inserts. Separation was performed on a 2.1 × 100 mm Speedcore Diphenyl column (Fortis Technologies, Cheshire, UK) using gradient separation at 0.4 mL/min with mobile phase A being 0.05% formic acid in water and mobile

phase B being 9:1 methanol: acetonitrile. Starting conditions of 30% B were held for 0.5 min, then %B was raised linearly to 53.3% at 4.5 min, then 97.5% at 4.7 min. The column was then flushed for 2 min and returned to starting conditions for 2.3 min for re-equilibration prior to the next injection. Injection volume was 10 µL. Buprenorphine eluted at 4.17 min and buprenorphine-d4 eluted at 4.14 min. A TSQ Quantiva triple quadrupole mass spectrometer (ThermoFisher Scientific, Waltham, MA) run in positive ion selected reaction monitoring mode with electrospray ionization was used for detection. Spray voltage was 2750 V, source fragmentation voltage 10 V, ion transfer tube temperature 350 °C, and vaporizer temperature 450 °C. Source sheath, auxiliary, and sweep gases were nitrogen and were set to 40, 15, and 2 arbitrary units, respectively. Buprenorphine was quantified using a precursor/product ion pair of 468.3/396.3 and confirmatory product ions at *m/z* 414.3 and 101.2 were also monitored. Buprenorphine-d4 was monitored with *m/z* pair 472.3/400.3. Cycle time was set to 0.4 seconds.

Calibration samples were prepared in serum from buprenorphine naïve mice. A 9-point calibration was used spanning from 0.05 to 50 ng/mL. A linear regression was applied with 1/X weighting. R^2 value was greater than 0.998. Calculated values were within ±15% of actual values at all calibration points, and the lower and upper calibration points were considered to be the lower and upper limits of quantification, respectively. Sample concentrations were adjusted from calculated concentrations when less than 50 µL of sample was available.

Study design for open field. The open field instrumentation (Opto Max, Columbus Instruments, Columbus, OH) consisted of 4 Plexiglas chambers (40 cm × 40 cm × 20 cm) surrounded by a row of photocells that emit and detect infrared beams. The infrared beams created a grid in each chamber that the software used to track the activity of the mouse. Six-wk old mice (n = 28 females and 25 males) were used for this study. Mice were assigned and acclimated as described above. Four males and 4 females were assigned to the saline control group (SAL). Seven male mice and 8 females were assigned to each of the BUP, SIM, and BSR groups. Females were studied 1 wk before the males. For each sex, the study was conducted over 3 d during h 0800 to 1200. One mouse from an assigned group was placed in one of the 4 acrylic chambers and allowed to acclimate in the chamber for 30 min. The mouse was then injected with saline (0.1 mL/10 g body weight) or with 1 of the 3 buprenorphine formulations. Activity was tracked in 5-min intervals for 40 min after injection.

Statistical analysis. Statistical analysis for serum concentrations of buprenorphine was done using R version 3.5.3. A test was considered statistically significant when a P value of less than 0.05 was achieved. Results are expressed as mean ± SD. Two-way ANOVA and the Tukey multiple comparison test were used to evaluate effects of time and treatment group. Linear regressions with second order polynomial in time were used to compare the changes of serum drug concentrations over time in the treatment groups. Due to the skewed distribution, log-transformed concentration values were used in ANOVA and linear regression analyses. A 2-sided test was used to generate P values for the regression analyses.

Statistical analysis for open field was performed with Prism version 7.02 (GraphPad, La Jolla, CA). A 2-way RM ANOVA and Tukey multiple comparisons tests were used to compare groups. Differences were considered significant when a P value of less than 0.05 was achieved.

Results

Serum concentrations of buprenorphine. The BSR group had an average buprenorphine concentration of 5.3 ± 1.5 ng/mL for the females at 1 h after injection. It increased to 7.6 ± 1.5 at 4 h and gradually decreased thereafter (Figure 1 A). At 24 h, concentrations were still above the 1 ng/mL (2.2 ± 0.6 ng/mL). The male BSR group had an average concentration of 6.3 ± 2.4 ng/mL of buprenorphine 1 h after injection (Figure 1 B) and was higher at 4 h (7.4 ± 2.4 ng/mL). The concentrations were similar at 8 h and then gradually fell, but remained greater than 1 ng/mL at 16 h (4.4 ± 1.6 ng/mL). None of the changes were statistically significant after Tukey adjustment. No significant differences were detected between males and females at any timepoint.

Males and females in the SIM and BUP groups both had very high concentrations of buprenorphine at 1 h. Buprenorphine concentrations in males were approximately 59.0 ± 6.4 in the SIM group and approximately 51.0 ± 24.3 in the BUP group. Buprenorphine concentrations in females were approximately 56.4 ± 13.6 in the SIM group and approximately 51.6 ± 4.7 in the BUP group. Values are approximate because some data points exceeded the upper calibration limit. For each of the SIM and BUP groups, the concentration rapidly fell and was below the therapeutic threshold of 1 ng/mL by 12 h for BUP and 16 h for SIM. The BSR group remained above the 1 ng/mL therapeutic threshold throughout the 24 h evaluation period.

The concentration in the SIM group was significantly higher than that in the BSR group at 1 h after injection in female mice (Figure 1 A; $P < 0.0001$) and male mice (Figure 1 B; $P < 0.0001$). The BUP group had significantly higher concentration at 1 h after injection in both females ($P < 0.0001$) and males ($P < 0.0001$) as compared with the BSR group. The differences between BSR as compared with SIM and with BUP were significant at 4 h in female mice (SIM, $P = 0.003$ and BUP, $P = 0.02$). In female mice statistically significant differences were detected between BUP and BSR at 8, 12, and 16 h and between SIM and BSR at 8, 16 and 24 h. In male mice, statistically significant differences were detected between BUP and BSR and SIM and BSR at 12 and 16 h and between SIM and BSR at 24 h

Linear regression (the slope of the curves) with second order polynomial in time was used to assess the change in concentration over time across different groups We identified significant differences in the slopes between BSR group and the BUP and SIM groups ($P < 0.0001$) in changes over time. Serum concentrations of BUP and SIM were similar over time

Behavioral changes that were noted in most male and female mice given SIM and BUP included mild ataxia, Straub tail reaction (dorsiflexion of the tail vertical to the orientation of the body), and a tiptoe gait for up to 1 h after injection. These behaviors appeared to abate within 1 h after injection.

Open field. RM ANOVA indicated a significant main effect of group on the number of beam breaks after injection of the male ($P = 0.0004$) and female ($P < 0.0001$) mice (Figure 2). Female mice also had a significant group x time interaction ($P = 0.0326$). Male mice treated with SIM had higher activity levels than their saline counterparts at 55 to 75 min ($P < 0.05$). Significant differences were detected between the female BUP and saline groups at 50 min ($P = 0.0009$) and between 65 to 75 min ($P < 0.05$), as well as between Simbadol and saline groups at 70 min ($P = 0.0438$).

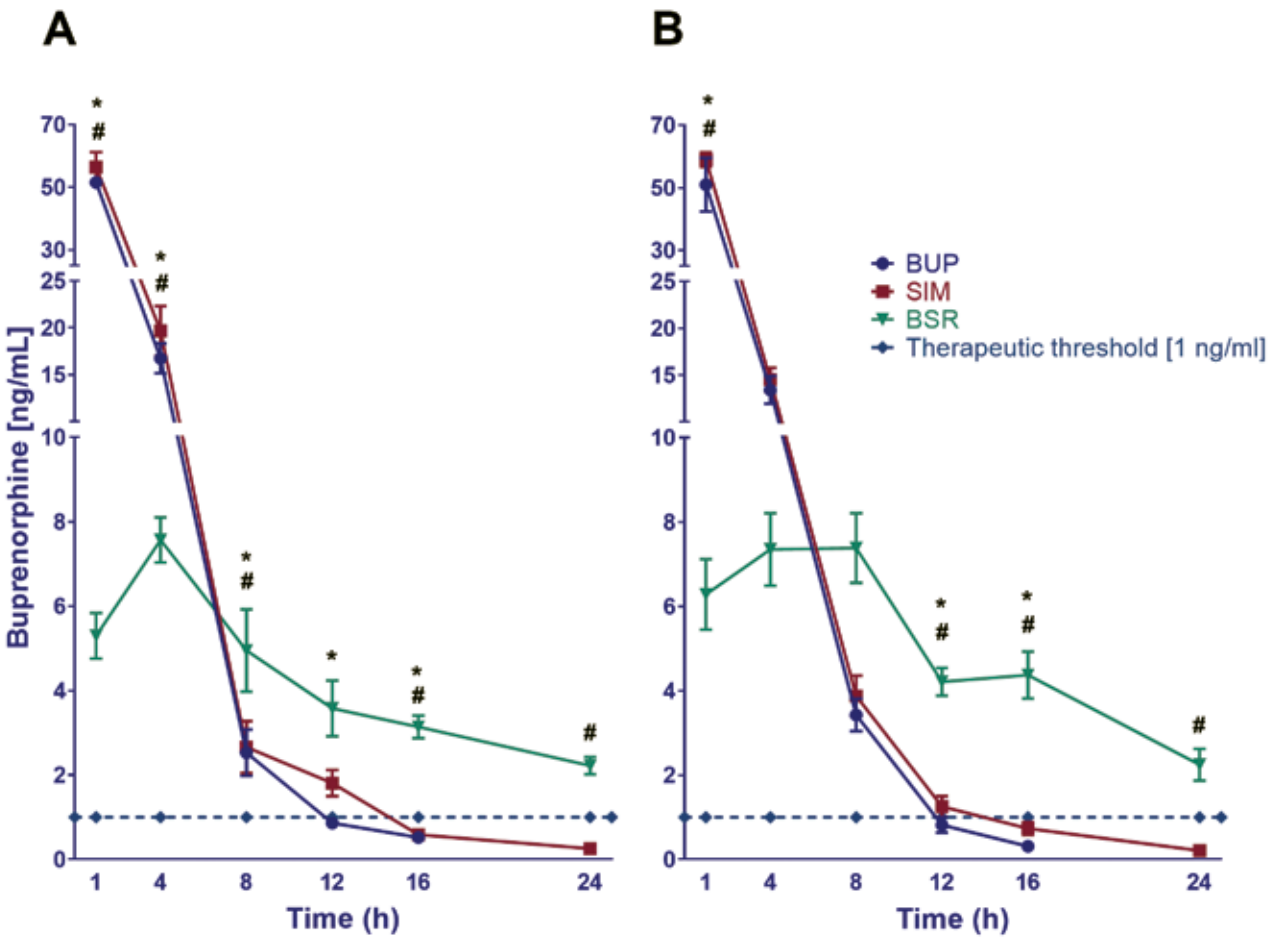

Figure 1. Blood serum concentrations of buprenorphine after a single 1 mg/kg subcutaneous injection of buprenorphine HCl (BUP), Simbadol (SIM), or Buprenorphine SR-LAB (BSR) in female (A) and male (B) mice. Both males and females in the SIM and BUP groups had higher concentrations of buprenorphine at one hour ($P < 0.0001$) than did the BSR groups. Significant differences in both sexes were also observed at later timepoints in the BSR group as compared with both the BUP and SIM groups. The slope of the BSR curve was significantly different from those of the BUP and SIM groups ($P < 0.0001$). *$P < 0.05$, comparison between BSR and BUP groups. #$P < 0.05$, comparison between BSR and SIM groups. Data points represent mean ± SEM; $n = 8$.

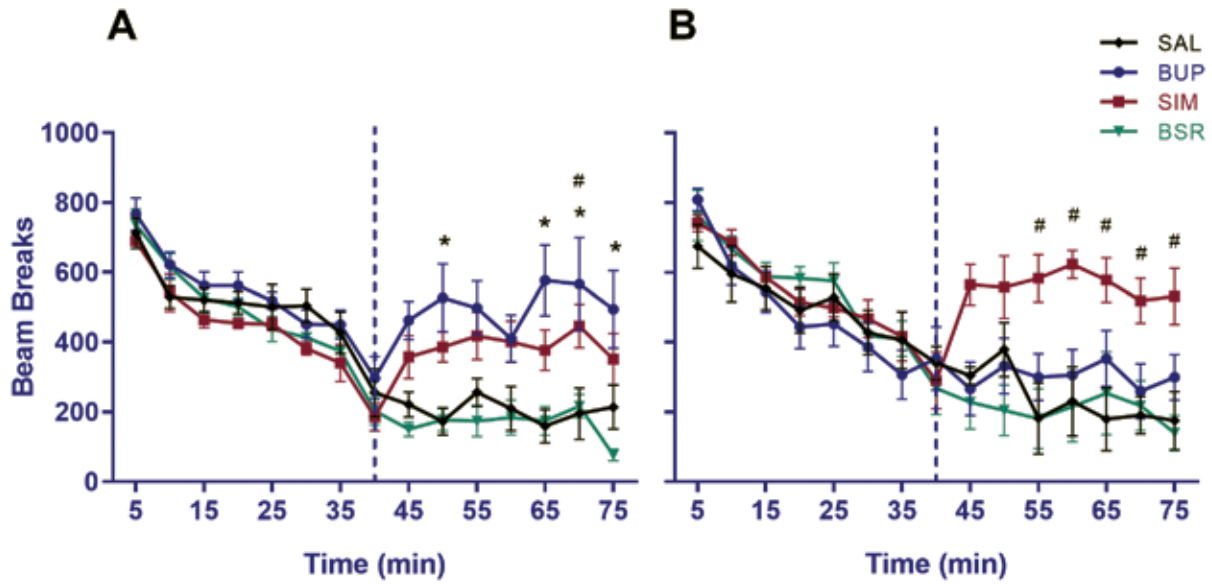

Figure 2. Activity measured by number of infrared beam breaks in an open field after a single 1 mg/kg subcutaneous injection of buprenorphine HCl (BUP), Simbadol (SIM), or Buprenorphine SR-LAB (BSR) in female (A) and male (B) mice after a 40 min acclimation period. Control mice (SAL) received a single subcutaneous injection of saline (0.1 mL/10 g body weight). Vertical line denotes time of injection (40 min). RM ANOVA indicated a significant main effect of group on the number of beam breaks after injection in the male ($P = 0.0004$) and female ($P < 0.0001$) mice. *$P < 0.05$, comparison between SAL and BUP groups. #$P < 0.05$, comparison between SAL and SIM groups. Data represent mean ± SEM; $n = 4$-8.

Discussion

Buprenorphine is widely used for postsurgical pain relief in laboratory mice. Buprenorphine HCl has been reported to be safe and effective in laboratory mice but does not provide long-acting analgesia beyond 4 to 6 h after injection at a standard dose of 0.1-0.3 mg/kg.[6,13,15,18,19] Sustained-release formulations provide continuous analgesia for up to 72 h in mice, allowing minimal handling. These formulations have been found to be safe and effective for moderate to severe pain.[6,12,17-20]

Simbadol has been reported to provide a therapeutic level of pain relief in cats for up to 24 h[9] with a buprenorphine serum concentration of 1 ng/mL or greater for the 24-h duration when given by the SC route at a dose of 0.24 mg/kg. One study compared the effect of Simbadol (0.3 mg/kg SC), sustained-release buprenorphine (1.2 mg/kg SC) and buprenorphine HCl (0.05 mg/kg SC) in rats.[1] While they did not evaluate serum concentrations, the authors reported self-injurious behavior in some rats with all the buprenorphine formulations tested.[1] In addition, hypoalgesia (measured using thermal sensitivity tests) was detected for only 1 h and was only observed with buprenorphine HCl and Simbadol. More recently, plasma buprenorphine concentrations were evaluated in Rhesus macaques given a single injection of Simbadol at either 0.24 mg/kg or 0.72 mg/kg SC.[24] That study reported 0.1 ng/mL as the therapeutic threshold in macaques, which is 10 fold lower than the 1.0 ng/mL therapeutic threshold reported in mice. Both the 0.24mg/kg and 0.72 mg/kg doses used in the macaques exceeded 0.1 ng/mL out to 48 h; at 72 h, the low dose fell below 0.1 ng/mL, whereas, the high dose was still above that threshold.[24]

The use of Simbadol has not previously been reported in mice. The current study sought to measure serum concentrations after subcutaneous administration of a single dose of Simbadol to mice and to compare those concentrations to both buprenorphine HCL and Buprenorphine SR-LAB given at the same dose and route. Specifically, we wanted to determine whether Simbadol would maintain serum buprenorphine concentrations above 1 ng/mL over time. LC-MS/MS was used to analyze serum concentrations of buprenorphine; however, some publications have used a forensic enzyme linked immunoassay (ELISA)[3,6,14] to determine if a therapeutic level was maintained in the sera/plasma.

When injected subcutaneously at 1 mg/kg, Simbadol maintained serum levels significantly above 1 ng/ml for 12 h. This dose of 1 mg/kg Simbadol administered subcutaneously was used to mirror the recommended dose of Buprenorphine SR-LAB. For comparison, a high dose of standard buprenorphine was injected subcutaneously at the same 1 mg/kg. Standard buprenorphine at 1 mg/kg demonstrated serum levels at 1 ng/mL for 12 h. The slopes of the Simbadol and standard buprenorphine serum concentrations curves were not significantly different. The serum concentration curves also showed that the rate of decline in serum concentration of the buprenorphine of these 2 products were similar. This indicates that the higher serum levels obtained from Simbadol and standard buprenorphine were solely due to the high dose (1 mg/kg) of product given and not due to any sustained release. Our laboratory reported similar results when evaluating a formulation of buprenorphine in Pluronic Gel.[3] A lower dose of Simbadol was not evaluated, but based on the shape of the curve, a more standard dose of 0.1 mg/kg would generate serum concentrations that would mirror standard buprenorphine concentrations.

Buprenorphine SR-LAB was the only formulation that delivered a serum concentration greater than or equal to 1 ng/mL for up to 24 h. Buprenorphine SR-LAB (0.6 mg/kg SC) has been previously reported to maintain a therapeutic plasma level of greater than 1.0 ng/mL for the first 24 h in mice, with levels dropping below this therapeutic level by 48 h.[19] Buprenorphine SR-LAB (1.2 mg/kg) has been reported to maintain this therapeutic level for up to 72 h in rats.[12] Our study did not evaluate serum concentrations beyond 24 h.

Behavioral changes that were noted in mice given Simbadol and standard buprenorphine included mild ataxia, Straub tail reaction, and a tiptoe gait. These behaviors resolved within the first hour after injection. Increased open field activity occurred in these 2 groups in the female mice and in the Simbadol group in males. Although all groups received 1mg/kg, the Buprenorphine SR-LAB releases buprenorphine over time, while the Simbadol and standard buprenorphine groups receive a bolus dose. Straub tail and hyperactivity have been reported in mice given opioids[2,17,23,25] and can interfere with pain observations.[3,8,16,18,27]

We conclude that Simbadol does not provide sustained serum concentrations of buprenorphine in mice. Although buprenorphine concentrations were maintained above 1 ng/mL at 12 h, this occurred after a 10-fold increase in the standard buprenorphine dose. At this high dose, mice exhibited behavioral changes immediately after injection; therefore, this dose is not recommended. As no long-acting benefit was observed, no other doses were tested. We speculate that a lower dose would be similar to giving standard buprenorphine, and thus Simbadol would be a viable alternative to standard buprenorphine. One study[24] concluded Simbadol is a cost-effective analgesic alternative to standard buprenorphine. The product is supplied as a 10 mL vial and expires 56 d after the vial is punctured, perhaps resulting in a potential increase in opioid waste. Although Buprenorphine SR-LAB also has a "use by" date once the bottle is pierced, it provided a more consistent, longer lasting blood serum concentration of buprenorphine in both females and males after injection and would be recommended over either Simbadol or standard buprenorphine in mice if long-term analgesia is needed.

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