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Authors

Houston, Elizabeth R
Tan, Sarah M
Thomas, Samantha M
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Pharmacokinetics and Efficacy of a Long-lasting, Highly Concentrated Buprenorphine Solution in Rats

Elizabeth R Houston,[1] Sarah M Tan,[1] Samantha M Thomas,[1] Ulana L Stasula,[1] Mollie K Burton,[1] Heather K Knych,[2] and Lon V Kendall[1,*]

Buprenorphine (Bup) is an opioid analgesic that is commonly used in laboratory rodents to provide postoperative analgesia. However, dosing every 4 to 6 h is necessary to maintain an analgesic plasma concentration of the drug. A long lasting, highly concentrated veterinary formulation of Bup (LHC-Bup) has been used to provide prolonged analgesia in cats and nonhuman primates. In the current study, we evaluated the duration of efficacy of LHC-Bup to determine if this formulation would provide a similarly prolonged analgesia in rats. Drug concentrations were measured after subcutaneous injection of 0.5 mg/kg LHC-Bup in both male and female rats. Plasma levels were measured at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, and 72 h. Male and female rats had peak plasma levels of LHC-Bup at 90 ng/and 34 ng/mL, respectively, at 15 min after administration, with a steady decrease by 24 h to 0.7 ng/mL in males and 1.3 ng/mL in females. Mechanical pain tolerance was evaluated after LHC-Bup administration using a Randall-Selitto analgesiometer to assess paw withdrawal. Male rats had a significantly longer paw withdrawal time for up to 12 h after administration, and females had longer paw withdrawal times for up to 24 h. An experimental laparotomy model was then used to assess the clinical efficacy of LHC-Bup at 0.5 mg/kg. LHC-Bup treatment was associated with a greater total distance traveled, reduced time to retrieve a food treat, and reduced grooming from 3 to 12 h after surgery as compared with saline controls. Groups receiving LHC-Bup showed coprophagy whereas other rats did not. These results suggest that administering LHC-Bup at 0.5 mg/kg provides therapeutic plasma concentrations for 12 to 24 h after administration and analgesic efficacy for at least 12 h after dosing. As such, LHC-Bup is a suitable alternative to Bup-HCl.

Abbreviations: Bup- buprenorphine, LHC- long lasting highly concentrated, SR- sustained release, ED- effective dose; SC, subcutaneous

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Pain management is a critical aspect in the use of animals in research, both to optimize animal welfare and achieve high-quality scientific results.[28] We are obliged as researchers to minimize pain and distress in animals, including rats, which are commonly used in biomedical research. Pain assessment in rats can be challenging and relies on observation of normal behaviors including activity, grooming, and facial expression.[19,30,31,35] Providing effective pain relief with minimal side effects is an important goal in research using rats.

Buprenorphine (Bup) is an analgesic that is commonly used for the management of postoperative pain in rodents. This partial μ agonist has a short duration of action and is administered by injection. A frequently published dosing regimen for rats is 0.01 to 0.1 mg/kg every 8 to 12 h[3,8,11,13,32,33] However, previously published pharmacokinetic data demonstrated that the duration of action is likely far less than 8 h.[10,12] When given subcutaneously (SC) at 0.05 mg/kg, the maximum plasma concentration was reached within 30 min and remained above the therapeutic level of 1.0 ng/mL for only 2 h.[12] A dose of 0.1 mg/kg SC reached a maximum plasma concentration at 4 h, but was below 1.0 ng/mL at 24 h.[10] These data suggest that commonly used dosing regimens do not maintain a therapeutic level between the dosing intervals, which may result in inadequate analgesia.

Sustained release (SR) Bup more effectively maintains therapeutic levels over time. When given to rats at 0.9 to 1.2 mg/kg SC, concentrations above 1.0 ng/mL were maintained for 24 to 48 h.[4,7,10,27,34] The SR formula improved analgesic coverage and decreased the need for handling for repeated injections. However, obtaining SR-Bup can be difficult in some places due to the emergence of state laws directed at the opioid crisis. An FDA-approved, long lasting, highly concentrated (LHC) veterinary formulation of Bup has a concentration of 1.8 mg/mL and is labeled as providing 24 h of analgesia in cats.[40] The LHC formulation is an effective option for prolonged postoperative analgesia that avoids the need for a compounding pharmacy. We have demonstrated in mice that LHC-Bup provided therapeutic plasma levels for up to 12 h, and is effective for postoperative pain in a laparotomy model.[18]

The objective of this study was to determine the pharmacokinetics of LHC-Bup in outbred male and female Sprague–Dawley rats to determine if the plasma levels could be maintained over a therapeutic threshold for 24 h, similar to what was seen in

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[1]Department of Microbiology, Immunology and Pathology, and Laboratory Animal Resources, Colorado State University, Fort Collins, Colorado
[2]Department of Veterinary Molecular Biosciences and K.L. Maddy Analytical Pharmacology Laboratory, School of Veterinary Medicine, University of California, Davis, California
[*]Corresponding author. Email: Lon.Kendall@colostate.edu

cats.[40] The analgesic efficacy was then evaluated using a paw withdrawal analgesiometric test and a laparotomy model to determine if LHC-Bup is a viable alternative for treating postoperative pain in rats. This study found that LHC-Bup at 0.5 mg/kg provides analgesic effects for at least 12 h after administration in analgesiometric and laparotomy tests with limited associated side effects.

Materials and Methods

Animals. Male and female, 6 to 8 wk-old, Sprague–Dawley rats (Crl:CD(SD)) were obtained from Charles River (Wilmington, MA). Based on vendor reports, rats received were free from Sendai virus, rat coronavirus, pneumonia virus of mice, lymphocytic choriomeningitis virus, Kilham rat virus, Toolan H-1 virus, rat parvovirus, rat minute virus reovirus, rat theilovirus, Hantaan virus, mouse adenovirus 1 and 2, and ecto- and endoparasites. Rats from the source colony were negative for bacterial pathogens, but positive for the opportunistic bacteria *Staphylococcus aureus*, β-*Streptococcus sp.* Group B, and *Pseudomonas aeruginosa*. For the pharmacokinetic study and paw withdrawal study, rats were pair-housed by sex in static Allentown rat caging (18 in. × 9.25 in. × 8 in, Allentown, Allentown, NJ). Rats were singly housed for the laparotomy and associated behavioral testing. Irradiated feed was provided ad libitum (Teklad 2918, Envigo, Indianapolis, IN) as was filtered, sterilized water. Rats were maintained on a 14:10 light:dark cycle at a temperature of 21 to 24 °C. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Pharmacokinetic study. The pharmacokinetics of LHC-Bup (Simbadol, Zoetis, Kalamazoo, MI) were evaluated in 20 6 to 8-wk-old Sprague–Dawley rats: 10 males and 10 females. Each rat was weighed and dosed with 0.5 mg/kg LHC-Bup SC in the interscapular region. The dose was based on allometric scaling of the cat dose of 0.24 mg/kg.[9] Three rats from each sex were randomly sampled at each time point (Table 1). Rats were manually restrained, and blood was collected from the jugular vein into a heparinized tube (Sarstedt AG and KG, Numbrecht, Germany) at baseline (before dosing), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, and 72-h after administration. Samples were centrifuged for 10 min at 3500 × g. Plasma was removed and stored at -80 °C prior to assessment of Bup levels.

Liquid chromatography tandem mass spectrometry of buprenorphine. Serum calibrators were prepared by dilution of the Bup working standard solutions (Cerilliant, Round Rock, TX) with drug free rat serum to concentrations ranging from 0.1 to 200 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (rat serum with Bup added at 3 concentrations within the standard curve) were included with each sample set as an additional accuracy check.

Table 1. Pharmacokinetic sampling schedule. M- male, F- female.

Hours after dosing	Animal identification
Baseline	M6, M8, M10, F2, F4, F8
0.25	M2, M4, M9, F1, F3, F5
0.5	M1, M3, M5, F7, F9, F10
1	M4, M7, M9, F1, F3, F8
2	M2, M6, M8, F2, F6, F9
4	M1, M3, M5, F4, F5, F7
8	M3, M4, M10, F3, F7, F8
12	M1, M5, M9, F2, F4, F5
24	M2, M7, M10, F1, F6, F9
36	M1, M2, M3, F4, F5, F7
48	M5, M6, M7, F1, F2, F8
72	M4, M8, M9, F6, F9, F10

Prior to analysis, 0.05 mL serum was diluted with 2.0 mL 0.1M pH 6 phosphate buffer and 0.1 mL water containing the d4-Bup (Cerilliant, Round Rock, TX) internal standard (40 ng/mL). All samples were vortexed gently to mix and subjected to solid phase extraction using C18UC columns 200 mg/3mL (UCT Bristol, PA). The columns were conditioned with 2.5 mL of methanol and 3 mL of water. The samples were loaded onto the column and given no less than 2 min for samples to pass through. The columns were rinsed with 2 mL 50% methanol in water before eluting with 2.5 mL methanol. Samples were dried under nitrogen, dissolved in 100 µL of 10% acetonitrile (ACN) in water with 0.2% formic acid and 40 µL injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

The analyte concentrations were measured in serum by LC-MS/MS using positive heated electrospray ionization. Quantitative analysis was performed on a TSQ Altis triple quadrupole mass spectrometer coupled with a Vanquish liquid chromatography system (Thermo Scientific, San Jose, CA). The spray voltage was 3500V, the vaporizer temperature was 400 °C, and the sheath and auxiliary gas were 40 and 15 respectively (arbitrary units). Product masses and collision energies of each analyte were optimized by infusing the analytes into the mass spectrometer. Chromatography employed an ACE 3 C18 10 cm × 2.1mm 3 µm column (Mac-Mod Analytical, Chadds Ford, PA) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.4 mL/min. The initial ACN concentration was held at 10% for 0.3 min, ramped to 95% over 5.6 min and held at that concentration for 0.3 min before re-equilibrating for 2.8 min at initial conditions.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for Bup (mass to charge ratio *(m/z)* 468.3) and the internal standard d4-Bup (*(m/z)* 472.3). The response for the product ions for Bup (*m/z* 101.0, 186.9, 243.0, 396.2, 414.2) and the internal standard (*m/z* 100.9, 186.9) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate analytes in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

The response for Bup was linear and gave correlation coefficients of 0.99 or better. Accuracy was 108% for 0.3 ng/mL, 93% for 5 ng/mL and 93% for 40 ng/mL. Precision was 5% for 0.3 ng/mL, 4% for 5 ng/mL and 4% for 40 ng/mL. The technique was optimized to provide a limit of quantitation of 0.1 ng/mL and a limit of detection of approximately 0.05 ng/mL for Bup.

Noncompartmental analysis for sparse data was performed on plasma buprenorphine concentrations using commercially available software (Phoenix Winnonlin v8.2, Certara, Princeton, NJ). With this sparse (naïve) data approach, plasma drug concentrations from all rats at each time points were analyzed simultaneously in a way that enabled estimation of the standard errors for C_{max} and AUC_{last}. Standard error of the mean AUC_{last} and C_{max} values were calculated as described previously,[26] with a modification.[15]

Analgesiometric Paw Withdrawal Assay. The nociceptive withdrawal threshold was performed using an electronic Randall-Selitto analgesiometer (IITC 2500 Digital Paw Pressure Meter, IITC Life Science, Woodland Hills, CA). Each rat was handled daily for a 7-d acclimation period. Rats were manually restrained with the handler supporting under the rat's body and the left hind limb draped over the handler's

hand. The analgesiometer was applied with gradually increasing mechanical force on the plantar aspect of the paw, between the paw pads of the third and fourth digit, until a withdrawal response was elicited or the analgesiometer read 1000 g. Baseline measurements were obtained in all rats immediately before dosing and both handler and female test performers were blind to treatment. Male and female rats (n = 4/sex) were randomly assigned to treatment groups and injected SC with 0.5 mg/kg LHC-Bup or saline in the interscapular region. The paw withdrawal test was performed at 1, 3, 6, 12, 24, and 30 h after administration. The rats were treated and tested again with the opposite treatment after a 7-d washout period.[14]

Midline laparotomy gonadectomy to evaluate efficacy of LHC-Bup. Male and female, 6 to 8 wk-old, Sprague–Dawley rats were randomly assigned to 4 groups of 14 rats (n = 7 per sex). The first group underwent a midline laparotomy for ovariectomy or orchiectomy with saline treatment. Another group had surgery with LHC-Bup treatment at 0.5 mg/kg. The third group received anesthesia without surgery with LHC-Bup, and the final group had anesthesia, no surgery, and saline. The anesthesia groups were induced and recovered at approximately the same time as their surgical counterparts. Surgery was performed between 0800 and 1000 and took 20 to 30 min per rat. Immediately before surgery, rats were given LHC-Bup or an equivalent volume of saline SC in the interscapular region. Anesthesia was induced and maintained on approximately 2.0% to 2.5% isoflurane delivered by oxygen at 1 L/min. The abdomen was shaved and prepared aseptically using alternating chlorhexidine and ethanol scrub. A 2.0 to 3.0 cm skin incision was made on midline, followed by a 1.5 to 2.0 cm incision through the abdominal wall. In females, ovaries were removed bilaterally by cautery of the ovarian pedicle and uterine horn with forceps heated in a microbead surgical sterilizer (Inotech Biosciences, Derwood, MD). The testicles of males were internalized into the abdomen, ligated with absorbable suture, and removed. The body wall was initially closed with 5-0 absorbable suture in a simple continuous pattern and the skin closed with 5-0 intradermal suture. Surgical glue was used if the incision site reopened. Rats were returned to their cages after recovery from anesthesia and monitored. LHC-Bup or saline administration was repeated every 24 h after operative, based on the pharmacokinetics data obtained in the first part of this study.

Behavioral Assessments. Each rat was scored for a 5-min period at every timepoint by 2 independent blind female observers, and the average pain score was recorded. Rats were acclimated to the ANY-maze apparatus for 10 min prior to beginning video and behavioral assessment. Baseline assessments were performed 24 h before surgery or anesthesia (time point 0), and postoperative pain was assessed at 3, 6, 12, 24, and 48 h. Interest in food was gauged by providing 3 yogurt treats at the start of the observation period in the corner of the testing cage and the latency to first interact with the treats and the overall number of treat interactions were recorded. A treat interaction was defined as movement to the treat. The frequency of grooming, wound licking, rearing, ataxia, hunched posture and coprophagy was tallied during a 5-min observation period. The total activity level of each rat was subjectively scored as 0 (no activity), 1 (decreased activity), or 2 (normal activity). Piloerection was scored as 0 (not present) or 1 (present). Orbital tightness scores were based on a modification of the rat facial grimace scale,[22] and were scored as 0 (no orbital tightening), 1 (mild orbital tightening), 2 (moderate), or 3 (severe). Behavioral data were recorded as the average score of frequency of each associated behavior.

General activity was assessed using ANY-maze video tracking software. The rat was identified with video focused on the head, mid region, and tail base to track the whole body and periods when the rat was immobile. The distanced traveled and time of activity were recorded with analysis performed on the average total distance per treatment group. Blind observers noted any additional observations, such as coprophagy or sedation, that were not captured in the previously described methods. Ultrasonic vocalizations as an indicator of pain or distress in rats,[2,16,17] were obtained using Avisoft-SAS Lab Pro Sound Analysis Software (Avisoft Bioacoustics, Nordbahn, Germany).

Statistical Analysis. Population size was determined using power analysis. Sample size was determined to be 7 per treatment group based on a power calculation on the main effect comparison between treatment groups using an α of 0.1, difference between means of 0.5, a standard deviation of 0.4, and a power of at least 0.8 from a 2-way ANOVA. Normality for the paw withdraw assay was confirmed using a Shapiro-Wilk test and treatment groups at each time point were compared with 2-way ANOVA using Tukey post hoc comparisons. Postoperative behavioral responses were compared with a mixed-effects model using Tukey post hoc comparisons. For all tests, values are expressed as mean ± SD. A P value less than 0.10 was considered statistically significant. The scores of the 2 blind observers were assessed for agreement using the Cohen k statistic (idostatistics.com, Giacomo Scarpelleni) and agreement assigned as follows: 0.01 to 0.2 slight agreement, 0.21 to 0.4 fair agreement, 0.41 to 0.6 moderate agreement, 0.61 to 0.8 substantial agreement, and 0.81 to 1.0 almost perfect or perfect agreement.

Results

Pharmacokinetics. LHC-Bup was administered at 0.5 mg/kg SC. and evaluated in 3 male and female rats at each time point. Male and female rats had a similar pharmacokinetic profile (Figures 1 and 2). The peak mean plasma concentration of Bup at the 15-min time point was 90 ng/mL in males and 34 ng/mL in females. The mean Bup plasma concentration in males at 24 h was 0.7 ± 0.3 ng/mL: just below the effective dose (ED)100 of 1 ng/mL, but still well above the ED50 of 0.5 ng/mL.[13] The Bup plasma concentrations in females stayed above the ED100 of 1 ng/mL[3,20] for over 24 h, with the plasma concentration measuring 1.3 ± 0.5 ng/mL at the 24 h measurement. The concentrations were well below the therapeutic plasma concentration after 36 h in both male and female rats. A noncompartmental analysis demonstrated the similarities and differences in the pharmacokinetic parameters of Bup in male and female rats (Table 2). The terminal half-life was shorter in males (8.3 h) compared with females (10.0 h) and the total concentration (AUC) was greater in males (158 ± 15 ng/mL) compared with females (139 ± 10 ng/mL).

Paw Withdrawal Response. Sprague–Dawley rats were dosed with either 0.5mg/kg LHC-Bup or saline, and mechanical pain tolerance tested on the hind paw using a Randall-Selitto analgesiometer. Male rats treated with LHC-Bup had a significant increase in mechanical pain threshold at 1 (P = 0.0002), 3 (P = 0.002), 6 (P = -0.02), and 12 (P = 0.09) h after administration, compared with a saline control. A maximal pressure of 818 ± 215 g was tolerated at the 1 h timepoint. The saline group did not tolerate more than 228 ± 115 g at any timepoint. The average pressure tolerance returned to baseline for the LHC-Bup treated male group by 24 h (Figure 3). Females showed similar results, with a significant elevation in pain threshold of the LHC-Bup treated group at 1 (P < 0.0001), 3 (P = 0.0007), 6 (P < 0.0001), and 12 h (P = 0.08) post-administration. The effect in females was also seen at 24 h (P = 0.04) after dosing, with a return to baseline at

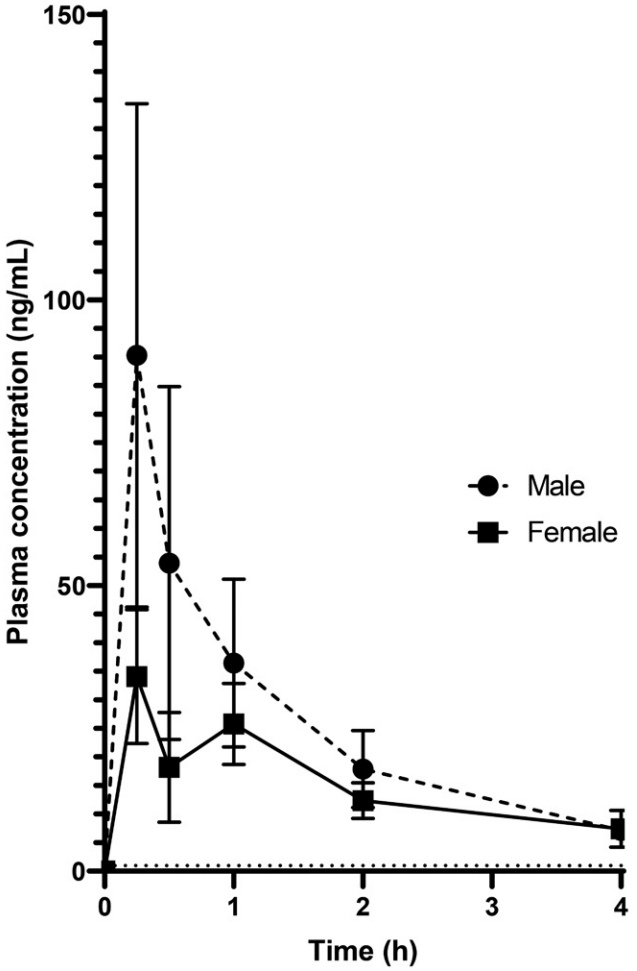

Figure 1. Pharmacokinetics of LHC-Bup in male and female Sprague–Dawley rats for first 4 h after administration. The dotted line indicates the ED100 of 1 ng/mL.

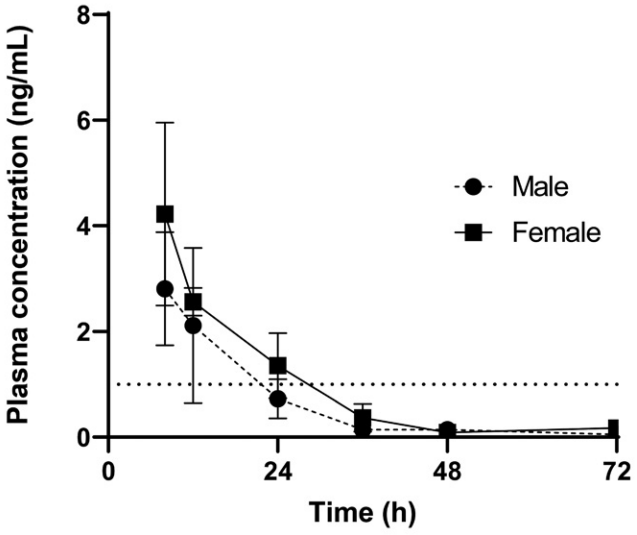

Figure 2. Pharmacokinetics of LHC-Bup in male and female Sprague–Dawley rats during h 8-24 after administration. The dotted line indicates the ED100 of 1 ng/mL.

30 h. The maximal pressure tolerated was 898 ± 204 g at 1 h after dosing. The saline group never tolerated more than 256 ± 116 g at any point during the experiment (Figure 3).

Table 2. Noncompartmental analysis of buprenorphine after SC. administration of LHC-Bup at 0.5 mg/kg to male and female rates.

Parameter	Unit	Male	Female
λ_z	1/h	0.08	0.07
$HL_{\lambda z}$	h	8.3	10.0
C_{max}	ng/mL	90	34
$C_{max\ SE}$	ng/mL	25	7
T_{max}	h	0.25	0.25
AUC_{last}	h*ng/mL	159	139
$AUC_{last\ SE}$	h*ng/mL	15	10
$AUC_{0\rightarrow\infty}$	h*ng/mL	161	143
$AUC_{\%Extrap}$	%	1.4	2.4

λ_z, elimination rate constant; HL λ_z, terminal half-life; C_{max}, maximum concentration; C_{max} SE, standard error of C_{max}; T_{max}, time of maximum concentration; AUC_{last}, area under the concentration-time curve from the time of dosing to the last measurable concentration; AUC_{last} SE, standard error of AUC_{last}; $AUC_{0\rightarrow\infty}$, area under the concentration-time curve from time 0 extrapolated to infinity; $AUC_{\%Extrap}$, percentage of $AUC_{0\rightarrow\infty}$ due to extrapolation from the last measured timepoint to infinity.

Postoperative efficacy of LHC-Bup. The efficacy of LHC-Bup as an analgesic was clinically tested using a laparotomy model in male and female rats given 0.5 mg/kg SC every 24 h based on the pharmacokinetic studies. One male was excluded from the study after developing a peritonitis secondary to ligation of an accessory sex gland, reducing the number of operated rats in the saline treatment group to 13. Three rats, 2 males and one female, required repair of the surgical skin incision with surgical glue.

Multiple behavioral measures indicative of pain were assessed. Fair to perfect agreement was seen between blind observers for the following parameters: total activity (k= 0.77), time to treat (k= 0.98), trips to treat (k= 0.47), grooming (k= 0.75), wound lick (k= 0.70), rearing (k= 0.34), hunch (k= 0.60), orbital tightening (k= 0.80), and coprophagy (k= 1). Baseline values did not differ between groups. Very few behavioral indicators suggested that pain was mitigated with LHC-Bup treatment (Table 3); however, the data revealed 2 key points. First, the surgery with saline treatment group displayed less total distance traveled at 12 h (P = 0.04), a longer time to the treat at 3 h (P = 0.08), and more grooming at 6 h (P = 0.06) as compared with the LHC-Bup treated group after surgery, suggesting that LHC-Bup had an analgesic effect. Otherwise, no significant differences were detected between the 2 surgical groups. Second, several behavioral parameters suggesting that the procedure was painful were the differences between the saline treated group after surgery and the anesthesia only groups with LHC-Bup or saline treatment. Compared with the anesthesia only with LHC-Bup treatment, saline treated rats after surgery had a lower distance traveled at 3 (P = 0.03) and 12 (P = 0.002) h; fewer trips to the treat at 3 (P = 0.09) and 12 (P = 0.01) h; less rearing at 12 h (P = 0.03); and more wound licking at 6 (P = 0.04), 12 (P = 0.05) and 24 (P = 0.05) h. Compared with the anesthesia only with saline treatment, saline treated rats after surgery had less total activity at 3 (P = 0.07) and 12 (P = 0.03) h; less distance traveled at 3 (P = 0.06) h; an increased time to treat at 6 h (P = 0.09); reduced trips to treat at 6 h (P = 0.08); reduced rearing at 3 h (P = 0.005); and increased wound licking at 6 (P = 0.04), 12 (P = 0.05) and 24 (P = 0.05) h; Coprophagy, indicative of a pica, a side effect of LHC-Bup treatment,[5,32,37] was higher in the LHC-Bup treated groups (with and without surgery) at 3, 6 and 12 h than in the saline treated group after surgery. Ataxia and piloerection were not observed. Ultrasonic vocalizations were not identified. No significant differences were found in the behavioral assessment of males and females.

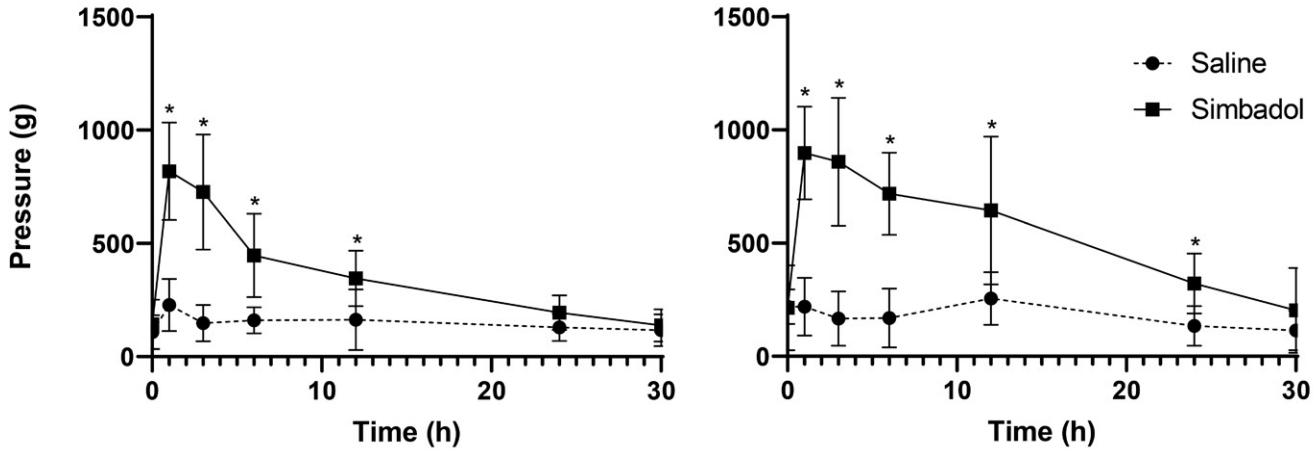

Figure 3. Mechanical pain tolerance measured by paw withdraw response after LHC-Bup in male and female Sprague–Dawley rats.

Table 3. Postoperative behavioral scores (mean ± 1 SD) in rats treated with saline or LHC-Bup after surgery, and rats that received anesthesia only plus LHC-Bup, or anesthesia only plus saline.

		Treatment group			
	Time (h)	Surgery + Saline	Surgery + LHC- Bup	Anesthesia + LHC-Bup	Anesthesia + saline
No. in group		13	14	14	14
Total activity	0	1.5 ± 0.7	1.5 ± 0.6	1.4 ± 0.8	1.6 ± 0.6
	3	0.7 ± 0.6[c]	0.9 ± 0.6	1.5 ± 0.5	1.5 ± 0.7
	6	1.2 ± 0.8	1.3 ± 0.6	1.5 ± 0.5	1.5 ± 0.7
	12	0.8 ± 0.6 [b,c]	1.4 ± 0.6	1.7 ± 0.4[c]	1.1 ± 0.7
	24	1.5 ± 0.5	1.4 ± 0.6	1.8 ± 0.4	1.7 ± 0.5
	48	1.2 ± 0.7	1.5 ± 0.8	1.5 ± 0.7	1.4 ± 0.8
Total distance (m)	0	1.4 ± 1.0	2.9 ± 2.8	1.7 ± 1.6	1.9 ± 1.5
	3	0.4 ± 0.4 [b,c]	1.1 ± 1.3	2.1 ± 2.1	1.4 ± 1.3
	6	1.8 ± 2.6	1.6 ± 1.5	2.2 ± 2.1	1.6 ± 1.8
	12	0.5 ± 0.7 [a,b]	2.1 ± 2.0	3.4 ± 2.3[c]	1.1 ± 0.9
	24	1.9 ± 1.5	3.2 ± 2.3	3.1 ± 2.0	2.2 ± 2.8
	48	1.9 ± 2.1	2.5 ± 2.3	2.9 ± 2.5	1.4 ± 1.0
Time mobile (s)	0	93.4 ± 95.4	88.3 ± 72.0	111.2 ± 105.9	77.1 ± 71.7
	3	130.4 ± 140.0	61.7 ± 83.3	64.2 ± 57.7	80.8 ± 96.9
	6	91.00 ± 95.0	58.0 ± 49.7	124.3 ± 101.4[c]	39.7 ± 33.5
	12	174.3 ± 141.9	96.4 ± 79.1	92.1 ± 55.5	99.0 ± 112.9
	24	119.9 ± 97.7	91.8 ± 74.6	78.7 ± 46.2	69.3 ± 68.4
	48	97.7 ± 100.9	57.7 ± 45.6	94.5 ± 76.2	68.3 ± 74.2
Time to treat	0	1.1 ± 1.0	0.8 ± 0.9	1.4 ± 0.9	0.9 ± 1.0
	3	1.5 ± 0.9[a]	0.6 ± 0.9	1.1 ± 1.0	0.7 ± 0.9
	6	1.6 ± 0.7[c]	1.4 ± 0.9	1.1 ± 0.9	0.8 ± 1.0
	12	1.4 ± 1.0	1.2 ± 1.0	0.8 ± 1.0	1.4 ± 0.9
	24	1.2 ± 0.8	0.9 ± 1.0	0.6 ± 0.9	0.7 ± 1.0
	48	0.9 ± 1.0	0.9 ± 1.0	0.5 ± 0.8	0.9 ± 1.0
Trips to treat	0	2.2 ± 1.8	2.0 ± 1.5	1.7 ± 1.9	3.0 ± 2.1
	3	0.8 ± 0.9[b]	1.8 ± 1.9	2.0 ± 1.5	2.2 ± 2.0
	6	0.8 ± 0.8[c]	1.1 ± 1.0	1.9 ± 1.5	2.1 ± 1.6
	12	0.9 ± 1.3[b]	1.7 ± 1.9	3.4 ± 2.4[c]	1.2 ± 1.5
	24	1.6 ± 1.0	1.7 ± 1.3	3.0 ± 2.1	2.2 ± 2.1
	48	1.7 ± 1.6	1.6 ± 1.4	2.4 ± 1.2	1.9 ± 1.8
Rearing	0	10.3 ± 8.3	13.2 ± 10.0	10.0 ± 6.9	13.9 ± 9.0
	3	1.6 ± 2.2[c]	2.5 ± 5.7[c]	7.0 ± 8.4	8.2 ± 5.9
	6	5.2 ± 7.0	5.0 ± 7.7	9.6 ± 10.6	6.4 ± 4.8
	12	2.2 ± 5.2[b]	5.9 ± 8.2	12.4 ± 10.9	4.8 ± 5.9
	24	7.9 ± 8.7	9.6 ± 10.5	11.4 ± 6.7	10.3 ± 10.3
	48	7.0 ± 7.6	8.5 ± 8.1	10.4 ± 8.8	9.1 ± 7.7

(*continued*)

Table 3. (Continued)

		Treatment group			
	Time (h)	Surgery + Saline	Surgery + LHC- Bup	Anesthesia + LHC-Bup	Anesthesia + saline
Grooming	0	1.7 ± 1.2	2.1 ± 1.6	1.8 ± 1.3	2.0 ± 1.4
	3	1.0 ± 1.3	0.5 ± 1.2	0.5 ± 1.1	1.3 ± 1.4
	6	2.8 ± 2.9[a]	0.4 ± 0.8[c]	1.9 ± 2.7	1.7 ± 1.4
	12	2.2 ± 2.3	1.1 ± 1.8	1.8 ± 2.3	1.5 ± 1.6
	24	1.8 ± 1.6	0.8 ± 1.0[c]	1.8 ± 0.9	1.9 ± 1.6
	48	1.5 ± 1.3	1.3 ± 1.2	1.6 ± 1.3	1.3 ± 1.4
Wound licking	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	0.9 ± 1.7	2.1 ± 3.4 [b,c]	0 ± 0	0 ± 0
	6	1.8 ± 2.3 [b,c]	1.8 ± 3.8 [b,c]	0 ± 0	0 ± 0
	12	145 ± 2.1 [b,c]	1.7 ± 3.2	0.4 ± 0.1	0.4 ± 0.1
	24	1.3 ± 1.7 [b,c]	0.5 ± 0.9	0 ± 0	0 ± 0
	48	0.9 ± 1.3	0.6 ± 1.3	0.1 ± 0.5	0.2 ± 0.6
Hunched	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	0.2 ± 0.7	0.3 ± 0.7	0.1 ± 0.4	0 ± 0
	6	0.4 ± 0.6	0.1 ± 0.5	0.1 ± 0.3	0 ± 0
	12	0.5 ± 1.2	0.0 ± 0.1	0 ± 0	0 ± 0
	24	0 ± 0	0.3 ± 0.6	0 ± 0	0 ± 0
	48	0.1 ± 0.3	0 ± 0	0 ± 0	0 ± 0
Orbital tightening	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	0.4 ± 1.0	0.1 ± 0.4	0 ± 0	0 ± 0
	6	0.0 ± 0.1	0.4 ± 1.3	0 ± 0	0 ± 0
	12	1.0 ± 3.5	1.4 ± 4.4	0 ± 0	0 ± 0
	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	48	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Coprophagy	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	3	0 ± 0	0.2 ± 0.4[c]	0.5 ± 0.5[c]	0.1 ± 03
	6	0 ± 0	0.2 ± 0.4[c]	0.5 ± 0.5[c]	0 ± 0
	12	0 ± 0	0.1 ± 0.4	0.3 ± 0.5[c]	0 ± 0
	24	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	48	0 ± 0	0 ± 0	0.1 ± 0.3	0 ± 0

a- value significantly different ($P < 0.10$) from the surgery + LHC-Bup group
b- value significantly different ($P < 0.10$) from the anesthesia + LHC-Bup group
c- value significantly different ($P < 0.10$) from the anesthesia + saline group

Discussion

Long-lasting analgesics are desirable because they provide adequate pain control while reducing the frequency of administration and need for repeated animal handling. In this study, we examined the use of LHC-Bup at 0.5 mg/kg SC as an analgesic for male and female Sprague–Dawley rats in a step wise manner, evaluating pharmacokinetics, mechanical pain thresholds and clinical efficacy using a laparotomy model. Plasma LHC-Bup levels remained above the therapeutic threshold of 1 ng/mL for just under 24 h in males and for over 24 h in females. The mechanical pain threshold, based on paw pressure withdrawal, was at least 12 h in males and at least 24 h in females. The efficacy of LHC-Bup in providing post-surgical analgesia was demonstrated in an experimental laparotomy model, as fewer pain behaviors occurred with LHC-Bup treatment for at least 12 h after surgery in both sexes as compared with saline treated rats.

LHC-Bup is a long-lasting, highly concentrated Bup formulation that is FDA approved for use in cats.[40] After subcutaneous dosing in cats, plasma levels remained above a therapeutic level for up to 72 h, and resulted in increased thermal nociception for over 24 h.[9,38] The pharmacokinetics of LHC-Bup have also been evaluated in nonhuman primates. Subcutaneous dosing in macaques resulted in therapeutic levels above 0.1 ng/mL for over 72 h.[23] In rodents, LHC-Bup has been tested as a long-lasting analgesic for mice.[18] While more rapid elimination occurred in mice, pharmacokinetics and behavioral assessments after a laparotomy indicate that dosing every 6 to 12 h is appropriate for postoperative management in mice.[18] Plasma samples collected from Sprague–Dawley rats after dosing with LHC-Bup revealed a peak plasma concentration of Bup 15 min after administration of 90 ng/mL in male rats and 34 ng/mL in female rats, with therapeutic levels above 1 ng/mL for over 12 h in male rats and for at least 24 h in female rats. This suggests that males may require more frequent dosing than females, as has been previously demonstrated when using opioids in rodents.[6]

The Randall Selitto analgesiometer was used as a preliminary assessment of analgesia to mechanical pain based on a paw withdrawal threshold.[1,29] Rats given 0.5 mg/kg LHC-Bup showed a higher threshold for at least 12 h in males and at least 24 h in females after dosing, indicating that LHC-Bup provided analgesia for an extended period as compared with the shorter acting Bup-HCl.[10] While this test does not directly

yield clinically relevant findings for postoperative pain, it does demonstrate efficacy of LHC-Bup as a long-lasting analgesic in rats. This assessment of mechanical pain threshold provides better translation to surgical pain than alternative pain assessment techniques because this method of evoking pain more closely mimics postoperative nociception as compared with thermal or chemical sensitivity, which elicit pain through exposure of the peripheral sensory nerves.[21,24] The positive analgesic effects in the paw withdrawal study prompted the progression to examining the clinical efficacy of LHC-Bup.

A surgical laparotomy to complete a gonadectomy was selected as the surgery used to evaluate the postoperative efficacy of LHC-Bup in male and female rats. No significant differences were found in the behavioral responses between the male and female rats, suggesting that the surgical pain was similar in both sexes. The only parameters that were statistically different, suggesting clinical analgesia for the first 3 to 12 h postoperatively, were the total distance traveled, the time to treat, and grooming. After 12 h, the behaviors evaluated for pain had returned to baseline. The other parameters measured were not statistically different. Clinical behaviors of pain are difficult to observe and assess, as prey species will often mask pain.[25,36] A great deal of individual variation occurred among animals, with each rat having a different collection of observed behaviors at baseline. Thus, many of the behaviors had not changed significantly when evaluated at the group level. Activity has been used as a common indicator of analgesic efficacy in rats.[30,31] The measurements of activity in this study included a subjective impression of overall activity, and digital recordings of distance traveled, total active time, and interactions with treats. Differences in the total distance at 12 h and the time to interact with the treat at 3 h were statistically different between the saline group and the LHC-Bup treated group after surgery. Measures of activity at other time points suggested less activity in the saline group after surgery, particularly during the 3 to 12 h postoperative period; total activity, total distance traveled, and interactions with treats were numerically reduced, whereas trips to and interactions with the treat were numerically higher. Grooming activity was similarly higher in saline treated rats after surgery, and rearing activity was lower. Although these measures were not individually statistically significant, all of these effects, considered together, suggest pain. The other parameters, including wound licking, hunched posture, and orbital tightening, were not informative with regard to potential pain.

Other behavioral parameters varied greatly between individual rats, making their use difficult in this experiment. We used an abbreviated version of the rat grimace scale (RGS) by evaluating only orbital tightening, without including scoring of nose and cheek flattening, ear position, and changes in whisker appearance[19,30,31,35] This single parameter is more readily visible and easy to assess than the remaining aspects of the full RGS. Although the RGS is generally viewed as a reliable indicator of pain,[39] it was not useful in this study. This could have been due to inadequate training of the observers (although we believe they were adequately trained), a "normal" appearance of the rats during the observation period, or failure of the surgical procedure to cause detectable pain. Other parameters such as rearing, ataxia and trips to treat may have not shown differences due to the nuanced and brief body positions that were observed, underscoring the need to use these methods cautiously when evaluating pain.

The lack of differences in the laparotomy model may be due to the sedative effects from the higher concentration of LHC-Bup. For example, total activity, distance traveled, time mobile and grooming were decreased in the LHC-Bup treated rats after surgery and in the anesthesia with LHC-Bup group at the 3 h time point. Subjectively, rats seemed easier to handle at the early time points of the pharmacokinetic study. Another side effect was an increase in coprophagy in the LHC-Bup treated rats. Coprophagy is not uncommon after administration of Bup in rats and has previously been described as a side effect of the administration of Bup to rodents.[5,32,37] While coprophagy was observed in association with LHC-Bup use, no clinically detrimental effects were observed, and all rats remained healthy beyond the span of the study. Taking the behavioral and activity findings together, rats appeared to experience pain after the laparotomy. Signs of pain were most evident in the first 12 h after surgery, suggesting that this is the most critical period for providing analgesia.

LHC-Bup is an FDA-approved product that can be used off-label in rats and provides an alternative to other Bup formulations. Side effects are minimal, and the analgesic response occurs in a sex dependent manner. Male rats maintain a therapeutic plasma level and demonstrate efficacy for at least 12 h, whereas females maintain therapeutic plasma levels and demonstrate efficacy for at least 24 h. Thus, males may require more frequent administration of LHC-Bup than do females to achieve continuous analgesia. The first 12 h after surgery appear to be the most critical period for treating pain in this model, and LHC-Bup provides analgesia in both male and female rats for the initial 12 h after surgery.

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