JOURNAL OF
Veterinary Pharmacology and Therapeutics

J. vet. Pharmacol. Therap. **39**, 356–362. doi: 10.1111/jvp.12297.

Subcutaneous meloxicam suspension pharmacokinetics in mice and dose considerations for postoperative analgesia

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Chen, P. H., Boyd, K. L., Fickle, E. K., Locuson, C. W. Subcutaneous meloxicam suspension pharmacokinetics in mice and dose considerations for postoperative analgesia. *J. vet. Pharmacol. Therap.* **39**, 356–362.

Meloxicam is a cyclooxygenase (COX) inhibitor with a higher selectivity for cyclooxygenase-2 (COX-2) than for cyclooxygenase-1 (COX-1). In the laboratory setting, this nonsteroidal anti-inflammatory drug (NSAID) is commonly selected for analgesia in mice and administered every 24 h. This study characterizes the plasma concentration achieved from a dose of 1.6 mg/kg of meloxicam administered once every 24 h subcutaneously for 72 h in male and female C57BL/6 mice. These values were compared, over time, to reference COX-2 inhibition constants for meloxicam. No significant differences in trough plasma concentrations were noted between genders. The plasma concentrations were below the COX-2 IC_{50} after 12 h. To maintain a plasma concentration at or above the COX-2 whole blood $IC_{50,}$ the study results suggest an administration frequency of every 12 h when using a dose of 1.6 mg/kg in C57BL/6 mice.

(Paper received 22 July 2015; accepted for publication 21 January 2016)

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INTRODUCTION

Meloxicam is a Food and Drug Administration approved product for daily oral and parenteral administration in dogs (Metacam® Boehringer Ingelheim; Loxicom® Norbrook). It is a nonsteroidal anti-inflammatory drug (NSAID) with a higher selectivity in several species for cyclooxygenase-2 (COX-2) inhibitory activity than for cyclooxygenase-1 (COX-1). This analgesic is commonly selected for research mice in compliance with The Guide for the Care and Use of Laboratory Animal 8th Edition (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and the current industry standard of practice of providing analgesia for procedures expected to cause more than momentary pain or distress. Based on the search of publically accessible university formularies for this analgesic, it is apparent that there is a wide dosage range in mice (0.3–20 mg/kg every 12–24 h), with a commonly used dosage of 1–2 mg/kg every 24 h (Animal Care and Use Program UCSF, 2015; Boston University, 2015; Johns Hopkins University, 2015; Portland VA, 2015; University of British Columbia, 2015; University of Pennsylvania, 2015). Recommended dosages in mice have been based on postprocedural physiologic and behavioral parameters, but to date, there is limited information correlating dose to plasma concentrations (Tubbs *et al.*, 2011; Miller *et al.*, 2012; Ratsep *et al.*, 2013; Kendall *et al.*, 2014).

A common practice in the research setting is to provide 48–72 h of postprocedural analgesia after invasive procedures. Meloxicam is a common drug of choice and is often administered at 24-h intervals. The purpose of this study was to characterize the plasma concentrations of meloxicam given at 24-h intervals for 72 h in male and female C57BL/6 mice and compare, over time, these values to reference COX-2 inhibition constants for meloxicam. Data from male and female mice were also compared because gender differences in meloxicam pharmacokinetics from subcutaneous administration are currently unknown. Sex-based considerations are important to address as stated in the 2014 National Institute of Health's policy for preclinical research (Clayton & Collins, 2014). The C57BL/6 mice were chosen as this strain represents the most commonly used in disease models.

MATERIALS AND METHODS

Animals and housing

This project was approved by the Institutional Animal Care and Use Committee for Vanderbilt University Medical Center,

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an Association for Assessment and Accreditation of Laboratory Animal Care International accredited institution. Three- to four-week-old C57BL/6 mice of both genders were purchased for this study. The excluded pathogens in the mouse colony included mouse parvoviruses, murine norovirus, mouse coronavirus, mouse rotavirus, mouse theilovirus, adenovirus types 1 and 2, reovirus types 1, 2, 3, and 4, pneumonia virus of mice, Sendai virus, lymphocytic choriomeningitis virus, ectromelia, Helicobacter, Citrobacter, *Mycoplasma pulmonis*, *Corynebacterium kutscheria*, *Corynebacterium bovis*, Giardia, Pneumocystis, *Spironucleus muris,* pinworms (*Myobia, Myocoptes, Radfordia*), and fur mites (*Aspiculuris, Syphacia*).

Mice were acclimated in the housing facility for 6 days prior to drug administration and group-housed by gender in three mice per ventilated cage. XJ mouse cages (Allentown, Incorporated, Allentown, NJ, USA) with Enrich-o'Cobs® bedding (The Andersons, Incorporated, Maumee, OH, USA) were autoclaved and individually ventilated at 50 air changes per hour with a humidity range of 35–53%. The room was maintained at 22–23 °C with a 12:12 light:dark cycle. The mice received irradiated food (LabDiet High Energy Mouse Diet 5LJ5, St. Louis, MO, USA) and autoclaved water *ad lib*.

Materials

Control male mouse plasma used in bioanalysis was acquired from BioreclamationIVT (Upstate, NY, USA). Liquid chromatography–mass spectrometry (LC/MS) grade acetonitrile (ACN), dimethyl sulfoxide, and formic acid were acquired from Fisher Scientific (Waltham, MA, USA). Meloxicam and piroxicam standards were obtained from LKT Laboratories (Saint Paul, MN, USA) and Sigma-Aldrich (St Louis, MO, USA), respectively.

Study design

The study design was longitudinal, randomized, and nonserial. Male and female mice were divided into four groups each (males: group 1–4; females: group 5–8). Each group contained a sample size of six mice. Three days prior to drug administration, mice were ear punched and body weights were measured. During the study, weights were obtained daily and at the time of sacrifice. Overall, the weights of male mice averaged 20 g (range of 18–23 g), and females averaged 16 g (range of 14–18 g). Meloxicam (Loxicom® 5 mg/mL Norbrook) was diluted with sterile saline to a concentration of 0.65 mg/mL. Mice anesthetized with isoflurane received up to three subcutaneous injections of meloxicam at 1.6 mg/kg (0.04–0.05 mL) at 0, 24, and 48 h time points. The subcutaneous injections were administered midline over the scapular region. Three blood samples were collected from each mouse at various time points up to 72 h following the first injection. The sequential blood collection time points for each mouse were based on maximizing the rest period between sample collections to address the individual animal's welfare.

Blood and skin sample collection

Each mouse underwent two survival and one terminal blood collection procedure. Survival samples up to 20 μL each (total 40 μL) did not exceed 15% of the animal's total blood volume. Submandibular survival samples were collected on conscious animals with a lancet (Goldenrod Animal Lancet 5 mm point length). Blood sample collection times were calculated from the first meloxicam administration. Samples were collected from six male and six female mice at 1, 2, 4, 8, 12, 24, 26, 28, 48, 50, 52, and 72 h. Blood was collected from groups one and five at 1, 12, and 24 h; groups two and six at 2, 50, and 72 h; groups three and seven at 4, 26, and 48 h; and group four and eight at 8, 28, and 52 h. Terminal blood samples were collected by cardiac puncture on mice anesthetized with isoflurane at time points 24, 48, 52, and 72 h. All samples were collected into ethylenediaminetetraacetic acid vials.

Skin samples were collected only at the 72-h time point which included groups two and six. The tissue samples were collected from the scapular area and fixed in 10% buffered formalin.

Histopathology analysis

Mice were humanely euthanized and evaluated grossly for skin lesions. Skin from the injection site was collected, oriented flat on an index card, and fixed in 10% neutral buffered formalin. The tissue was then processed routinely, embedded in paraffin, sectioned at four microns, stained with hematoxylin and eosin stain, and evaluated by a pathologist (KLB), board certified by the American College of Veterinary Pathologists, experienced in the examination of murine skin samples.

Statistical analysis

Plasma concentrations between time points were compared using the *t*-test with a two-tailed *P*-value. Table 1 is a comparison of the $C_{max,obs}$ and $C_{min,obs}$ mean plasma concentration values. Table 2 is a comparison between genders of each $C_{max,obs}$ and $C_{min,obs}$ mean plasma concentration values. Paired *t*-tests were used for serial data points when comparing 2–50 h (Table 1); unpaired *t*-tests with Welch's correction were utilized for nonserial data which involved all other time point comparisons (Tables 1 and 2).

Plasma analysis of meloxicam concentrations

Standards of meloxicam were prepared by diluting a fresh 1 mg/mL dimethyl sulfoxide solution into blank mouse plasma followed by further plasma dilutions to give a range of 0.5–10 000 ng/mL. Twenty microliter volumes of standard and study plasma samples (diluted 10-fold into mouse plasma) were then precipitated with four volumes of ACN containing 50 nM piroxicam as an internal standard. Samples were next centrifuged at 4000 ***g*** in a refrigerated centrifuge (4 °C) for 5 min. Sixty microliter volumes of the supernatant were then

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Table 1. Comparison of overall $C_{max,obs}$ and $C_{min,obs}$ mean meloxicam plasma concentration values

	Time point	Plasma concentration (ng/mL)	SD	Comparison	Time point	Plasma concentration (ng/mL)	SD	*P*-value
$C_{max,obs}$	2	920	189	vs.	26	3448	1156	<0.0001*
	26	3448	1156	vs.	50	3364	1422	0.8776
	50	3364	1422	vs.	2	920	189	<0.0001*,†
$C_{min,obs}$	24	1.35	1.55	vs.	48	1.57	0.765	0.6762
	48	1.57	0.765	vs.	72	1.14	0.695	0.2014
	72	1.14	0.695	vs.	24	1.35	1.55	0.6792

Unpaired *t*-test Welch's correction. SD, standard deviation. *Significant difference $P < 0.05$. †Paired *t*-test, serial sample.

Table 2. Comparisons of $C_{max,obs}$ and $C_{min,obs}$ mean meloxicam plasma concentration between genders following subcutaneous injection *q* 24 h

		Male mean values			Female mean values			
	Time point	Sample size	Plasma concentration (ng/mL)	SD	Sample size	Plasma concentration (ng/mL)	SD	*P*-value
$C_{max,obs}$	1	5	806	418	5	2000	342	0.0017*
	2	6	924	242	6	917	142	0.9493
	26	6	2828	981	5	4192	934	0.0462*
	50	6	2135	449	6	4593	789	0.0003*
$C_{min,obs}$	24	6	1.30	2.24	6	1.40	0.522	0.9134
	48	6	1.35	0.526	4	1.89	1.03	0.3955
	72	6	0.802	0.484	5	1.55	0.729	0.0970

Unpaired *t*-test Welch's correction. SD, standard deviation. *Significant difference $P < 0.05$.

added to an equal volume of water in a 96-well injection plate, sealed, and placed into a refrigerated autosampler for analysis.

Samples (10 μL) were analyzed by LC/MS/MS by injecting them onto a Shimadzu liquid chromatography system with Fortis Technologies Ltd 2.1 × 50 mm, 3 μm C18 column (Cheshire, UK) coupled to an AB Sciex 5500 mass spectrometer operating in positive mode (Framingham, MA, USA). The initial mobile phase conditions were 40:60 0.1% v/v formic acid in water:acetonitrile. After injection, a solvent gradient was initiated from 40% to 95% acetonitrile from 1.3 to 2.0 min, held for 0.5 min, and followed by a return to the starting conditions for equilibration. Mass spectrometer conditions were dwell time = 50 ms, declustering potential = 200 V, entrance potential = 10 V, collision energy = 30 eV, collision cell exit potential = 10 V, collision gas = 6, curtain gas 20 psi, ion spray voltage = 5000 V, and probe temperature = 500 °C. Retention times were 1.32 min for meloxicam (m/z 352→115; thiazole-amine ion) and 1.23 min for the internal standard piroxicam (m/z 332→121; pyridinyl amide acylium ion).

Three standard curves and three sets of quality controls samples (low and high) were run during study sample analysis (beginning, middle, and end of the run). The quality control samples of 50 and 500 ng/mL were within 10% of nominal values. The standard curves (0.5–5000 ng/mL) were fit to a quadratic function with $1/x^2$ weighting ($r = 0.987$). The lowest tested standard concentration was the lower limit of quantitation (signal-to-noise >10). Carryover was assessed by comparing peak areas of analyte and internal standard with a double blank injection and was found to be <1%.

PK analysis and simulation

Phoenix™ v6.2 (Pharsight/Certara, Princeton, NJ, USA) was used to perform pharmacokinetic (PK) analysis and simulation. Although the actual dose range was 1.4–1.8 mg/kg, PK analysis was conducted using the mean normalized dose of 1.6 mg/kg (Table 3) due to the need to perform the study in nonserial fashion. Extravascular noncompartmental PK was used to determine area under the curve ($AUC_{0\text{-}24}$) with the linear–log trapezoidal method. The mean elimination rate constant used to calculate half-life was determined from the 4, 8, 12, and 24 h samples after the first dose, and 2, 4, and 24 h for the second and third doses. Noncompartmental analysis of the observed single exponential plasma PK was conducted using the results from the first meloxicam dose where blood sampling was more intensive. Simulations of different absorption rates were conducted using the determined elimination rate constant of 0.312/h and V/F of 977 mL/kg.

RESULTS

All mice appeared healthy with no significant weight change throughout the 3-day study period. The skin at the injection site remained normal in appearance with no gross lesions evident, and microscopic examination revealed no lesions in the skin associated with drug administration.

Although blood samples were collected from six males and six females at each time point, insufficient sample volumes occurred

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Table 3. Pharmacokinetic parameters of meloxicam in mice receiving 1.6 mg/kg every 24 h

	AUC (ng·h/mL)			$C_{max,obs}$ (ng/mL)			$T_{max,obs}$			$t_{1/2}$ (h)		
Dose (h)	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female
1st (0 h)	5242	4228	6299	1403	924	2000	1.0	2.0	1.0	2.22	2.34	2.25
2nd (24 h)	13302	9532	16867	3448	2828	4192	2.0	2.0	2.0	1.98	2.03	1.97
3rd (48 h)	11891	6550	17208	3364	2135	4593	2.0	2.0	2.0	1.92	1.99	1.91

in several instances. At the 1-h time point, five male and five female samples were analyzed; at 2 h, six males and six female samples; at 4 h, six male and four female samples; at 8 h, six male and six female samples; at 12 h, five male and five female samples; at 24 h, six male and six female samples; at 26 h, six male and five female samples; at 28 h, five male and six female samples; at 48 h, six male and four female samples; at 50 h, six male and six female samples; at 52 h, six male and six female samples; and at 72 h, six male and five female samples.

The maximum observed concentration occurred 1 h after administration, and the lowest concentration occurred 24 h following injection (Fig. 1). In the samples collected 2 h after injection, the mean plasma concentration after the first dose was found to be significantly lower than the second (26 h) and third doses (50 h) (Table 1).

Male mice had a significantly lower plasma concentration than female mice at the 1, 26, and 50 h time points (Table 2). These concentrations were similar at the 2 h time point; however, there were no significant differences between the groups at the 24, 48, and 72 h (Table 2).

The area under the curve (AUC) was also found to be consistently higher for female mice compared to male mice (Table 3), but no differences were found in the trough concentrations (Table 2).

DISCUSSION

Meloxicam is an established analgesic agent for mice used in laboratory research. Despite the common use of subcutaneous (s.c.) injections of meloxicam in mice, s.c. pharmacokinetics information is currently limited (Kendall *et al.*, 2014). The reported s.c. doses have been found to have a roughly 10-fold variation, with a range of approximately 2–20 mg/kg being employed depending on the institution and surgical procedure (Leach *et al.*, 2012; Miller *et al.*, 2012). Dosing intervals as long as 24 h have been used for s.c. treatment of mice, although this species has demonstrated rapid plasma clearance from other dosing routes. The previously reported intravenous and oral plasma clearances of meloxicam in mice are approximately 10-fold higher than those found for rats and humans (Busch *et al.*, 1998). While rats and humans have half-lives that allow once-daily dosing, the clearance displayed by mice may require more frequent dosing to maintain analgesia. By characterizing the pharmacokinetics of a regimen of *q*24 h s.c., dosing of meloxicam for 3 days in mice, it is our intent to provide information that will enable investigators to achieve effective s.c. drug exposures. To this end, the pharmacokinetics were used to simulate meloxicam s.c. dosage regimens that provide coverage of the primary pharmacological target, cyclooxygenase-2 (COX-2), as suggested by published mouse efficacy studies.

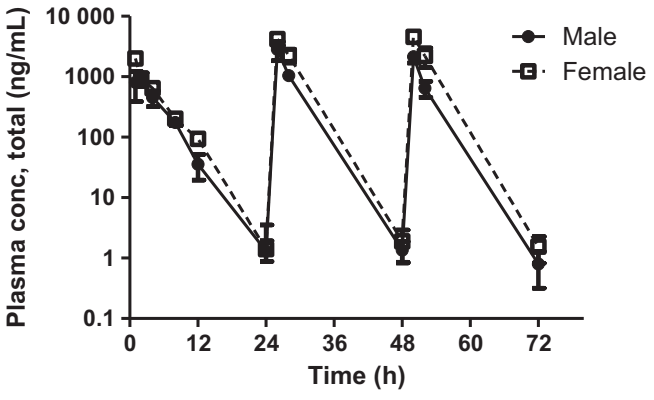

Fig. 1. Meloxicam plasma time–concentration (mean ± SD) plots for male and female mice following three 1.6 mg/kg subcutaneous doses given every 24 h.

Mouse PK with s.c. meloxicam was not unlike the published oral PK (Ingrao *et al.*, 2013) in that there was rapid absorption and a short half-life. A single elimination phase was observed. Dose normalized exposure was within~ twofold of published oral PK. This suggests good s.c. absorption as the published oral bioavailability is 94% (Busch *et al.*, 1998).

The most distinguishing PK characteristic of s.c. meloxicam in mice was its rapid plasma clearance (*CL*). This agrees with previous studies where mice were shown to have the highest reported i.v. and p.o. clearance when compared to rats, dogs, mini-pigs, baboons, and rabbits (Busch *et al.*, 1998; Carpenter *et al.*, 2009). Meloxicam is actually a low-extraction drug in all species, including mice, as defined by the calculated extraction ratio (*ER*) of 0.029 based on the i.v. *CL* and a hepatic blood flow of 90 mL/min/kg (Davies & Morris, 1993). However, the mouse *CL* is 10-fold higher than the CL in rats, dogs, and humans. At least one explanation for higher clearance, other than the high metabolic rate of mice, is the lower degree of plasma nonspecific binding. (Busch *et al.*, 1998).

With respect to gender, the results from this study showed that male mice had an overall trend of lower plasma concentrations compared to females at the C_{max}, but the half-life (Table 3) and trough (Table 2) concentrations were similar. These results suggest that the higher clearance rate in male mice did not alter the trough concentrations. This information

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implies that a gender-based dose adjustment would not be required if the trough concentration is used as the pharmacokinetic/pharmacodyamic index. Rats demonstrated a similar gender trend from oral dosing where male rats showed a lower plasma concentration, but unlike the mice, meloxicam in male rats had a shorter half-life when compared to that in female rats (European Agency for the Evaluation of Medicinal Products EMEA, 1997).

This study also suggests that the subcutaneous administration of meloxicam is well tolerated in C57BL6 mice, similar to dogs (Norbrook, 2015), rabbits (Stei *et al.*, 1996), and cattle (Vetoquinol, 2015). Pathologic changes were not observed at the injection site in the mice used in this study. These results were quite different than those documented in cats in which histologic changes of 'hemorrhage and inflammation, myofiber atrophy, panniculitis, fibrin deposition, and fibroblast proliferation' resulted from a single injection (Norbrook, 2015). It is important to note that the dilution of the commercial meloxicam drug product in our study may have contributed to the lack of injection site reaction observed in the study mice.

Once the s.c. PK and pathology were defined, a review of meloxicam pharmacology was initiated to determine if there is agreement between known efficacious doses, *in vivo* drug exposures, and coverage of COX-2. The challenge of performing such an assessment is that the *in vivo* translation of *in vitro* and *ex vivo* COX inhibition lacks standardization, and differences in assay methodology have led to interlaboratory variation in the determination of inhibition constants (Vane & Botting, 1995; Brooks *et al.*, 1999; Blain *et al.*, 2002). For this reason, whole blood assay approaches have been favored as they appear to be superior to cellular assays in predicting efficacy of drug exposures. For example, the efficacious doses of several COX inhibitors produce total *in vivo* plasma concentrations that are greater than the whole blood COX-2 IC_{80} (Warner *et al.*, 1999). Because the COX-2 enzyme sequence identity is 90% conserved across species (Guan *et al.*, 1997), human whole blood inhibition constants were used in the comparison of mouse dosage regimens and mouse efficacy studies (Table 4).

In fact, human whole blood COX-2 inhibition constants appeared to parallel published studies evaluating the expected range of plasma drug concentrations in mouse inflammation and pain. In one study, an ID_{50} oral meloxicam dose of 1.36 mg/kg/day (two doses, *q*8 h) was determined against zymosan that was used to induce inflammatory markers in the peritoneum (Engelhardt, 1996). Single-dose ED_{50} values for intraperitoneal (IP) meloxicam in mice have also been reported for writhing induced by acetic acid (2.6–6.5 mg/kg) (Santos *et al.*, 1998; Miranda *et al.*, 2006). If only modest differences (≤2-fold) in plasma concentrations related to dosing route and formulation are assumed, s.c. meloxicam in these dose ranges is expected to inhibit COX-2 as the whole blood IC_{50} (230–250 nM) would be achieved for approximately 12 h at only 1.6 mg/kg s.c. (Fig 2A). While we acknowledge some important assumptions in this retrospective analysis, it is proposed that known effective meloxicam doses for mice are reflective of a correlation between our observed plasma drug exposures and published whole blood inhibition constants.

Table 4. Reported *in vitro* COX-2 potency values for meloxicam

In vitro COX-2 assay	IC_{50} (nM)	Reference
WHMA[†] assay	230	Warner *et al.* (1999)
Human whole blood assay	250	Pairet *et al.* (1998) Inflammation Research, **47**, 270–276

[†]William Harvey human modified whole blood assay.

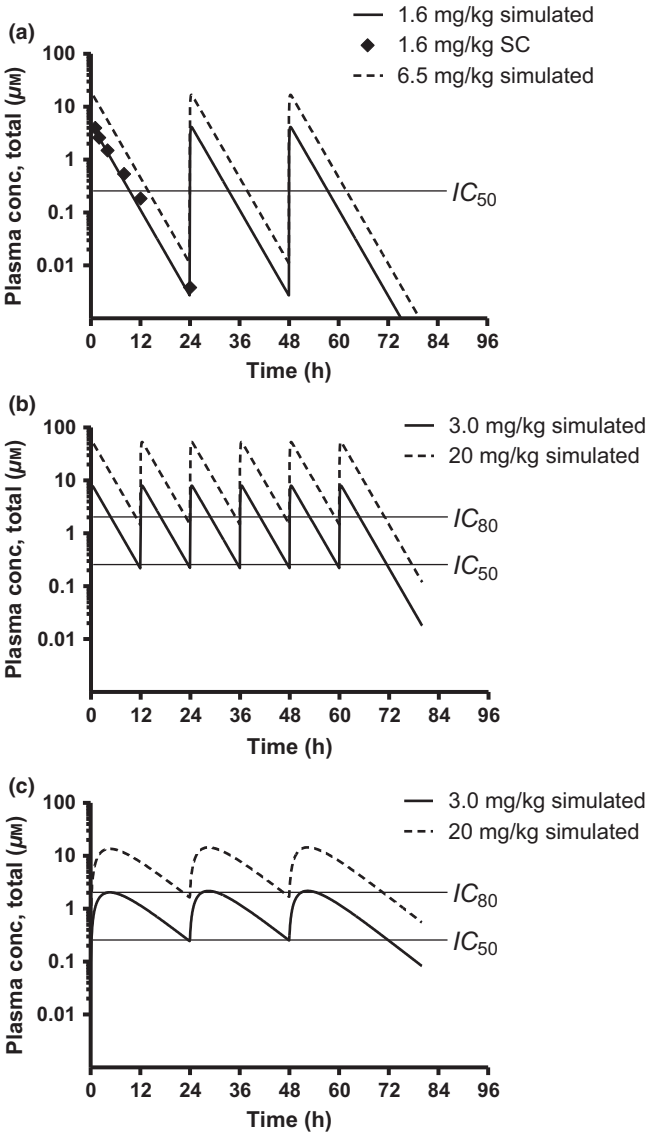

Fig. 2. PK simulation of meloxicam in the mouse based on mean plasma concentrations from 1.6 mg/kg SC injections. (a) Once-daily doses bracketing the effective doses (ED_{50} and ID_{50}) from published efficacy studies. (b) Twice-daily doses predicted to cover whole blood COX-2 IC_{50} and IC_{80}. (c) Once-daily doses with 24 h COX-2 coverage that would result from a theoretical delayed release formulation (T_{max} = 4.5 h). IC_{50} and IC_{80} values were taken from Warner *et al.* (1999).

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Pharmacokinetic simulation further suggests that to maintain analgesia, more than one dose per 24 h may be required (Fig. 2). It should be noted that these projections may only apply to the formulation studied here. The alternative of giving a higher dose once a day would lead to high C_{max} values that may be tolerated for acute treatment, but may also contribute to increased risk of adverse events. Simulation of the top reported s.c. dose of 20 mg/kg suggests the time above the whole blood IC_{50} would be extended to ~18 h assuming that the absorption rate remains unchanged at such a high dose (Leach *et al.*, 2012). Subcutaneous doses of 3 and 20 mg/kg delivered $q12$ h are estimated to cover the IC_{50} (12 h/dose) and IC_{80} (11 h/dose), respectively (Fig. 2B). Alternatively, the development of new formulations that modestly delay absorption without affecting the onset of action could allow for daily dosing. PK simulation suggested delaying T_{max} to just 4.5 h could provide coverage of COX-2 IC_{50} for 24 h/dose and IC_{80} for 23 h/dose when using s.c. doses of 3 and 20 mg/kg, respectively (Fig. 2C).

In summary, meloxicam remains a valuable analgesic agent in comparative medicine. Because the pharmacokinetic profile in mice is very different from that in rats and other species, the typically used dosage regimen should be further assessed. Mouse analgesic therapy would benefit from additional pharmacokinetic investigation.

Postsurgical analgesia often requires more than 1 day of therapeutic coverage, but we have shown that s.c. meloxicam is rapidly eliminated in mice and that $q24$ h regimens may not provide the sustained analgesia observed in rats. Admittedly, our conclusions weigh heavily on the use of human whole blood COX-2 inhibition, although COX-2 is a highly conserved enzyme across species. As a wide range of s.c. meloxicam doses are currently used in practice, we propose researchers consider modifying their preferred meloxicam dosing to $q12$ h if the increase in animal handling is tolerated. It is anticipated that any s.c. inflammatory response from a short-term increased dosing frequency would be minimal as no pathologic lesions were noted in the skin with daily s.c. administration over a 3-day period. While an efficacy endpoint was not included in our studies, these findings have highlighted a potential deficiency in the way meloxicam analgesia is provided to mice. Future multidose efficacy studies that confirm analgesia over multiple days will help solidify postoperative dosing guidelines for meloxicam in mice.

COMPETING INTERESTS

The authors state that there are no completing interests related to this study.

AUTHORS CONTRIBUTIONS

PHC secured funding, designed the study, performed data collection, and assisted with the draft of the manuscript. CWL performed all pharmacokinetic analysis, the statistical analysis, and assisted with the draft of the manuscript. EKF provided project design input and performed data collection. KLB conducted the histopathology analysis and contributed the pathology description in the manuscript. All the authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

Wildlife Pharmaceuticals, Inc. and Vanderbilt University Medical Center departments provided funding for this study. Dr. Scott Daniels provided guidance on the pharmacokinetic analysis and project design. Atef Khalil and Anne Pate provided pathology support. Printha McCallum and Sherry Smith provided administrative support. Elizabeth Merritt provided manuscript editing support.

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