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

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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 Animals 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 for providing analgesia for procedures expected to cause more than momentary pain or distress. Based on the search of publicly 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.
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 thiovirus, 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, Sigphacia).

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 were collected from the scapular area and fixed in 10% buffered formalin. 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.

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 $C_{\text{max obs}}$ and $C_{\text{min obs}}$ mean plasma concentration values. Table 2 is a comparison between genders of each $C_{\text{max obs}}$ and $C_{\text{min obs}}$ 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 ng 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
added to an equal volume of water in a 96-well injection plate, sealed, and placed into a refrigerated autosampler for analysis. Samples (10 \( \mu L \)) were analyzed by LC/MS/MS by injecting them onto a Shimadzu liquid chromatography system with Fortis Technologies Ltd 2.1 × 50 mm, 3 \( \mu m \) 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 (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 (AUC0-24) 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 1.** Comparison of overall \( C_{\text{max,obs}} \) and \( C_{\text{min,obs}} \) mean meloxicam plasma concentration values

| Time point | Plasma concentration | SD | Comparison | Time point | Plasma concentration | SD | \( P \)-value |
|------------|----------------------|----|------------|------------|----------------------|----|-------------|
|            | \( \text{ng/mL} \)   |    |            |            | \( \text{ng/mL} \)   |    |             |
| \( C_{\text{max,obs}} \) | 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* |
| \( C_{\text{min,obs}} \) | 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 \( P < 0.05 \).

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

| Time point | Sample size | Plasma concentration | SD | Sample size | Plasma concentration | SD | \( P \)-value |
|------------|-------------|----------------------|----|-------------|----------------------|----|-------------|
| \( C_{\text{max,obs}} \) | 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 | 4939 | 789 | 0.0003* |
| \( C_{\text{min,obs}} \) | 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 \( P < 0.05 \).
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

| 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 |

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

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.
implies that a gender-based dose adjustment would not be required if the trough concentration is used as the pharmacokinetic/pharmacodynamic 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 C57BL/6 mice, similar to dogs (Norbrook, 2015), rabbits (Stein 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 IC50 (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 ID50 oral meloxicam dose of 1.36 mg/kg/day (two doses, q8 h) was determined against zymosan that was used to induce inflammatory markers in the peritoneum (Engelhardt, 1996). Single-dose ED50 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 IC50 (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 | IC50 (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 (ED50 and ED80) from published efficacy studies. (b) Twice-daily doses predicted to cover whole blood COX-2 IC50 and IC80. (c) Once-daily doses with 24 h COX-2 coverage that would result from a theoretical delayed release formulation (Tmax = 4.5 h). IC50 and IC80 values were taken from Warner et al. (1999).
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 Cmax 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 IC50 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 IC50 (12 h/dose) and IC80 (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 Tmax to just 4.5 h could provide coverage of COX-2 IC50 for 24 h/dose and IC80 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 competing 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.

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