ORIGINAL ARTICLE

Pharmacology of enflicoxib, a new coxib drug: Efficacy and dose determination by clinical and pharmacokinetic-guided approach for the treatment of osteoarthritis in dogs based on an acute arthritis induction model

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Abstract

Enflicoxib is a newly developed NSAID of the coxib class. The optimal therapeutic dose to be confirmed in the field studies was established using a combination of pharmacokinetic (PK) modelling and pharmacodynamic (PD) studies. First, a PK study was performed to determine the plasmatic profile of enflicoxib and its active pyrazol metabolite in dogs. Thereafter, two studies using a urate crystal-induced acute arthritis model allowed to correlate efficacy with plasmatic concentrations. Finally, a population PK model was developed to establish the Minimum Effective Concentration (MEC) and the Maximum Tolerated Concentration (MTC). Enflicoxib plasma concentrations were highest for the first 48 h. Thereafter, pyrazol metabolite concentrations were higher and persisted up to the end of the study. No reduction on the lameness (CLS) or pain scores (PS) was observed in the first hours after enflicoxib administration so no MEC could be established for the parent compound. Both CLS and PS were greatly reduced when the pyrazol metabolite achieved concentrations of 411 ng/ml or higher, so this concentration was established as the MEC for the pyrazol metabolite. Enflicoxib MTC was established at 6723 ng/ml whereas for the pyrazol metabolite it was 4258 ng/ml. The population PK model showed that a loading enflicoxib dose of 8 mg/kg followed by weekly maintenance doses of 4 mg/kg would achieve stable concentrations of the pyrazol metabolite within the therapeutic window (between the MEC and the MTC), and it was considered the most adequate posology to be confirmed in the field clinical studies for the treatment of canine osteoarthritis.

KEYWORDS
dogs, enflicoxib, NSAID, osteoarthritis, pharmacokinetic, synovitis, urate
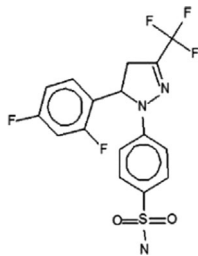
FIGURE 1 Chemical structure of enflicoxib

1 | INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are an essential therapeutic class both in human and veterinary medicine. It is now well established that most side effects of NSAIDs, especially those of the gastrointestinal tract, are related to their anti-cyclooxygenase-1 (COX-1) activity while the therapeutic effects are mainly associated with cyclooxygenase-2 (COX-2) inhibition. This has led to the development of preferential or selective COX-2 inhibitors (coxib class) to improve its safety profile (Brune & Patrignani 2015).

Osteoarthritis (OA), also referred to as degenerative joint disease, is a commonly encountered condition or disease process in canine veterinary practice. Therapeutic strategies against canine OA are multimodal and directed towards slowing the progression of the disease and controlling pain and inflammation. In this strategy, NSAIDs are frequently used as a first-line treatment due to their rapid palliating effect on pain caused by the condition (Innes et al., 2010).

Enflicoxib (also known by its research acronym E-6087) is a new pyrazoline derivative NSAID of the coxib class (see Figure 1). It has been described in rats, that the pyrazoline ring of enflicoxib is oxidised forming an active persistent pyrazol metabolite, suggesting the later to be the main contributor to the long-term activity of enflicoxib (Reinoso et al., 2001).

The selection of the therapeutic dosage of enflicoxib to be tested in the field clinical studies for the treatment of pain and inflammation associated with canine osteoarthritis has been achieved using the existing information on its safety, pharmacokinetics (PK) and pharmacodynamics (PD) in combination with the results of the new studies described herein.

The safety of enflicoxib was studied in a 4-week toxicity study at three dose levels administered daily to Beagle dogs (data not shown). Clear signs of toxicity, mainly gastrointestinal, were seen when enflicoxib was orally administered at 15 mg/kg/day. At 4 mg/kg/day, only occasional presence of occult blood in faeces and a reversible decrease in erythrocyte count, proteins and haematocrit was observed. The dose of 1 mg/kg/day was identified as the Non-Observed Adverse Effect Level (NOAEL). In this study, a toxicokinetic analysis of enflicoxib was performed but blood levels of the pyrazol metabolite were not determined. Therefore, in this article, it is described how safe pyrazol metabolite levels were estimated using population PK modelling.

In a PK study in Beagle dogs (Reinoso et al., 2001), enflicoxib peak plasma concentration (C_{max}) was achieved between 4 and 8 h (T_{max})

after oral administration at 5 mg/kg, and the PK profile was characterised by a long terminal half-life ($T_{1/2}$) (27.8 h). Pyrazol metabolite levels were not measured in this study, so no information on the PK profile in dogs was available at this stage of research. A new PK study is described here that includes determination of the parent and the pyrazol metabolite and the results are used to establish the population PK model.

In the PD studies, it was described that both enflicoxib and its pyrazol metabolite are potent inhibitors of the COX-2 enzymatic activity in vitro. Enflicoxib is more than 100-fold selective for COX-2 versus COX-1 (IC_{50} 2.93 μM and 334 μM, respectively), and the pyrazol metabolite is more than 250-fold selective (IC_{50} of 0.5 μM and 134 μM, respectively), as measured in COX-2 enzymes isolated from sheep placenta and COX-1 enzymes isolated from rat seminal vesicles (Wagemakers et al., 2009). The efficacy and selectivity of both compounds was also demonstrated in vivo by measuring prostaglandin E_2 (PGE$_2$) production in rat carrageenan-induced inflammatory exudates and in rat gastric mucosa samples (Wagemakers et al., 2009; Iñiguez et al., 2010).

This information, combined with the new PK data generated, allowed the selection of the tentative dose used in the anti-inflammatory/analgesic activity studies in dogs with experimentally induced arthritis.

Experimentally, a four-step process was followed. First, a PK study in dogs was performed to determine plasma levels of enflicoxib and its pyrazol metabolite. Second, two studies using an experimentally induced acute arthritis model in Beagle dogs, with determination of plasma levels of enflicoxib and its pyrazol metabolite were performed, and PK and efficacy relationships were established. Third, a PK model was developed by means of population approach (population PK model) to characterise the PK profile of enflicoxib and its pyrazol metabolite using plasma samples from the previous studies in dogs. Finally, the efficacy and population PK model were applied to define the therapeutic window and ultimately to establish the optimal therapeutic dose and treatment interval of enflicoxib to be used in the field clinical studies with dogs with naturally occurring OA.

2 | MATERIALS AND METHODS

All study procedures described herein were checked and approved by the Animal Experimentation Ethics committee of the research centres involved.

2.1 | Pharmacokinetic study

Considering the results of the previous toxicity study in dogs, a single dose PK study was performed at two dose levels, 1 and 4 mg/kg. The study followed current European guidelines for PK studies (EMA, 2000) and was designed to obtain a profile of the plasmatic concentrations of enflicoxib and, particularly, of its pyrazol metabolite, in Beagle dogs.

Eight healthy Beagle dogs (2 males and 2 females per each dose group) of approximately 2 years old and bodyweight between 7 and 11 kg were housed indoors in climate-controlled facilities in accordance with accepted laboratory animal care and use guidelines. Enflicoxib was administered in gelatine capsules containing the adjusted quantity of micronised product in the morning of day 1 at a single oral dose of 1 or 4 mg/kg. Adequate swallowing of the capsule was ensured by administering 5 ml of water after product administration. The animals had free access to water but were fasted overnight and until 4 h post-administration.

Blood samples (3 ml) were collected from the jugular vein on day 1 at pre-dose, 0.25, 0.5, 1, 2, 4, 8 and 12 h post-administration. From days 2 to 21, one sample was taken each day at 24 h intervals from the pre-dose sampling time of day 1. Blood was collected into K_3-EDTA tubes and centrifuged at 2500 g for 15 min at 4°C. The resulting plasma was frozen at −80°C until analysis.

The PK parameters were obtained for each animal by non-compartmental approach using WinNonlin Professional version 5.3 (Pharsight Corporation. 1699 S. Hanley Road, St. Louis, MO 63144, USA). C_{max} and T_{max} were observed values. $T_{1/2}$ was calculated by linear regression of the terminal phase of the curve (log concentration vs. time). The area under the concentration curve of plasma levels versus time from zero to 24 h (AUC_{24h}) or to last measurable concentration (AUC_{last}) were calculated by the linear trapezoidal method. The AUC from zero to infinity (AUC_{inf}) was the sum of AUC_{last} and the extrapolation after the last measurable concentration (dividing last measurable concentration by terminal rate constant).

2.2 | Anti-inflammatory/analgesic activity in an acute arthritis induction model in dogs

Two studies, one single dose and one repeated dose, were performed to evaluate the anti-inflammatory/analgesic activity of enflicoxib, by using a well-established in vivo arthritis induction model for testing NSAIDs efficacy in dog (Toutain et al., 2001; Borer et al., 2003, Dauteloup et al., 2017).

Acute arthritis was induced through inoculation of a sodium urate crystal suspension in the stifle (femorotibial) joint of the dog. The intra-articular injection results in a transient inflammatory/painful reaction leading to a severe lameness detectable from 2 h post-inoculation for a total duration of 8–10 h, approximately, and an average time of maximum lameness 2–3 h after induction (Borer et al., 2003). After an NSAID administration, partial or total inhibition of pain and lameness during this period would be attributable to its pharmacological effect.

On each day of arthritis induction, the anti-inflammatory/analgesic activity of the treatments was assessed at different time points (just before the inoculation, and 0.5, 1, 2, 3, 4, 6, 9 and 12 h later). Two clinical signs of arthritis were investigated: lameness (while standing and walking) and pain during stifle joint palpation.

Signs of pain during palpation were scored (PS) as described for efficacy evaluation of other NSADs (Millis et al., 2002; Borer et al., 2003; Dauteloup et al., 2017). Lameness was scored while standing accord-

TABLE 1 Semi-quantitative scale for scoring lameness (CLS) and pain (PS)

Lameness while standing	Score
Full weight bearing	0
Partial weight bearing	1
No weight bearing to toe touching	2
Lameness while walking	**Score**
Full weight bearing, no lameness	0
Slight lameness (including intermittent) with partial weight bearing (75%)	1
Severe lameness with partial weight bearing (≥50%)	2
Severe lameness with no weight bearing	3
Signs of pain	**Score**
No sign of pain during palpation	0
Signs of mild pain (e.g. turned head in recognition)	1
Signs of moderate pain (e.g. pulled limb away)	2
Signs of severe pain (e.g. vocalised or became aggressive)	3
Did not allow limb palpation	4

ing to semi-quantitative scales previously described (Dauteloup et al., 2017; de Salazar Alcalá et al., 2019). The combined visual lameness score (CLS), determined as the sum of the standing and walking lameness scores, was also calculated. All investigators responsible for the assessments were blinded to treatment groups. The scoring system is described in Table 1.

The anti-inflammatory/analgesic activity was assessed comparing the intensity and duration of pain and lameness obtained after each induction to those described in the published literature (Dauteloup et al., 2017; de Salazar Alcalá et al., 2019). Blood samples were drawn from the jugular vein at different time points (including the time of each intra-articular injection of urate crystals) for the determination of enflicoxib and pyrazol metabolite concentrations and processed as described for the PK study.

The experimental design allowed to correlate the obtained activity scores with the plasma concentrations of enflicoxib and the pyrazol metabolite at different time points (see Minimum Effective Concentration calculation in the Therapeutic window section).

To avoid interferences, dogs did not receive any anti-inflammatory or opiate drugs during the 2 weeks prior to the in-life phase. The intra-articular injections were performed with a sufficient recovery period between injections as required for welfare reasons. Likewise, injections were performed alternatively in the right and the left stifle at different days.

2.3 | Single dose arthritis induction study

The study was performed with 10 male Beagle dogs 23–36 months old and weighing from 9 to 12 kg. Six dogs were allocated to initially receive 4 mg/kg of enflicoxib (Daxocox®, Ecuphar/Animalcare) on day 0, and

three dogs to receive 2 mg/kg of mavacoxib (Trocoxil®, Zoetis) as reference product, on days 0 and 14, as recommended by the manufacturer. One dog was left untreated to confirm the model induced pain and lameness according to previous experience of the experimental team and to what is described in the published literature.

The intra-articular injections of urate crystals were performed on days 0, 2, 7, 14 and 21. On day 0, the injection was performed 2 h after product administration. The enflicoxib treated animals received a second dose of 4 mg/kg on day 12 to evaluate the effect of the accumulation of both doses following intra-articular injections on days 14 and 21.

2.4 | Repeated dose arthritis induction study

The results of the previous study suggested that the administration of an initial higher dose (loading dose) on the first day of treatment could help to rapidly achieve effective metabolite blood concentrations, which could be maintained by a 1-week dosing interval.

This hypothesis was tested in this second study, conducted with 12 male Beagle dogs from 13 to 35 months old and weighing from 9 to 13 kg. Dogs received a double dose of enflicoxib on day 0 (8 mg/kg) as loading dose and thereafter weekly maintenance doses of 4 mg/kg on days 7, 14 and 21. During the study, intra-articular injections of urate crystals were performed on days 0 (2 h after treatment administration), 2, 4, 9, 14, 21, 23 and 28.

2.5 | Analytical method

Plasma concentrations of enflicoxib and its pyrazol metabolite were measured by a validated HPLC- method coupled with MS/MS detection (API4000 triple mass quadrupole from AB Sciex).

Briefly, enflicoxib and its metabolite with the internal standard celecoxib were extracted from dog plasma by solid phase extraction (SPE) on Oasis MCX 30 μm 96-well plate, 10 mg (Waters). The SPE method consists in conditioning of sorbent with methanol (500 μl) and water (500 μl). The wells were loaded with 200 μl of sample and 300 μl of 4% phosphoric acid. The samples were washed with 2% formic acid in water and with water/methanol 60/40 (v/v), eluted with 250 μl of methanol and dilute with 100 μl of 0.1% formic acid in water and mix for 5 min.

The compounds were then separated on Luna PFP(2), 50 × 4.6 mm, 3 μm column (Phenomenex), in gradient mode elution using 0.1 % formic acid in water/methanol as a mobile phase at a flow rate of 1 ml/min. The peaks corresponding to enflicoxib, its metabolite and celecoxib were quantified by MS/MS detection.

Enflicoxib, its pyrazol metabolite and the internal standard (celecoxib) were detected by a API4000 triple quadrupole mass spectrometer in negative electrospray ionisation using multiple reaction monitoring (MRM) mode. Monitored analyte transitions were m/z 404.0 > 169.9, 402.1 > 318.0 and 380 > 316.1, respectively. The ion spray voltage was set at −4500 V, the temperature for the solvent

evaporation was established at 550°C. The entrance potential was −10 V and the dwell time was 250 ms for the analytes and 100 ms for the internal standard. The declustering potentials, collision energies and collision exit potentials were: −105, −36 and −11 V for enflicoxib, −90, −32, −7 V for the metabolite and −100, −30 and −17 V for celecoxib, respectively. The purity of enflicoxib, pyrazol metabolite and celecoxib ranges from 92.2% to 100%. The validation of the analytical method described that it is selective, accurate, precise, reproducible and linear over the concentration range of 5.0–1000 ng/ml for enflicoxib and 2.5–1000 ng/ml for the pyrazol metabolite with an overall precision below 14.2% and overall accuracy (expressed as % of nominal value) ranges from 85.0% to 116.1%. The concentrations of analytes in each sample were calculated from the regression equation of the calibration curve analysed with each analytical batch.

2.6 | Population PK approach

The development of a population PK model of enflicoxib and its pyrazol metabolite included all available PK data in dogs. The population PK model allowed to establish the 'Therapeutic Window' by defining the Minimum Effective Concentration (MEC) and Maximum Tolerated Concentration (MTC) and, in combination with the efficacy/safety profile at different dose schemes, provided an efficacy and PK rationale to select the optimal oral posology for the clinical development of enflicoxib.

Three different datasets were used:

- Toxicokinetic (TK) dataset: The time course of enflicoxib levels on days 1 and 28 from a toxicity study at both the NOAEL daily dose of 1 mg/kg and the reasonably safe daily dose of 4 mg/kg/day were used to establish the MTC of parent drug. A total of 228 PK samples of enflicoxib from 12 Beagle dogs were included in the dataset (data not shown). The pyrazol metabolite exposure was not measured in this study.
- Pharmacokinetic (PK) dataset: The time course of enflicoxib and pyrazol metabolite plasma concentrations from the PK and the two efficacy studies described above were included in the PK dataset. Thus, a total of 341 samples for both parent and pyrazol metabolite from 26 Beagle dogs were included in this dataset.
- Efficacy dataset, which was used to determine the MEC, corresponds to the response of combined lameness visual scores on each day of experimentally induced acute arthritis versus the observed plasma levels obtained on the same day. A total of 18 enflicoxib-treated animals, which had at least one pair of enflicoxib or pyrazol metabolite levels and a lameness score at the same time, were included in the efficacy dataset, providing a total of 71 paired PK/Efficacy observations.

Enflicoxib and pyrazol metabolite concentrations were log-transformed to handle non-normality distribution of residuals. Concentrations below the limit of quantification (BLQ) of both compounds were retained as censored data, to implement the M3 approach in

NONMEM version 7 (Beal et al., 2009). This approach allowed to fit simultaneously the continuous (measurable levels) and categorical data (BLQ), using likelihood estimation only for BLQ data.

The PK data of enflicoxib and the pyrazol metabolite along with covariate information (weight, age, sex and dose level) were analysed by NONMEM, using PsN software (Lindbom et al., 2004) as an interface, to estimate the population mean parameters, inter-individual and residual random effects. Pharmacokinetic data from all animals were fitted simultaneously but preserving their individuality.

All models were fitted using the First Order Conditional Estimation method with INTERACTION (FOCEI) or LAPLACIAN estimation method when BLQ data were included in the PK analysis. Outputs and graphical plots were processed using Xpose (Jonsson & Karlsson, 1999) or R-language (Free Software Foundation, Boston, MA, USA).

Model selection was guided by plausibility of the estimates, visual inspection of diagnostic plots, the precision of parameter estimates and the difference in the Minimum Value of the Objective Function (MVOF). A model was declared superior to an alternative nested model at the levels of significance of 5% or 0.5% when difference in MVOF was reduced by ≥ 3.84 or 7.88 points, respectively (Likelihood Ratio Test). In addition, the shrinkage, which should be below 30%–40%, was determined for all random effects to ensure that there was no overfit (ε-shrinkage) and to inform about the relevance of employing empirical Bayesian estimations during covariate analysis (η-shrinkage).

Enflicoxib and pyrazol metabolite disposition was characterised by compartmental models parameterised in terms of apparent volumes of distribution along with distribution and elimination clearances. In addition, due the volume of distribution of the pyrazol metabolite was not identifiable, it was assumed to be the same as that of enflicoxib, considering the structural similarity between compounds

In the PK models, the clearance of enflicoxib was supposed to occur from the central compartment by two elimination pathways (as first order processes): a fraction of enflicoxib was irreversibly converted to the pyrazol metabolite; meanwhile the main fraction of enflicoxib was eliminated by other metabolic and/or excretion pathways.

Inter-individual variability (IIV) associated to the PK parameters was modelled assuming a log-normal distribution as follows:

$$P_i = P_{pop} \cdot e^{\eta},$$

where P_i represents the value of PK parameter for individual i; P_{pop} is the typical population estimate for the PK parameter and η is a random variable that accounts for the deviation between individual value (P_i) and typical population value (P_{pop}). The set of η are assumed to be independent and symmetrically distributed around 0 and variance ω^2. The magnitude of IIV was expressed as coefficient of variation (%CV).

Residual variability, which accounts for random unexplained discrepancies between observed and individual predicted concentrations, was modelled using a log error model as shown below:

$$\ln C_{ij} = \ln C_{pred,ij} + \varepsilon_j,$$

where C_{ij} and $C_{pred,ij}$ represent the jth observed and model predicted enflicoxib or pyrazol metabolite concentrations, respectively, for individual i. ε_{ij} denotes the additive residual random error for individual i and observation j. The ε random effects are assumed to be independent and symmetrically distributed with mean of zero and variance equal to σ^2. The magnitude of residual variability was expressed as %CV.

Covariate analysis was performed to identify additional variables that were able to explain part of the inter-individual variability seen in the parameter estimates of the base PK model. In this population PK analysis, the available continuous covariates were weight and age, and the available categorical covariates were gender and the dose level.

The development of covariate PK model was performed in two stages. The first stage consisted in including the total body weight in all PK parameters that corresponds to clearances and volume of distributions using the following allometric equation:

$$P_{POP} = \theta_1 \cdot \left(\frac{WGT}{9.9} \right)^{\theta_2},$$

where P_{POP} represents the typical value of a PK parameter, WGT represents the individual weight, 9.9 is the median total body weight; and θ_1 and θ_2 corresponds to the allometric coefficient and allometric exponent, respectively. The allometric exponent (θ_2) was estimated, but it could be fixed to the physiological value (0.75 or 1.00 for clearances and volumes of distribution, respectively) if the estimation of exponent (θ_2) was similar to physiological value.

The second step consisted to investigate if any of the PK parameters was affected by other animal covariates. These covariates were initially screened for inclusion using covariates plots and applying Generalised Additive Modelling (GAM) implemented in Xpose. The covariates found significant were then tested in NONMEM using the forward and backward procedure. Continuous covariates were normalised by the corresponding median value, and the PK parameters were regressed on the continuous covariates using a variety of models (linear model, power model, etc.). On the other hand, the inclusion of categorical covariates was formulated to obtain its own parameter estimate for each category using a fractional change model in case of categorical and dichotomous covariates.

The decision to include a covariate in a final model was based on (i) statistical significance of reduction of MVOF (3.84 and 7.88 points for forward and backward step, respectively); (ii) clinical relevance of the relationship; (iii) the relationship was required to be biologically plausible; (iv) an improvement in the precision of the parameter estimate and (v) reduction in inter-individual and/or residual variability.

Different strategies were used to evaluate the validity of the final population PK model:

- Diagnostic plots used to assess model goodness-of-fit included observed concentrations (OBS) versus population predictions (PRED), OBS versus individual predictions (IPRED), etc.
- Visual Predictive Check (VPC): Pharmacokinetic data of enflicoxib and its pyrazol metabolite for 1000 animals were simulated for each dosing schedule and compared with the observed dataset. The

median and 90% prediction intervals of simulated concentrations at times corresponding to the observed concentrations were calculated and plotted against observed levels, and a judgement was made about adequacy of the final model.

- Bootstrap: The confidence intervals of the final model parameter values were estimated using the non-parametric bootstrap approach in PsN. For the final PK model, 200 bootstrap datasets were generated by sampling randomly from the original dataset with replacement. Subsequently, data sets were fitted one at the time to the final population PK model. Then, the mean, median, standard error, coefficient of variation and the lower and upper 95% confidence intervals of the bootstrap PK parameter were calculated and compared with the estimates obtained from the final PK model.

Once the model evaluation was completed, the final PK model of enflicoxib and its pyrazol metabolite was established and then several Monte Carlo simulations were performed in order to reflect the expected range of variability in concentration values after administration of different dosing schedules with or without administration of the loading dose.

The 90% prediction intervals, the typical population PK profiles, the MEC for the pyrazol metabolite and the MTC for enflicoxib and pyrazol metabolite were displayed in one plot, in order to visually and numerically evaluate the viability of each dosing schedule checking additionally the percentage of the predicted profiles that were inside the therapeutic window. An ideal dosing schedule should provide high percentage of individual PK profiles above the MEC (> 90%) during the entire dosing interval at steady-state without exceeding both MTC (enflicoxib and pyrazol metabolite).

3 | RESULTS

3.1 | Pharmacokinetic study

The average PK parameters of enflicoxib and the pyrazol metabolite are summarised in Table 2, and drug concentrations versus time profiles after single oral doses of 1 or 4 mg/kg are shown in Figure 2.

After single oral administration, at either dose, enflicoxib plasma concentrations were higher than those observed for the pyrazol metabolite during the first 48 h and lower from 48 h to the end of the study.

For the pyrazol metabolite, T_{max} was 132 h (5.5 days) for both administered doses, indicating that the formation of pyrazol metabolite from enflicoxib was very slow. The overall mean C_{max} values were lower than for the parent compound, but the extent of exposure AUC_{last} was much higher attending to its much slower elimination. Due to the limited sampling times, the $T_{1/2}$ for the pyrazol metabolite could not be adequately determined, but the graphic profile shows a very slow terminal half-life (around 14 days).

When characterised by AUC_{24h}, the extent of systemic exposure of both enflicoxib and the pyrazol metabolite increased at increasing dose, suggesting dose-proportionality.

3.2 | Anti-inflammatory/analgesic activity in an acute arthritis induction model in dogs

High lameness scores and moderate pain was observed in the untreated dog. Scores ranging from 1 to 5 for CLS and from 1 to 2 for PS were observed from 2 to 6 h after induction on day 0, from 2 to 12 h on day 14 and from 2 to 12 h on day 28, thus confirming the induction model gave the expected level and duration of pain and lameness and there was no habituation effect. The average scores of the three induction days on this animal were taken as reference to compare the evolution of the scores obtained in the treated dogs (see Figure 3).

3.3 | Single dose arthritis induction study

On day 0, pain and lameness while standing and while walking was observed in all enflicoxib treated dogs from 3 to 6 h after induction (5 to 8 h after treatment), with a maximum intensity observed 3–4 h post-induction (median CLS: 4 to 5 at 3–6 h post-induction and median PS: 1 to 2 at 3–9 h post-induction). All parameters scored similarly after the inductions on days 2 and 7, with no significant effects attributable

TABLE 2 Mean (±SD) pharmacokinetic parameters of parent and pyrazol metabolite after single oral administration of enflicoxib to dogs

Parameter	Enflicoxib		Pyrazol metabolite	
Dose (mg/kg)	1	4	1	4
$T_{1/2}$ (h)	31.0 ± 13.6	29.3 ± 16.2	ND	ND
T_{max}[†] (h)	6.0 (4.0–24.0)	18.0 (1.0–24.0)	132.0 (96–168)	132.0 (96–168)
C_{max} (ng/ml)	384.5 ± 208.2	872.1 ± 162.0	165.8 ± 48.5	577.0 ± 203.4
T_{last}[†] (h)	108.0 (96-312)	192.0 (96–408)	480	480
AUC_{24h} (ng·h/ml)	5279 ± 2301	$12,274 \pm 4334$	1104 ± 502	2647 ± 1322
AUC_{last} (ng·h/ml)	$14,465 \pm 10,796$	$46,653 \pm 35,341$	$40,784 \pm 10,228$	$132,329 \pm 33,065$
AUC_{inf} (ng·h/ml)	$14,924 \pm 10,860$	$47,047 \pm 35,377$	ND	ND

[†]Median value (range).
ND: not determined.
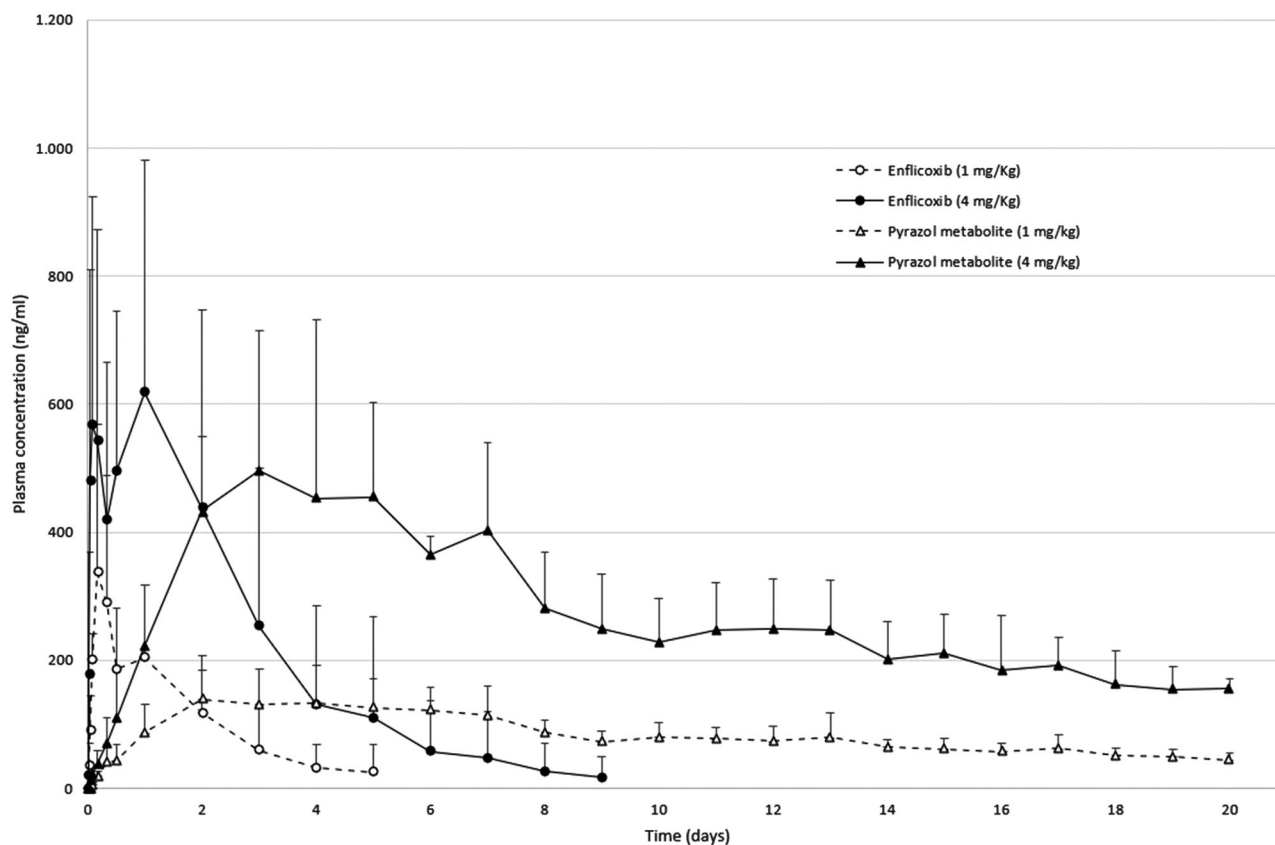
FIGURE 2 Graphical representation of plasma levels of parent and its active pyrazol metabolite after a single oral dose administration of enflicoxib at 1 or 4 mg/kg, in dogs (average ± SD)

to the treatment. Plasma concentrations within this time interval following single 4 mg/kg administration of enflicoxib were (average ± SD) 544 ± 327 ng/ml, 274 ± 189 ng/ml and 59 ± 41 ng/ml for enflicoxib, and 59 ± 34 ng/ml, 207 ± 191 ng/ml and 321 ± 241 ng/ml for the pyrazol metabolite, on days 0, 2 and 7, respectively.

After the second 4 mg/kg enflicoxib administration on day 12, none or very mild lameness or pain was observed after the induction on days 14 and 21, which was associated with higher plasma levels of the pyrazol metabolite (average ± SD: 492 ± 240 ng/ml and 398 ± 172 ng/ml on days 14 and 21, respectively). Plasma levels of enflicoxib on day 14 were 658 ± 496 ng/ml but, as expected, decreased sharply on day 21 (74 ± 85 ng/ml).

Likewise, in the mavacoxib group, a similar behaviour was observed. All dogs showed some degree of lameness and pain on days 0 and 7, and after the second administration on day 14 none or mild lameness or pain was observed after the induction on days 14 and 21.

3.4 | Repeated dose arthritis induction study

A severe reaction (median CLS: 2–5 between 3 and 6 h post-induction and median PS: 2 between 3 and 9 h post-induction), was observed after the arthritis induction on day 0, 2 h after the administration of

the 8 mg/kg loading dose. However, a great reduction in lameness scores was observed on days 2 and 4. Continuing the treatment regimen with the 4 mg/kg weekly maintenance doses, lameness progressively decreased in intensity and duration from 2 days after the first maintenance dose. Pain scores in treated animals remained similar to those in the control dog for a longer period, but 7 days after the fourth dose (day 21) both pain and lameness virtually disappeared (maximum effect). The duration and intensity of lameness (CLS) and pain (PS) during this study is shown in Figure 3.

Blood concentrations of enflicoxib were higher after the first loading dose and fell almost to basal levels before each weekly maintenance dose administration. Pyrazol metabolite levels tended to increase after each weekly administration reaching maximum values of 1748 ± 334 ng/ml on day 23. At the end of the experimental period (day 56, that is 28 days after last dose administration) pyrazol metabolite levels decreased to 348 ± 152 ng/ml.

No adverse reactions related to the enflicoxib administration were observed throughout the studies.

3.5 | Population PK model

The pharmacokinetics of enflicoxib and its pyrazol metabolite was best described using a two-compartment model for the parent compound
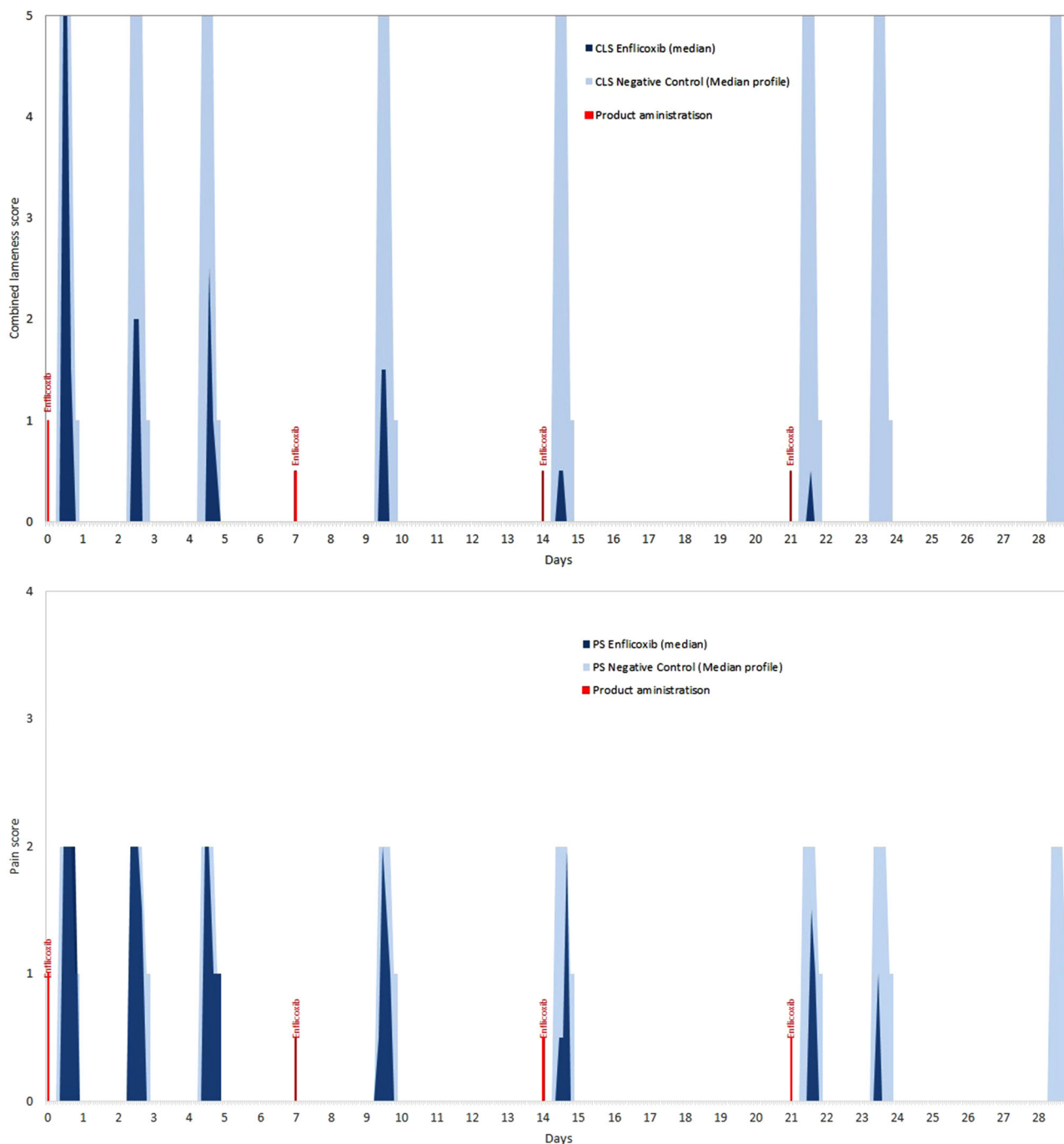
FIGURE 3 Combined Lameness Score (CLS) and Pain Score (PS) at different arthritis induction intervals (shadow areas) after administering enflicoxib at a loading dose of 8 mg/kg and maintenance dose of 4 mg/kg/week for 28 days

with first-order elimination and absorption (with lag time) along with a three-compartment model for the pyrazol metabolite with first order elimination (see scheme in Figure 4). Apart from total body weight, none of the remaining covariates showed a significant influence on parent or pyrazol metabolite PK behaviour.

The population PK parameters of the final PK model along with 95% confidence interval generated from the bootstrap are shown in Table 3. Bootstrap results confirm an adequate precision of the parameter estimates.

A total apparent enflicoxib clearance of 0.713 L/h (CL/F = CL1/F+CL2/F) was predicted by the PK model. The apparent clearance of pyrazol metabolite (CLM/F) provides the lowest value of elimination clearance (0.111 L/h), and thus became the rate-limited step. The estimated terminal half-life for both compounds was 1.4 and 13.8 days for parent and pyrazol metabolite, respectively. This result agrees with the assumption that the decline of pyrazol metabolite levels is controlled by its elimination and not governed by the elimination of the parent compound.
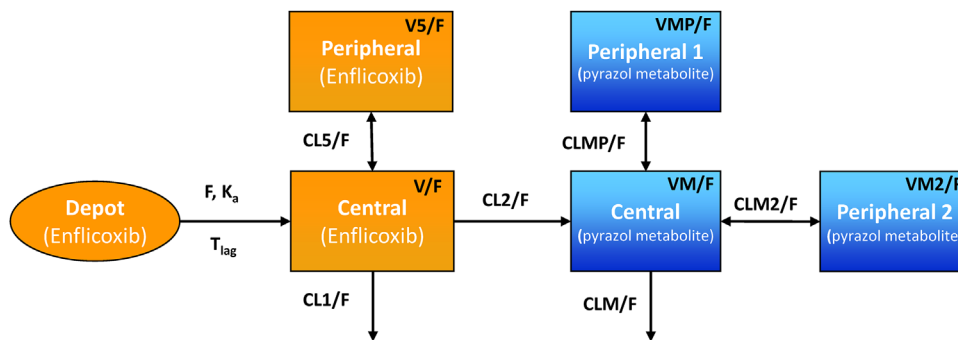
FIGURE 4 Schematic representation of the combined parent-pyrazol metabolite population pharmacokinetic model after oral administration of enflicoxib in dogs. [†] For parent enflicoxib: relative bioavailability (*F*); absorption rate (*K*ₐ); lag time (*T*lag); apparent volume of distribution in the central compartment (*V/F*); apparent volume of distribution in the peripheral compartment (*V5/F*); apparent clearance to other elimination pathways (*CL1/F*); apparent clearance of irreversible biotransformation to pyrazol metabolite (*CL2/F*) and apparent distribution clearance between central and peripheral compartment (*CL5/F*). [‡] For the pyrazol metabolite: apparent volume of distribution in the central compartment (*VM/F*); apparent volume of distribution in the shallow peripheral compartment (*VMP/F*); apparent volume of distribution in the deep peripheral compartment (*VM2/F*); apparent clearance (*CLM/F*); apparent distribution clearance between central and shallow peripheral compartments (*CLMP/F*) and apparent distribution clearance between central and deep peripheral compartments (*CLM2/F*)

Figure 5 shows that the influence of total body weight to increase PK exposure was more relevant for the pyrazol metabolite than for enflicoxib.

Overall, the established PK model adequately described the parent and pyrazol metabolite data. The basic goodness-of-fit plots revealed good agreement between the observed and the population- and individual-predicted values (PRED and IPRED, respectively), showing that most of the data points of parent and pyrazol metabolite were symmetrically distributed around the line of identity (Figure 6).

All terms of variability were reasonably distributed around the zero value (data not shown) with an adequate extent of shrinkage (lower than 40% for all parameters except for IIV on *T*lag).

The final PK model was further explored by means of VPC in Figure 7, where enflicoxib and pyrazol metabolite PK profiles were simulated and compared to the observed values. The model was considered adequate as most of the observations of both compounds fell within the prediction intervals and were randomly distributed throughout the typical PK profiles.

The benefits to include a loading dose in a weekly dosing schedule was also evaluated by PK simulations, as shown in Figure 8. While the steady state achievement (%ss > 90%) of enflicoxib is not affected by a loading dose, the pyrazol metabolite steady state is attained sooner when an initial enflicoxib loading dose is administered compared to dosing schedule without loading dose (6 and 7 weeks, respectively). In addition, the loading dose provided pyrazol metabolite levels much higher during the first 4 weeks of treatment.

3.6 | Therapeutic window

Combination of established population PK model with the available TK, PK and efficacy data allows to define the 'therapeutic window' of enflicoxib and the pyrazol metabolite, determining the MEC and the MTC.
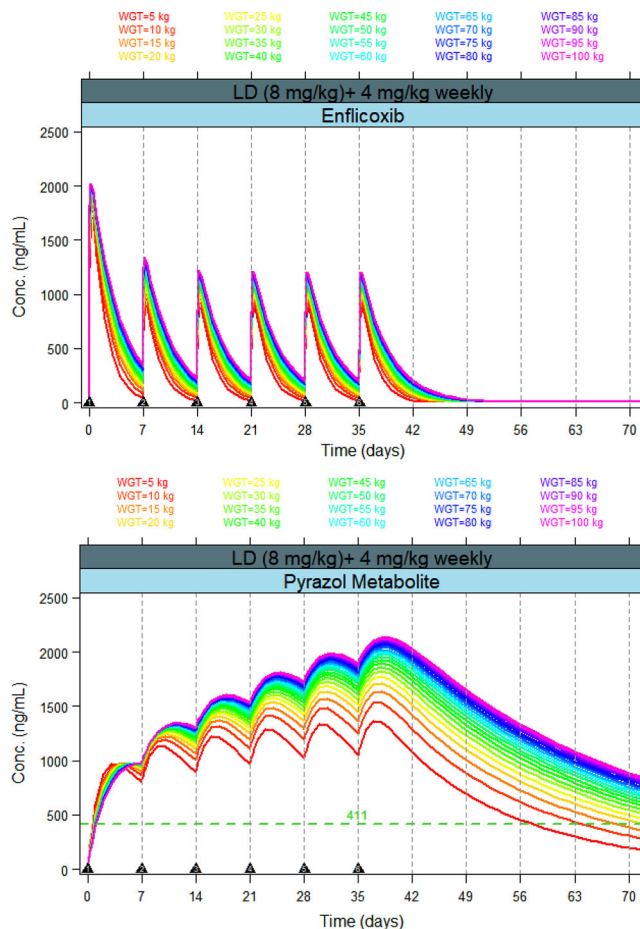
FIGURE 5 Influence of total body weight on the PK behaviour of enflicoxib (left panel) and pyrazol metabolite (right panel) after administration of loading dose of 8 mg/kg followed by a weekly maintenance dose of 4 mg/kg for 6 weeks

TABLE 3 Pharmacokinetic parameter estimates and bootstrap results for the final PK model of enflicoxib and pyrazol metabolite

Parameter	Units	Fixed parameters			Random parameters			
			Bootstrap			Bootstrap		
		Value	Median	(2.5%–97.5%)	Value	Median	(2.5%–97.5%)	Shr (%)
Enflicoxib								
Absorption								
Relative bioavailability (F)	–	1 FIX	–	–	–	39%	38%	(32%–41%)	-1%
Lag time (T_{lag})	h	0.249	0.248	(0.215–0.285)	68%	68%	(64%–83%)	51%
Absorption rate (k_a)	h^{-1}	0.220	0.220	(0.190–0.255)	101%	101%	(90%–130%)	38%
Distribution								
$V/F = \theta_4 \cdot (WGT/9.9)^{1.0}$					94%	94%	(81%–106%)	16%
θ_4	L	6.59	6.60	(6.15–8.13)				
$V5/F = \theta_{11} \cdot (WGT/9.9)^{1.0}$					–	–	–	–	–
θ_{11}	L	27.70	27.68	(23.63–31.39)				
$CL5/F = \theta_{10} \cdot (WGT/9.9)^{0.75}$					–	–	–	–	–
θ_{10}	L/h	12.1	12.17	(10.91–15.48)				
Elimination								
$CL2/F = \theta_6 \cdot (WGT/9.9)^{0.75}$					49%	49%	(46%–56%)	-5%
θ_6	L/h	0.520	0.513	(0.435–0.559)				
$CL1/F = \theta_5 \cdot (WGT/9.9)^{0.75}$					–	–	–	–
θ_5	L/h	0.193	0.193	(0.174–0.221)				
Pyrazol metabolite								
Distribution								
$VM/F = \theta_4 \cdot (WGT/9.9)^{1.0}$					–	–	–	–	–
θ_4	L	6.59	6.60	(6.15–8.13)				
$VMP/F = \theta_9 \cdot (WGT/9.9)^{1.0}$					–	–	–	–	–
θ_9	L	29.3	29.33	(26.33–32.89)				
$VM2/F = \theta_{13} \cdot (WGT/9.9)^{1.0}$					–	–	–	–	–
θ_{13}	L	13.4	13.5	(12.1–15.9)				
$CLMP/F = \theta_8 \cdot (WGT/9.9)^{0.75}$					–	–	–	–	–
θ_8	L/h	13.2	13.2	(12.3–15.5)				
$CLM2/F = \theta_{12} \cdot (WGT/9.9)^{0.75}$					–	–	–	–	–
θ_{12}	L/h	0.131	0.132	(0.116–0.164)				
Elimination								
$CLM/F = \theta_7 \cdot (WGT/9.9)^{0.75}$					31%	31%	(28%–39%)	28%
θ_7	L/h	0.111	0.111	(0.098–0.129)				
Residual variability								
Parent drug	%	–	–	–	–	34%	34%	(29%–39%)	8%
Pyrazol metabolite	%	–	–	–	–	20%	20%	(19%–23%)	

IIV, inter-individual variability; Shr, shrinkage; WGT, weight.

Although enflicoxib shows an evident in vitro COX-2 inhibition activity, the MEC could not be determined in this analysis, as no evidence of efficacy was observed on the first day of treatment in the arthritis induction trials described above, when levels of pyrazol metabolite are not yet significant. As the activity in further time points was mainly related to pyrazol metabolite levels, the MEC was only established for the pyrazol metabolite.

The pyrazol metabolite MEC was estimated by relating the combined lameness visual scores with the pyrazol metabolite levels obtained in dogs with experimentally induced acute arthritis. The
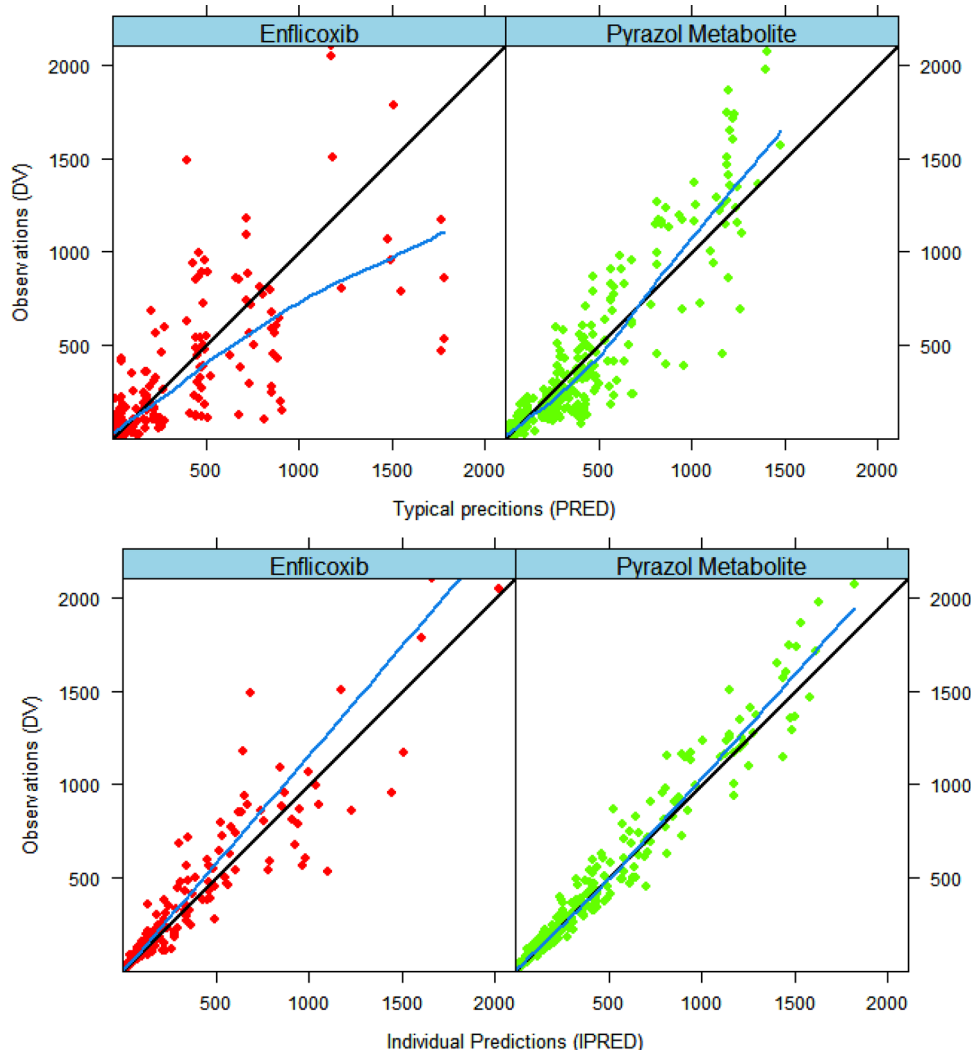
FIGURE 6 Basic goodness-of-fit plots for enflicoxib and pyrazol metabolite. Plotted lines show the line of identity (black) and the non-parametric smooth curve (blue)

pyrazol metabolite concentrations, sorted in ascending order, were divided in 5 bins (groups) containing approximately the same number of observations per group ($n = 14$–15). Then, the proportion of lameness for all scores could be determined for each bin (see Figure 9), considering that score values from 0 to 5 corresponded to absence, very low, low, mild, moderate and severe lameness, respectively (ordered categorical variable). The two panels of this figure show that lameness scores of 0 and 5 increase and decrease, respectively, until reaching a plateau as the pyrazol metabolite levels increase. The observed probabilities for the absence of lameness increase up to pyrazol metabolite concentrations of 536 ng/ml. At higher pyrazol metabolite concentrations, probabilities of absence of lameness do not increase, which suggests that a maximal response in this acute arthritis model is achieved. Considering that the concentration range observed in the bin corresponding to this concentration was between 411 and 704 ng/ml, the MEC for the pyrazol metabolite was established in 411 ng/ml.

The population PK model along with the TK dataset were used to establish the MTC for enflicoxib and the pyrazol metabolite. A VPC demonstrated that the established population PK model for enflicoxib and the pyrazol metabolite was able to describe the PK profile of enflicoxib after daily oral administrations at 1 and 4 mg/kg/day (according to the toxicity study design, data not shown). Considering that in this toxicity study, no relevant toxicological effects were observed at 4 mg/kg/day, the MTC for enflicoxib was established as the upper 90% confidence interval of the maximum enflicoxib level predicted by the PK model on day 28 after the administration of 4 mg/kg/day, that is 6723 ng/ml.

However, as the pyrazol metabolite levels were not measured in this TK study, it was not feasible to confirm versus real data that the PK model was able to adequately predict the pyrazol metabolite accumulation after daily doses of enflicoxib. For this reason, the MTC of the pyrazol metabolite was established estimating the upper 90% confidence interval of the maximum pyrazol metabolite level predicted by the PK model on day 28 after daily oral administrations of enflicoxib at 1 mg/kg/day (NOAEL in dogs). Thus, a conservative pyrazol metabolite threshold of 4258 ng/ml was established as MTC.
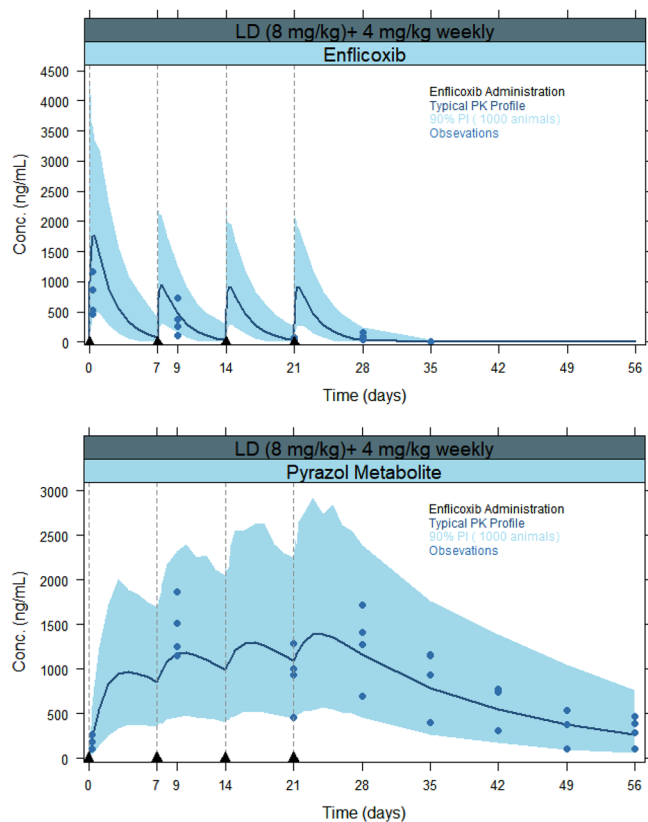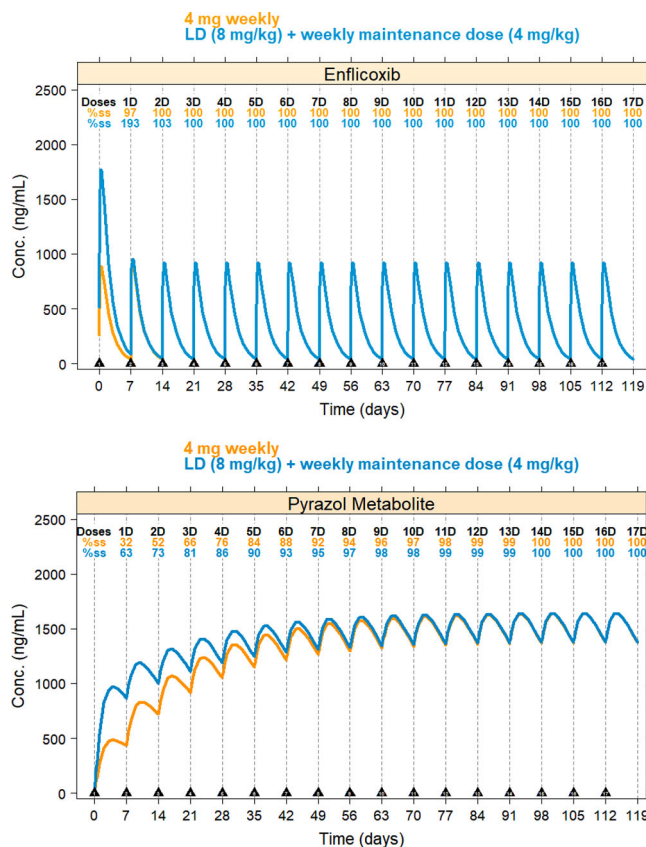
FIGURE 7 Visual predictive check for parent (left) and pyrazol metabolite (right). Typical PK profile of predicted concentration (solid line), 90% prediction intervals (coloured area) vs. time after administration of a loading dose of 8 mg/kg followed by 3 weekly maintenance doses of 4 mg/kg of enflicoxib. Observed enflicoxib and pyrazol metabolite concentrations in the repeated dose arthritis induction study were also superimposed (circles)


FIGURE 8 Predicted PK profiles of parent (left panel) and pyrazol metabolite (right panel) after two enflicoxib oral weekly dosing schedules of 4 mg/kg, with and without loading dose of 8 mg/kg. The percentage of steady state achieved after each dose administration for the different dosing schedules is numerically superimposed

Accordingly, the Therapeutic Window was set at levels of pyrazol metabolite between 411 (MEC) and 4258 (MTC) ng/ml, but without exceeding the threshold of 6723 ng/ml (MTC) for enflicoxib.

3.7 | Selection of optimal dosing schedule

Several weekly dosing schedules with and without loading dose where explored using Monte Carlo simulation techniques implemented in the NONMEM program by means of VPC, with the aim to achieve pyrazol metabolite levels above the MEC during the whole dosing interval but without exceeding the MTC for both enflicoxib and the pyrazol metabolite: (i) loading dose of 8 mg/kg followed by 4 mg/kg weekly; (ii) two consecutive daily doses of 4 mg/kg followed by 4 mg/kg weekly and (iii) loading dose of 10 mg/kg followed by 5 mg/kg weekly. In all cases, 6 weekly drug administrations were simulated.

A loading dose of 8 mg/kg followed by weekly maintenance doses of 4 mg/kg provided the best safety and efficacy profiles as seen in Figure 10. This posology gives a very low risk for dogs to achieve levels of enflicoxib above the MTC (6723 ng/ml) during the first hours after the administration of the initial loading dose (8 mg/kg). For the subsequent

weekly doses of 4 mg/kg, it is not expected that any animal reaches drug levels higher than the MTC for enflicoxib. Regarding the pyrazol metabolite profile, less than 1 day (approximately 21 h) is needed to achieve the efficacy level (MEC = 411 ng/ml) in 50% of treated animals after loading dose administration. After 6 weekly administrations of enflicoxib at 4 mg/kg, more than 95% of treated dogs will reach pyrazol metabolite levels higher than the MEC at C_{min} and none of them will achieve pyrazol metabolite levels higher than 4258 ng/ml (MTC for the pyrazol metabolite).
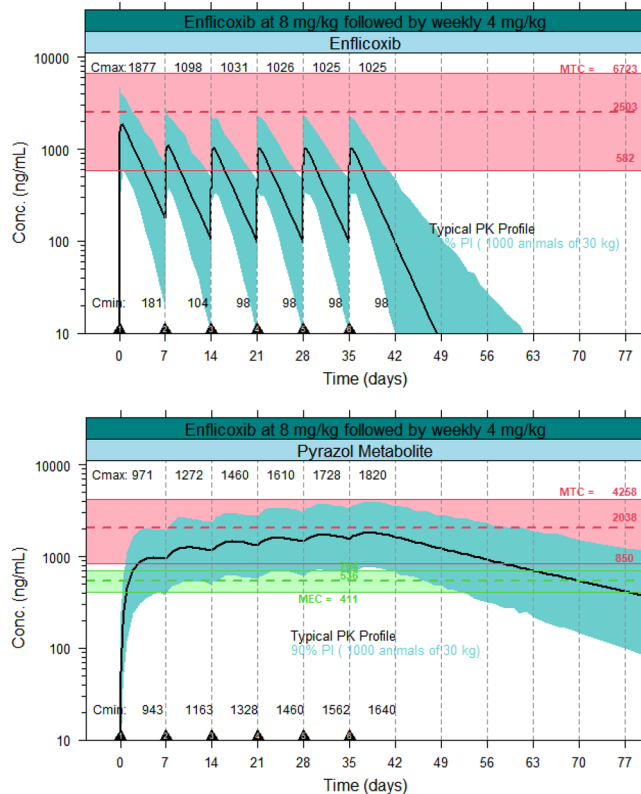
4 | DISCUSSION

This is the first PK and efficacy evaluation of enflicoxib in dogs in which both parent and its active pyrazol metabolite are measured. Although the number of dogs used was limited, due to its prospective nature, a higher variability on the PK profiles was observed for the parent compound in comparison with the pyrazol metabolite, mainly during terminal phase. The PK characteristics of enflicoxib were like those described by Reinoso et al. (2001). No obvious relationship between dose-normalised concentrations and dose was detected, suggesting that the dose does not modify the PK behaviour of both the parent and
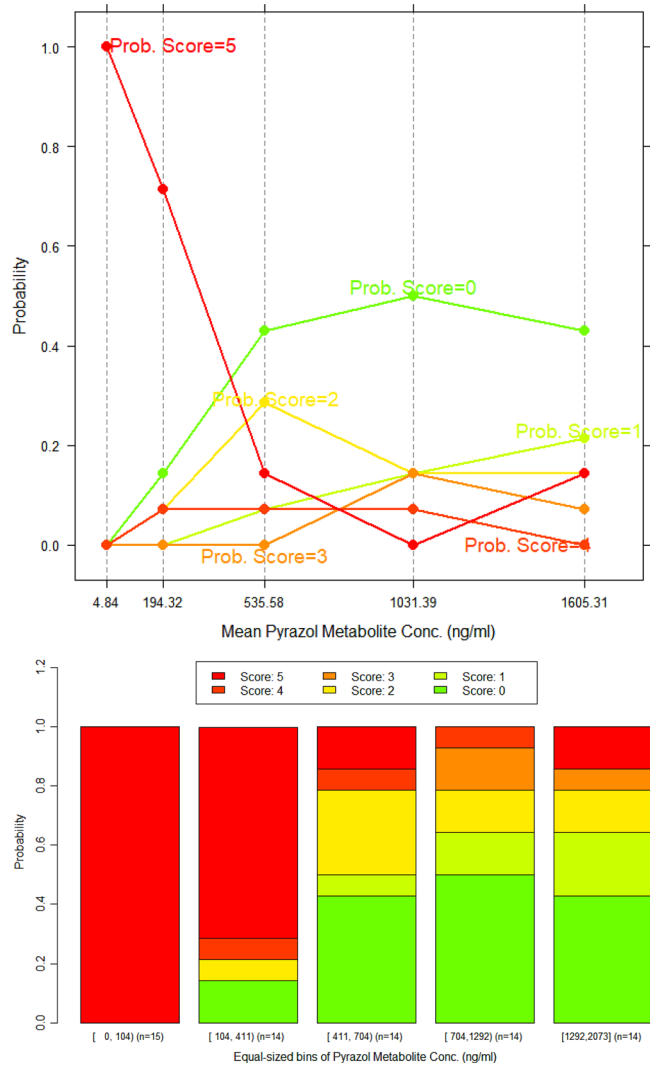
FIGURE 9 Observed probability curves for discrete lameness scores across mean pyrazol metabolite levels (left panel) and bar plot comparing proportion of combined lameness visual scores across equal-sized bins of pyrazol metabolite levels (right panel)

FIGURE 10 Scatter plot of typical PK profile along with the 90% prediction intervals from simulated data (left panel for parent drug and right panel for pyrazol metabolite) after administration of loading dose of 8 mg/kg followed by weekly 4 mg/kg doses of enflicoxib after six product administrations

the pyrazol metabolite in this dose range (from 1 to 8 mg/kg). Plasma levels declined slower for the pyrazol metabolite than for the parent compound, suggesting that the decline of the pyrazol metabolite is controlled by its elimination half-life and not governed by the elimination of enflicoxib. The long terminal half-life of the pyrazol metabolite confirms the findings described in rat (Reinoso et al., 2001) as being the responsible for the long-term efficacy of enflicoxib.

The arthritis induction model used in these studies has been largely used in the development of many NSAIDs for dogs (Toutain et al., 2001; Borer et al., 2003, Dauteloup et al., 2017). However, it could be considered inappropriate for a long acting product as the duration of the inflammatory reaction is short (about 16 ± 24 h) and does not persist throughout the time-course of the pyrazol metabolite disposition. However, this model allowed repeated pain and inflammation induction at different times in the same animal, which allowed a more accurate evaluation of the onset of action compared to a chronic inflamma-

tion model such as Freud's adjuvant. Likewise, vertical force was not recorded with a force plate. While it could have guaranteed objectivity of the measure itself, it is subjected to numerous perturbations of spurious behavioural origin. Only quiet dogs can be selected, and a long control training period is necessary to ensure appropriate repeatability of the procedure (Botrel et al., 1994; Toutain et al., 2001). In addition, by using blinded investigators, the lack of objectivity of the clinical evaluations was reduced.

A single 4 mg/kg dose was initially intended to be used in the first efficacy study of canine acute synovitis induction model. This dose was expected to show efficacy and to be well tolerated considering the results of the PK study in dogs and the available information from the studies performed in vitro and in laboratory animals. The lack of efficacy observed after the inductions on days 1, 2 and 7 evidenced that the concentrations achieved in blood did not exert enough analgesic/anti-inflammatory activity. Accordingly, a second dose of 4 mg/kg of enflicoxib was administered on day 12 to evaluate the effect of the accumulation of both doses. The administration of this second dose allowed pyrazol metabolite plasma concentrations to reach efficacy levels, as seen in the inductions on days 14 and 21. In order to achieve these therapeutic concentrations rapidly, a loading dose was proposed, and this strategy proved its efficacy in the second study, where pain and lameness were reasonably well controlled

with increasing efficacy as subsequent doses were administered. The lack of efficacy after the first induction performed 2 h after the loading dose administration confirmed that the blood concentrations of the parent compound are far from its COX-2 IC_{50} as described in isolated enzymes by Wagemakers et al. (2009) and by our team in whole dog blood assay (4.734 for enflicoxib and 1.145 ng/ml for the pyrazol metabolite, unpublished data).

The established population PK model of enflicoxib and its pyrazol metabolite in healthy Beagle dogs describes a linear PK behaviour in the dose range tested, including the total body weight as a covariate in all disposition PK parameters by means of allometric exponents. This allometric approach allows to extrapolate the PK behaviour in dogs of a wide range of weights, as it would be expected in future clinical studies that will include dogs of any breed. In addition, it should be considered that an increase of the exposure of both compounds at increasing weights was predicted by the model, anticipating a greater increase of exposure for the pyrazol metabolite than for enflicoxib. This model also predicts that the 90% of steady sate of pyrazol metabolite was achieved after 6 weekly administrations of enflicoxib with an initial loading dose. This initial loading dose (doubling the maintenance dose) on the first day of treatment was necessary for the pyrazol metabolite to reach the therapeutic levels and the PK steady state condition sooner.

Based on available TK, PK and efficacy data on an acute arthritis induction dog model, it seemed to be adequate to set the therapeutic levels of the pyrazol metabolite between 411 and 4258 ng/ml, but without exceeding the enflicoxib threshold of 6723 ng/ml. Thus, it was essential to ensure that pyrazol metabolite levels fluctuate within the range of plasma concentrations associated with safety and efficacy to establish an appropriate dosing interval of enflicoxib in dogs.

The blood samples taken in these studies yielded the PK and efficacy information to define the MEC under an arthritis induction model and to test, using population PK modelling, different dosing schedules (including loading and maintenance doses) that could maintain plasma concentrations within the therapeutic window for the highest proportion of the canine population. The major advantage of this clinical and PK-guided approach over a dose titration is to replace the independent variable (the dose) by the plasma concentration versus time profile, which can be used during the drug development process to predict a response whenever the drug formulation is modified, or the route of administration changed. The time interval between administrations cannot be easily determined from the classical dose titration either. In contrast, the independent variable in the PK approach (i.e. the plasma concentration vs time profile) provides time information and is the most appropriate tool for selecting a suitable dose interval (Toutain et al., 2001). Using this clinical and PK-guided approach, an enflicoxib posology of a loading dose of 8 mg/kg followed by weekly maintenance doses of 4 mg/kg was considered as the most adequate to be used in further clinical studies.

As described for other long acting coxibs, such as mavacoxib, sustained levels of active compound in blood, and therefore prolonged COX-2 inhibition, combined with a low frequency dose interval would be very beneficial in terms of efficacy and treatment compliance (Lees et al., 2015). The signs of osteoarthritis are commonly intermittent rather than continuous, and a continuous analgesic therapy breaks the cycles of acute flares providing pain control and increased mobility, leading to maintenance of muscle mass, increased joint stability and possible slowing of the disease process (Sanderson et al., 2009). Non-compliance with number of doses and/or treatment interval may raise welfare/efficacy issues for short-acting NSAIDs, while a delay of 2–3 days in administering the next dose of enflicoxib should not have significant implications for efficacy due to the prolonged terminal half-life of the pyrazol metabolite.

These efficacy and compliance considerations offer clear advantages of the posology selected for enflicoxib using clinical and PK-guided approach. Further clinical trials in naturally occurring cases of canine osteoarthritis should confirm the efficacy and safety of the proposed dosage.

ANIMAL WELFARE AND ETHICS STATEMENT

The authors confirm that the animal housing and care in the studies described herein comply with the recommendations of Directive 2010/63/EU. The least number of animals were used in compliance with current regulations and scientific integrity. The welfare of the animals was taken into account in terms of number and extent of procedures to be performed. All study procedures were checked and approved by the Animal Experimentation Ethics committee of the research centres involved.

AUTHOR CONTRIBUTIONS

All authors discussed and designed this project. JMC developed the population pharmacokinetic model, established PK and efficacy relationships and presented the data as tables and figures. JH designed the PK study and reported the results graphically. MS designed and reported the results of the arthritis induction model in Beagle dogs. GE validated and analysed all plasma samples and made the PK interpretations. JMV evaluated and consolidated the conclusions in each of the steps described in this project. All authors contributed to data interpretation and drafted parts of the manuscript. All authors approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.670

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