ORIGINAL ARTICLE

Co-formulation of ketoprofen with tulathromycin alters pharmacokinetic and pharmacodynamic profile of ketoprofen in cattle

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Abstract

The current studies aimed to evaluate the pharmacokinetic (PK) and pharmacodynamic (PD) profile and to establish a PK-PD model for ketoprofen in a new fixed combination product containing tulathromycin (2.5 mg/kg) and ketoprofen (3 mg/kg) to treat bovine respiratory disease associated with pyrexia in cattle. Firstly, the effect of different ketoprofen doses as mono-substance (1, 3, and 6 mg/kg subcutaneous) on lipopolysaccharide-induced fever was evaluated which indicated that rectal temperature reduction lasted longer in the calves receiving 3 and 6 mg/kg ketoprofen. Secondly, the PK profile of the combination product was compared with mono-substance products (3 mg/kg subcutaneous and intramuscular). The PK profile of ketoprofen in the combination product was characterized by longer $t_{1/2}$, lower C_{max} and increased AUC in comparison with mono-substance products. Due to prolonged ketoprofen exposure in the combination product, the pyrexia reducing effect of the combination product lasted longer in a second lipopolysaccharide challenge study in comparison with mono-substance products. Finally, a PK-PD model for the anti-pyretic effect of ketoprofen was developed based on the data from the different studies. The PK-PD model eliminated the need for additional animal experiments and indicated that a 3 mg/kg ketoprofen dose in the combination product provided optimal efficacy.

KEYWORDS
3Rs, cattle, ketoprofen, pharmacodynamics, pharmacokinetics

1 | INTRODUCTION

In cattle (*Bos taurus*), infectious disorders of the respiratory tract occur frequently and have an important impact on animal welfare and economic return of a farm (EFSA Panel on Animal Health, 2012). In the case of bovine respiratory disease (BRD), the mounted immune response, despite being necessary to heal lesions and restore tissue homeostasis, is thought to be an important contributor to the

severity of clinical symptoms and local lung lesions (McGill & Sacco, 2020; Mosier, 2014; Thacker, 2006). Treatment of these disorders with antibiotics targets the etiological agent and adjunct therapy with NSAID controls local and systemic inflammatory processes thereby decreasing pyrexia and the duration and severity of clinical symptoms (EFSA Panel on Animal Health, 2012; Lekeux, 2007).

NSAID reduce the production of pro-inflammatory molecules by inhibiting COX activity thereby exerting their local and central

anti-inflammatory therapeutic effect (Lees, Landoni, et al., 2004). Different NSAID as mono-substance or in combination products are available as adjunct therapy for BRD. PK-PD models of these NSAID as mono-substance in cattle have been developed to evaluate the anti-inflammatory properties at the molecular level by directly measuring the production of anti-inflammatory molecules (thromboxane, prostaglandin, leukotriene, and bradykinin) in tissue cage studies (Landoni et al., 1995b, 1995c, 1996; Lees, Giraudel, et al., 2004). These studies provide valuable information about the mechanism of action, the COX-1:COX-2 selectivity of NSAID and the time course of drug action at tissue level (Lees, Giraudel, et al., 2004). Albeit the magnitude of COX inhibition is related to the clinical outcome of reduced inflammation, pain, and fever (Toutain & Lees, 2004), to the authors' knowledge, PK-PD models of NSAID have not been applied to clinical outcome measures (fever) in cattle.

The aim of the current studies was to evaluate the PK and PD profile of ketoprofen and to establish a PK-PD model for ketoprofen in a new fixed combination product containing tulathromycin and ketoprofen (Draxxin® Plus or Draxxin® KP, same formulation different brand names, 100 mg/ml tulathromycin and 120 mg/ml ketoprofen, Zoetis) to treat BRD associated with pyrexia in cattle. Based on the PK-PD model, an efficacious dose was selected thereby eliminating the need to conduct additional animal experiments. A stepwise approach was taken to create the PK-PD model. In a first step, the effect of different ketoprofen doses on LPS-induced fever was investigated in combination with the PK profile. The second step involved the comparison of the PK profile of the combination product with the respective mono-products. In a third step, the effect of the combination product on LPS-induced fever was determined. Finally, a PK-PD model, for the effect of ketoprofen, as mono-substance and in the combination product, on the reduction of LPS-induced pyrexia, was developed based on the data from the different studies.

2 | MATERIALS AND METHODS

During the development of a new fixed combination product containing tulathromycin and ketoprofen, three studies were conducted to determine the optimal dose of ketoprofen. The results of these studies were used to evaluate the PK-PD profile and to develop a PK-PD model for the effect of ketoprofen treatment on rectal temperature following an LPS challenge in cattle.

All studies complied with all applicable animal welfare regulations and were reviewed and approved prior to study initiation by the ethical review committee of Zoetis. All studies were conducted in the United States of America and animals were housed according to the requirements in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). Animals had ad libitum access to water and were fed age-appropriate rations. Statistical analyses of rectal temperatures were done using SAS Release 9.2 (SAS Institute, Cary NC). Estimation of PK parameters was done using Watson LIMS v7.4.1 (Thermo Fisher Scientific, Inc).

2.1 | Study 1: First LPS challenge study

A first lipopolysaccharide (LPS) challenge study was conducted to evaluate the anti-pyretic effect of the mono-substance ketoprofen (Ketofen® Sterile Solution; Zoetis). The LPS challenge material was derived from *E. coli* 0111:B4 (Sigma-Aldrich). On study day 0, 80 healthy Holstein steer calves (approximately 6 to 11 weeks of age and approximately 100 kg of body weight) were challenged by subcutaneous (SC) injection of 5.0 μg/kg LPS. Immediately after the LPS challenge, all animals were treated with one of five treatments (*n* = 16 per treatment group): saline control, 1 mg/kg ketoprofen, 3 mg/kg ketoprofen, 6 mg/kg ketoprofen, or 2.2 mg/kg flunixin meglumine (Banamine® Injectable Solution; Schering-Plough Animal Health Corp). All ketoprofen doses and saline were administered SC in the neck while flunixin was given intravenous in the jugular vein.

Power calculations indicated that 11 animals per treatment group were required to detect 0.56°C difference in rectal temperature between treatment groups with power >80% and a two-sided significance level α = .10. Animals were randomly assigned to pens (16 pens of each five animals). Assignment to treatment using a computer-generated randomization list was done in a way so that each treatment group was represented within each pen. The study followed a randomized complete block design with one-way treatment structure. The blocking factor was based on pen location. Masking was accomplished by separation of functions of study personnel. Personnel collecting rectal temperatures and performing clinical observations were masked to treatment assignment. Time of challenge was defined as time point 0. Rectal temperatures were measured prior to LPS injection, and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h post-challenge. Prior to challenge on study day 0 and at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, and 48 h post-challenge, blood samples were collected by jugular venipuncture from 8 randomly selected calves from each of the ketoprofen treated groups. Blood was collected into K_3-EDTA anticoagulant tubes and placed on ice until centrifugation. Plasma was transferred to 96-well tube racks and frozen at ≤ −10°C until analyzed.

Rectal temperatures were analyzed using a general linear mixed model with repeated measures with treatment, time point and the interaction between treatment and time point as fixed effects and pen and animal as random effects. The animal was the experimental unit. If the main effect of treatment or the interaction of treatment by time was significant (*p* ≤ .05), then pairwise treatment comparisons (*p* ≤ .10) were made at each time point. Least squares means and SEM were reported for each treatment at each time point.

Ketoprofen PK parameters were estimated using non-compartmental techniques. The linear trapezoidal method was used for AUC determination. Estimates of the PK parameters C_{max}, t_{max}, $AUC_{0-t(last)}$, $AUC_{0-\infty}$, and $t_{1/2}$ were made for each animal. C_{max} is the peak concentration, and t_{max} is the time at which the peak occurs. $AUC_{0-t(last)}$ is the AUC from time 0 to the last sampling time associated with quantifiable drug concentration. The log transformed (ln) plasma ketoprofen concentrations were modeled using a general linear mixed model for repeated measures with treatment, time

point and the interaction between treatment and time point as fixed effects and pen and animal as random effects. Back-transformed least squares means and 90% confidence intervals were reported for each treatment and sample time. $AUC_{0-t(last)}$, $AUC_{0-\infty}$, C_{max}, $t_{1/2}$, and t_{max} were determined using a general linear mixed model. $AUC_{0-t(last)}$, $AUC_{0-\infty}$, and C_{max} were log transformed (ln) prior to analysis. The model included treatment as fixed effect. The random effects included block and error. Back-transformed LS means, 90% CI, and minimums and maximums were reported for each variable by treatment. If more than half of the animals in a group had ketoprofen concentration values that were below the limit of quantitation (BLQ) at a time point, data from that time point were not used in the statistical analysis. Where more than or equal to half of the animals in a group had values above the lower limit of quantitation (LLOQ) of the assay at a time point, BLQ values were included in the statistical analysis as half of the LLOQ.

2.2 | Study 2: Pharmacokinetic Study

A PK study was conducted to evaluate the pharmacokinetics of the combination tulathromycin–ketoprofen product relative to the mono-substance tulathromycin and two ketoprofen products. In this study, 80 healthy male Holstein calves (age 7.9–8.2 months and weighing between 174 and 286 kg at dose administration) were randomly allocated to four different treatment groups ($n = 20$ per treatment group): SC injection of 2.5 mg/kg tulathromycin (Draxxin®; Zoetis), intramuscular (IM) injection of 3.0 mg/kg ketoprofen (Ketofen® 10%; CEVA), SC injection of 3.0 mg/kg ketoprofen (Ketofen® Sterile Solution; Zoetis), or SC injection of the combination product at 2.5 mg/kg tulathromycin and 3.0 mg/kg ketoprofen.

Power calculations indicated that 20 animals per treatment group were required to detect 25% difference in AUC between tulathromycin and tulathromycin–ketoprofen with power >80% and a two-sided significance level α = .10. Animals were randomly assigned to pens (10 pens of each eight animals). Assignment to treatment using a computer-generated randomization list was done in a way so that two animals per treatment group were represented in each pen. The study followed a generalized randomized block design with a one-way treatment structure. Pen and day of dosing were the blocking factors. Due to the large number of animals and blood sampling required, dosing was conducted over multiple days. Each pen was randomly assigned to a dosing day, and all animals in a pen were dosed on the same day.

All animals received a single injection of the assigned treatment in the neck. Blood was collected by jugular venipuncture into K_3-EDTA anticoagulant tubes. Blood was collected within 24 h prior to treatment administration and at 20 and 40 min, 1, 1.5, 2, 3, 4, 6, 10, 24, 28, 32, 48, 52, 56, 72, 120, 168, 216, 264, 336, and 360 h following treatment administration. Blood was centrifuged, and plasma was transferred to 96-well tube racks and frozen at ≤ −10°C until analyzed. Ketoprofen PK parameters were estimated as described in study 1 (first LPS challenge study).

2.3 | Study 3: Second LPS challenge study

A second lipopolysaccharide (LPS) challenge study was conducted to evaluate the anti-pyretic effect of the combination tulathromycin–ketoprofen product relative to mono-substance ketoprofen products. On study day 0, 80 healthy male Holstein calves (100 to 120 kg in body weight) were challenged SC with 5.0 μg/kg LPS (the same challenge material as in the 1st LPS challenge study). Immediately after challenge, all animals were treated with one of four treatments ($n = 20$ per treatment group): saline control (SC), 3 mg/kg SC injection of ketoprofen (Ketofen® Sterile Solution; Zoetis), 3 mg/kg IM injection of ketoprofen (Ketofen® 10%; CEVA) and SC injection of the combination product at 2.5 mg/kg tulathromycin and 3.0 mg/kg ketoprofen (Draxxin® Plus or Draxxin® KP).

Power calculations indicated that 14 animals per treatment group were required to detect 0.56°C difference in rectal temperature between treatment groups with power >80% and a two-sided significance level α = .05. Animals were randomly assigned to pens such that there were 20 pens with four animals in each pen. Assignment to treatment using a computer-generated randomization list was done in a way so that each treatment group was represented within each pen. The study followed a randomized complete block design with a one-way treatment structure. Pen was the blocking factor, and animal was the experimental unit. Time of challenge was defined as time point 0. Treatment administration occurred within 30 min of challenge. Rectal temperatures were measured approximately 17 h and 2 h prior to challenge administration and continued 1, 2, 4, 6, 8, 10, 12, and 24 h post-challenge. No blood samples were taken in this study. Masking was accomplished by separation of functions of study personnel. Personnel collecting rectal temperatures were masked to treatment assignment.

Rectal temperatures were analyzed using a general linear mixed model with repeated measures with treatment, time and the interaction between treatment and time as fixed effects and block and block by treatment interaction as random effects. Pairwise treatments comparisons were made between treatments ($p \le .05$) at each time point for a significant ($p \le .05$) treatment or treatment by time interaction. Least squares means and SEM were reported for each treatment at each time point.

2.4 | Analytical methodology

Ketoprofen (total of R[−] and S[+] enantiomers) was extracted from bovine plasma by adding 200 μl of working internal standard solution (50 ng/ml flunixin in acetonitrile) to 50 μl of plasma (standards, QCs, and samples) in a 0.5 ml 96-well polypropylene plate. Blank (control) plasma was added to wells containing acetonitrile for double blanks. The plates were sealed and vortexed and then centrifuged at 3220 g for approximately 10 min to precipitate the proteins. Following centrifugation, 50 μl of supernatant was diluted with 250 μl of 0.1% formic acid in 5 mM ammonium formate in water in a separate 0.5 ml polypropylene plate and mixed well. The plate

was sealed and placed in the autosampler set at 10°C for analysis by LC-MS/MS.

A 6 μl volume was injected onto a Waters Acquity UPLC® BEH C_{18}, 1.7 μm, 2.1 × 50 mm column heated to 40°C and eluted at 0.600 ml/min using an isocratic method on a Waters Acquity Ultra Performance LC. The UPLC® mobile phases were A: 0.1% formic acid in 5 mM ammonium formate in water and B: aceto-nitrile. The LC method was 60% A / 40% B, and the total run time was 1.5 min. Detection was performed using a Sciex API 4000 mass spectrometer using negative electrospray ionization (ESI-) with multiple reaction monitoring of the transitions 253 → 209 amu for ketoprofen and 295 → 251 amu for the internal stan-dard (flunixin). The retention times were approximately 0.9 min for ketoprofen and 1.1 min for the internal standard. Acquisition and peak integration data were collected in Analyst (v1.6.2, AB Sciex). Integrated peak areas were imported into Watson LIMS for standard regression. Analyte/IS peak area ratios were used for quantitation with quadratic fit ($1/x^2$ weighting). The validated concentration range of the calibration curve was 10 ng/ml to 10,000 ng/ml.

2.5 | Pharmacokinetic–pharmacodynamic model

A PK-PD model of the anti-pyretic effect of ketoprofen was developed.

2.5.1 | PK model

The first step in the PK-PD analysis was to fit a PK model to the means of each of the ketoprofen treated groups in the 1st LPS chal-lenge study and the PK study. A two-compartment linear PK model with Weibull absorption was fit to the mean data. The model easiest written in differential equations:

$$\frac{dA_1}{dt} = -k_w \cdot A_1$$

$$\frac{dA_2}{dt} = k_w \cdot A_1 - \frac{Q}{V_c} \cdot A_2 + \frac{Q}{V_p} \cdot A_3 - \frac{CL}{V_c} \cdot A_2$$

$$\frac{dA_3}{dt} = \frac{Q}{V_c} \cdot A_2 - \frac{Q}{V_p} \cdot A_3$$

where A_1, A_2, and A_3 is the amount of ketoprofen in the dosing, cen-tral and peripheral compartments, respectively; CL is the systemic clearance; Q is the distributional clearance; V_c is the volume of cen-tral compartment; and V_p is the volume of the peripheral compart-ment. The absorption term, k_w, takes the form: $k_w = \left(\frac{\beta}{\lambda}\right)\left(\frac{t}{\lambda}\right)^{\beta-1}$, which is based on the Weibull cumulative distribution function $\left(1 - e^{-(t/\lambda)^\beta}\right)$ with t being time after dose, λ is the scale parameter and represents the time at which 63.2% of the dose is absorbed and β is the shape parameter.

Covariates based on route of administration (IM, SC) and for-mulation (mono-substance, combination) were considered for the λ parameter as well as for the relative bioavailability:

$$\lambda_i = e^{\left(\log(\lambda) \,+\, IM \cdot \log(\theta_{IM,\lambda}) \,+\, CB \cdot \log(\theta_{CB,\lambda})\right)}$$

$$F_i = e^{\left(IM \cdot \theta_{IM,F} \,+\, CB \cdot \theta_{CB,F}\right)}$$

where IM is 0 for SC and 1 for IM dosing and CB is 0 for mono-substance and 1 for combination formulations, and θ_x are the es-timated parameters and λ is the estimated scale parameter for SC dosing of mono-substance product. Because the two studies en-rolled cattle with approximately 100 kg body weight difference, the clearance and volume parameters were varied through allometric scaling with estimated power coefficients (one for clearance terms and one for volume terms):

$$P_i = P \cdot \left(\frac{BW}{BW_m}\right)^{\theta_{p,pow}}$$

where P represents one of the parameters (CL, Q, V_c, or V_p); BW is the body weight and BW_m is the median body weight across the studies (=150 kg); and $\theta_{p,pow}$ is the allometric power term with one estimate for CL and Q and one for V_c and V_p. The PK model was fit simultaneously across the groups allowing for a single set of param-eters. NONMEM version 7.4 was used for the model fitting.

2.5.2 | Pharmacodynamic model

To account for the increase in rectal temperature after LPS chal-lenge, the treatment effect in the PK-PD model was (rectal tem-perature treated) − (rectal temperature placebo) + (baseline rectal temperature), the baseline value was added to avoid non-positive PD values. The time of the greatest treatment effect occurred ap-proximately at 4 h which is after the ketoprofen t_{max} value for the mono-substance groups. This lag in peaks was accounted for by using an indirect response model (Dayneka et al., 1993). The indirect response model chosen was an inhibition of k_{in}, that is, an inhibition of the zero-order rate constant for production of pyrexia due to LPS challenge (model I of Dayneka et al., (1993)). The driving differential equation was as follows:

$$\frac{dR}{dt} = k_{in}\left(1 - \frac{I_{max} \cdot C_p^\gamma}{IC_{50}^\gamma + C_p^\gamma}\right) - k_{out} \cdot R$$

where C_p is the ketoprofen plasma concentration. Model param-eters are k_{out} (first-order rate constant for loss of the response), I_{max} (proportional maximum inhibitory effect), IC_{50} (the plasma concentration that produces half-maximal effect), and γ (gamma, controlling the slope and sigmodicity of the inhibition curve). As a stationarity condition of the model (i.e., starts and ends at the same value) $k_{in} = $ baseline$\cdot k_{out}$, where baseline was a constant in

TABLE 1 Least squares means rectal temperature (°C) from the first lipopolysaccharide (LPS) challenge study

Treatment group	Time post-challenge (h)											
	0	0.5	1	2	3	4	5	6	8	10	12	24
Saline control (SC)	38.6[a]	38.9[a]	39.3[b]	39.9[c]	40.0[b]	40.4[b]	40.1[b]	40.1[c]	40.0[c]	39.5[b]	39.5[c]	38.6[ab]
Ketoprofen (1 mg/kg SC)	38.7[a]	38.9[a]	38.8[a]	39.0[a]	38.9[a]	39.3[a]	39.2[a]	39.7[b]	39.8[bc]	39.6[b]	39.5[bc]	39.2[c]
Ketoprofen (3 mg/kg SC)	38.7[a]	38.9[a]	38.7[a]	38.8[a]	38.8[a]	39.1[a]	39.1[a]	39.2[a]	39.4[a]	39.4[ab]	39.2[ab]	38.9[b]
Ketoprofen (6 mg/kg SC)	38.7[a]	38.9[a]	38.8[a]	39.0[a]	38.9[a]	39.2[a]	39.2[a]	39.4[a]	39.6[ab]	39.2[a]	39.0[a]	39.2[c]
Flunixin (2.2 mg/kg IV)	38.6[a]	38.7[a]	38.9[a]	39.4[b]	39.8[b]	40.1[b]	40.0[b]	39.9[bc]	39.7[bc]	39.4[ab]	39.2[abc]	38.6[a]

Note: [a,b,c] Unlike superscripts within a column are significantly different ($p < .10$). $n = 16$ at each time point for each treatment group.

the model set at the mean of pre-challenge temperatures across groups within study. NONMEM version 7.4 was used for the model fitting.

The PK-PD model was fitted to the data of the different studies. Because the 2nd LPS challenge study did not have a PK component, the treatment group means were paired with the data from the PK study which included an identical set of treatment groups. This required that the PK-PD integration was performed on the treatment group means. Finally, the PK-PD model was used to evaluate the effect of altering the ketoprofen dose (1, 3, and 6 mg/kg) in the combination tulathromycin–ketoprofen product.

3 | RESULTS

The focus of this manuscript is to describe the PK-PD profile and the PK-PD model of ketoprofen in a new fixed combination product containing tulathromycin and ketoprofen; therefore, only ketoprofen data are shown and discussed while data related to tulathromycin are not presented.

3.1 | Study 1: First LPS challenge study

Peak rectal temperature for the saline-treated animals ranged from 40.0 to 41.3°C, and the rectal temperature of all saline-treated animals increased compared to their pre-challenge rectal temperature. This indicates that the LPS challenge was valid. There was an overall treatment effect ($p < .0001$) on rectal temperatures, and comparisons between the treatment groups are presented in Table 1 and Figure 1. In the control saline treatment group, peak temperatures occurred between 2 and 4 h post-challenge. In the ketoprofen treatment groups, the peak was reduced and delayed to 8 to 10 h post-challenge. Relative to saline-treated controls, treatment with 1 mg/kg ketoprofen significantly ($p < .10$) reduced rectal temperatures at 1 through 6 h post-challenge. Treatment with 3 mg/kg ketoprofen significantly ($p < .10$) reduced rectal temperatures compared with saline-treated controls starting 1 h post-challenge through 8 h and again at 12 h. Dosing with 6 mg/kg ketoprofen significantly ($p < .10$) reduced temperatures starting at 1 h post-challenge through the end of the study at 24 h compared to the saline-treated controls. Flunixin administered intravenously at 2.2 mg/kg demonstrated significantly ($p < .10$) reduced rectal temperatures compared with saline-treated controls at 1 h and 2 h after challenge. Relative to flunixin treated positive controls, treatment with 3 and 6 mg/kg ketoprofen significantly ($p < .10$) reduced rectal temperatures from 2 h through 6 h post-challenge and at 24 h post-challenge. Up to 5 h post-challenge, the temperature reduction of all three ketoprofen doses (i.e., 1, 3, and 6 mg/kg bw) exhibited no statistically significant differences. Thereafter, the two higher doses of ketoprofen resulted in a better temperature reduction compared to the dose of 1 mg/kg and no obvious difference were detected between the 3 and 6 mg/
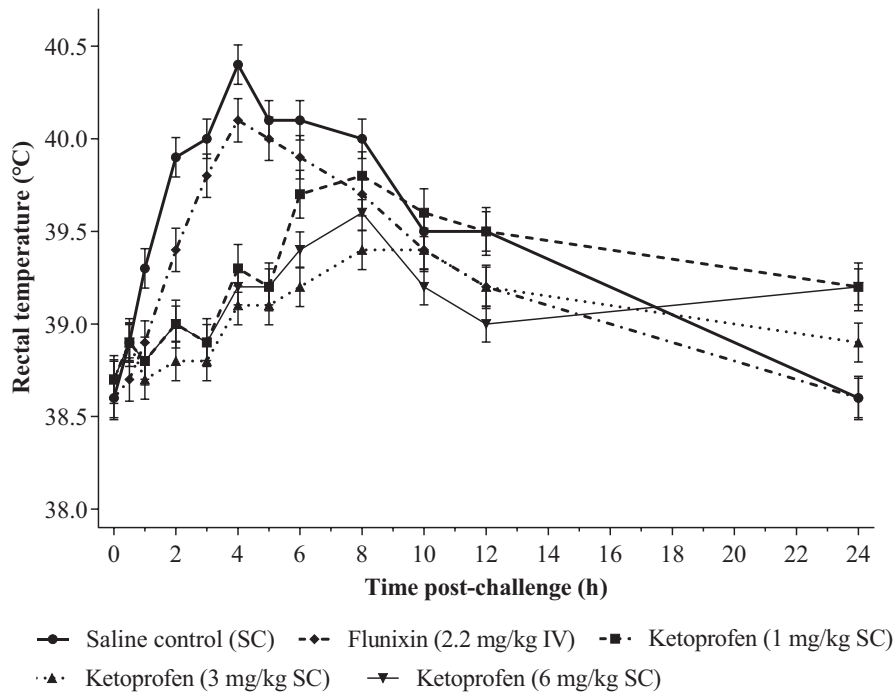
FIGURE 1 Rectal temperature (°C, LS means ± SEM) for the different treatment groups after lipopolysaccharide (LPS) challenge (time 0 h) and treatment in the first LPS challenge study
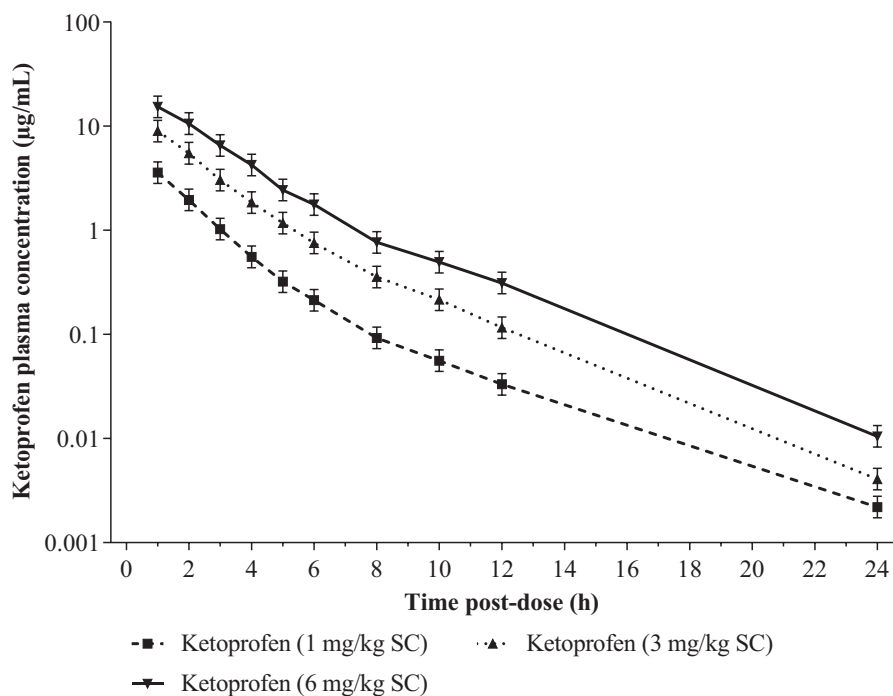
Saline control (SC) — Flunixin (2.2 mg/kg IV) — Ketoprofen (1 mg/kg SC)
Ketoprofen (3 mg/kg SC) — Ketoprofen (6 mg/kg SC)

FIGURE 2 Ketoprofen plasma concentration (μg/ml, LS means ± 90% CI) for the different ketoprofen treatment groups after lipopolysaccharide (LPS) challenge (time 0 h) and treatment in the first LPS challenge study

Ketoprofen (1 mg/kg SC) — Ketoprofen (3 mg/kg SC)
Ketoprofen (6 mg/kg SC)

kg dose except at the 24 h time point at which the 3 mg/kg treatment group had a significantly ($p < .10$) lower rectal temperature compared to the 6 mg/kg treatment group.

Summarized plasma ketoprofen concentration data are shown in Figure 2. Summarized pharmacokinetic variables are shown in Table 2. The peak plasma concentration occurred at the first sample point (1 h) for every animal, and thus, C_{max} has not been well characterized. The amount of extrapolation required to go from $AUC_{0-t(last)}$ and $AUC_{0-\infty}$ was at most 0.22.

## 3.2 | Study 2: Pharmacokinetic Study

The ketoprofen PK results indicated that the IM and SC administration of the two mono-substance products gave similar bioavailability and terminal half-life (Figure 3 and Table 3). The C_{max} following the IM administration was higher than following SC administration (8.92 vs 6.31 μg/ml, respectively) with most IM animals (17/20) having a t_{max} at the first sample time (20 min). The ketoprofen PK following the combination formulation differed from the results of both IM

TABLE 2 Summary statistics for the ketoprofen pharmacokinetic (PK) parameter estimates from the first lipopolysaccharide (LPS) challenge study

PK variable	Treatment group	n	LS mean	90% CI		Min	Max
$AUC_{0-\infty}$ (µg h/ml)	Ketoprofen (1 mg/kg SC)	8	8.42	7.51	9.45	7.49	11.60
	Ketoprofen (3 mg/kg SC)	8	24.10	21.50	27.00	17.40	31.90
	Ketoprofen (6 mg/kg SC)	8	47.10	42.00	52.80	35.70	60.10
$AUC_{0-t(last)}$ (µg h/ml)	Ketoprofen (1 mg/kg SC)	8	8.41	7.50	9.44	7.49	11.60
	Ketoprofen (3 mg/kg SC)	8	24.10	21.50	27.00	17.40	31.80
	Ketoprofen (6 mg/kg SC)	8	47.10	42.00	52.80	35.70	60.10
C_{max} (µg/ml)	Ketoprofen (1 mg/kg SC)	8	3.58	3.37	3.81	3.21	4.16
	Ketoprofen (3 mg/kg SC)	8	8.97	8.35	9.64	7.66	10.30
	Ketoprofen (6 mg/kg SC)	8	15.30	12.90	18.20	10.20	20.60
t_{max} (h)	Ketoprofen (1 mg/kg SC)	8	1	NA	NA	1	1
	Ketoprofen (3 mg/kg SC)	8	1	NA	NA	1	1
	Ketoprofen (6 mg/kg SC)	8	1	NA	NA	1	1
$t_{1/2}$ (h)	Ketoprofen (1 mg/kg SC)	8	3.02	2.85	3.20	2.71	3.85
	Ketoprofen (3 mg/kg SC)	8	2.48	2.30	2.65	2.24	2.80
	Ketoprofen (6 mg/kg SC)	8	2.57	2.40	2.75	2.22	2.98

Note: $AUC_{0-\infty}$ = the AUC extrapolated to infinite time. $AUC_{0-t(last)}$ = the AUC from time 0 to the last sampling time associated with quantifiable drug concentration. C_{max} = the peak concentration. t_{max} = the time at which the peak occurs. $t_{1/2}$ = the terminal half-life.

FIGURE 3 Ketoprofen plasma concentration (µg/ml, LS means ± 90% CI) for the different treatment groups after treatment (time 0 h) in the pharmacokinetic (PK) study
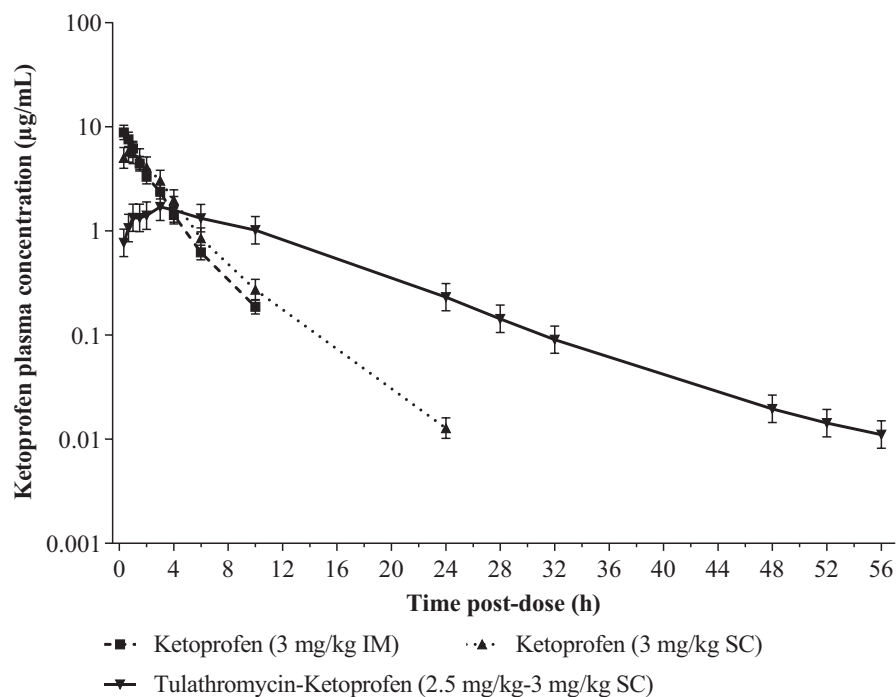
and SC mono-substance products (Figure 3 and Table 3). The AUCs were higher, C_{max} was lower, t_{max} was later, and $t_{1/2}$ was longer for the combination product than for either mono-substance product. Compared to the SC ketoprofen mono-product, the combination product resulted in a geometric mean ratio (GMR: combination product/mono-product) for $AUC_{0-\infty}$ of 1.15 (90% CI: 1.07,1.25) and for C_{max} of 0.327 (90% CI: 0.269, 0.396). The difference in t_{max} was 3.2 h (90% CI: 2.0, 4.3) and $t_{1/2}$ was 4.05 h (90% CI: 3.23, 4.87).

3.3 | Study 3: Second LPS challenge study

Peak rectal temperature for the saline-treated animals ranged from 39.4 to 40.9°C, and the rectal temperature of all saline-treated animals increased compared to their pre-challenge rectal temperature. This indicates that the LPS challenge was valid. There was an overall treatment, time and treatment by time interaction effect ($p < .0001$) on rectal temperatures and comparisons

TABLE 3 Summary statistics for the ketoprofen pharmacokinetic (PK) parameter estimates from the PK study

PK variable	Group	n	LS Mean	90% CI		Min	Max
$AUC_{0-\infty}$ (µg h/ml)	Ketoprofen (3 mg/kg IM)	20	21.3	20.1	22.5	16.0	31.1
	Ketoprofen (3 mg/kg SC)	20	22.9	21.6	24.2	18.7	29.8
	Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	20	26.4	25.0	28.0	19.3	31.4
$AUC_{0-t(last)}$ (µg h/ml)	Ketoprofen (3 mg/kg IM)	20	21.0	19.8	22.2	15.9	31.0
	Ketoprofen (3 mg/kg SC)	20	22.6	21.3	24.0	18.2	29.7
	Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	20	26.2	24.7	27.8	19.0	31.1
C_{max} (µg/ml)	Ketoprofen (3 mg/kg IM)	20	8.92	8.02	9.92	4.61	14.3
	Ketoprofen (3 mg/kg SC)	20	6.31	5.79	6.88	3.82	8.49
	Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	20	2.06	1.73	2.46	1.08	8.08
t_{max} (h)	Ketoprofen (3 mg/kg IM)	20	0.38	0.33	0.43	0.33	0.67
	Ketoprofen (3 mg/kg SC)	20	0.83	0.63	1.0	0.33	2
	Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	20	4.0	2.9	5.1	1	10
$t_{1/2}$ (h)	Ketoprofen (3 mg/kg IM)	20	2.38	2.05	2.71	1.42	4.07
	Ketoprofen (3 mg/kg SC)	20	2.72	2.38	3.07	1.52	4.36
	Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	20	6.78	6.02	7.53	4.18	11.3

Note: $AUC_{0-\infty}$ = the AUC extrapolated to infinite time. $AUC_{0-t(last)}$ = the AUC from time 0 to the last sampling time associated with quantifiable drug concentration. C_{max} = the peak concentration. t_{max} = the time at which the peak occurs. $t_{1/2}$ = the terminal half-life.

between the treatment groups at different time points are presented in Table 4 and Figure 4.

Least squares means rectal temperatures were significantly ($p < .05$) lower among all treated groups compared with saline-treated controls, beginning 1 h post-challenge and continuing through 8 h post-challenge. From 10 h to 24 h after challenge, rectal temperatures of animals receiving the mono-products were not significantly different ($p > .05$) or significantly ($p < .05$) higher compared with the saline-treated control. Animals receiving the tulathromycin–ketoprofen combination product displayed significantly ($p < .05$) lower rectal temperatures compared to the mono-products from 10 h to 24 h post-challenge (for ketoprofen 3 mg/kg IM also at 8 h post-challenge) and saline-treated control from 10 h to 12 h post-challenge.

3.4 | Pharmacokinetic–pharmacodynamic model

The 2-compartment linear PK model with Weibull absorption was fit simultaneously to the treatment group means of the 1st LPS challenge study and the PK study. The parameter estimates are provided in Table 5. The 2-compartment linear PK model fits the mean data well (Figure 5) with no discernable systematic lack of fit. The combination formulation had a time to 63.2% absorbed of 6.73 h compared to 0.388 h for the SC dosing of the mono-substance. In the PK study, the AUCs were slightly higher for the combination than for the mono-substance (Table 3). Combining the data from the 1st

LPS challenge study with the PK study, the 2-compartment linear PK model estimated the relative bioavailability of the combination to be 93.1% of the SC dosing of the mono-substance (Table 5).

The parameter estimates of the PK-PD model are provided in Table 6. The IC_{50} was estimated to be 0.796 µg/ml, and the I_{max} parameter was estimated to be 0.0337. All ketoprofen doses tested in the different studies peaked above the IC_{50}. With a baseline rectal temperature of 38.5°C, the maximum effect of ketoprofen is a difference of 1.3°C from placebo. The PK-PD model provides a reasonable fit to the observed mean difference in rectal temperature between ketoprofen treated and placebo treated groups (Figure 6). The model perhaps shows some unaccounted-for study to study variability. There is also some lack of fit in the late time points due to the stationarity restriction (the model must finish at the baseline value).

Using the PK-PD model, the predicted effects on rectal temperature for ketoprofen doses of 1, 3, and 6 mg/kg as part of the combination tulathromycin–ketoprofen product are presented in Figure 7.

4 | DISCUSSION

Determination of an efficacious dose of new pharmaceutical compounds involves studies testing different dosages to characterize the dose–response relationship. Classically, dose determination has been based on dose titration studies (Toutain & Lees, 2004).

TABLE 4 Least squares means rectal temperature (°C) from the second lipopolysaccharide (LPS) challenge study

Treatment group	Time post-challenge (h)									
	-17	-2	1	2	4	6	8	10	12	24
Saline control (SC)	38.6[b]	38.3[a]	39.2[b]	39.5[b]	39.8[b]	39.5[b]	39.6[c]	39.6[bc]	39.6[b]	38.8[a]
Ketoprofen (3 mg/kg SC)	38.4[ab]	38.1[a]	38.7[b]	38.4[a]	38.6[a]	38.5[a]	38.9[a]	39.6[b]	39.7[bc]	39.3[b]
Ketoprofen (3 mg/kg IM)	38.4[ab]	38.2[a]	38.7[a]	38.3[a]	38.5[a]	38.6[a]	39.2[b]	39.9[c]	39.9[c]	39.5[b]
Tulathromycin–Ketoprofen (2.5 mg/kg–3 mg/kg SC)	38.4[a]	38.1[a]	38.6[a]	38.3[a]	38.5[a]	38.5[a]	38.8[a]	38.8[a]	39.0[a]	38.6[a]

Note: [a,b,c] Unlike superscripts within a column are significantly different ($p < .05$). $n = 20$ at each time point for each treatment group.

However, by combining PK and PD data in a PK-PD model, the dose–response effect can be determined and can be used as a tool in the determination of the optimal dose (Riviere et al., 2016; Toutain & Lees, 2004). A PK-PD model describes mathematically the relationship between the dose of a drug and how the drug behaves in the body (PK) in conjunction with the pharmacological effect of the drug (PD) (Baggot, 2008; Riviere et al., 2016; Toutain, 2011). An important advantage of PK-PD modeling is that the expected response of every dose can be computed thereby reducing the need for animal studies (Riviere et al., 2016). The PK-PD model established in the current manuscript supports the dose selection of ketoprofen in a fixed combination product containing tulathromycin and ketoprofen.

Ketoprofen is a non-selective COX-1 and COX-2 inhibitor and has a rapid onset of action and short half-life (Kantor, 1986; Papich & Messenger, 2015). Veterinary products containing ketoprofen are racemic mixtures of S(+) and R(−) enantiomers. The S(+) enantiomer has been shown to be a much more potent inhibitor of PGE_2 production in comparison with the R(−) enantiomer and following dosing of the R(−) enantiomer in cattle, 31% was inverted to the S(+) enantiomer (Landoni & Lees, 1995, USP Monographs, 2004, Plessers, Watteyn, et al., 2015). The ketoprofen products used in the studies discussed herein, dosed a racemic mixture of R(−) and S(+) enantiomers. The assay used to measure ketoprofen in the plasma was nonchiral and only measured the total ketoprofen content. The half-life of ketoprofen as mono-substance has been reported to range from 0.42–1.55 h to 3.40 h after intravenous or intramuscular administration, respectively (Landoni et al., 1995c; Singh et al., 2014). These values are similar to the half-life of ketoprofen observed in the first LPS challenge study ($t_{1/2}$ ranging from 2.48 to 3.02 h after SC administration) and the PK study ($t_{1/2}$ 2.38 and 2.72 h after IM and SC administration, respectively). The results of the first LPS challenge study indicated that a ketoprofen dose between 3 and 6 mg/kg was efficaciously controlling pyrexia in calves. This agrees with the currently authorized 3 mg/kg ketoprofen dose in mono-substance products for cattle.

The temperature reducing effect of flunixin in the first LPS challenge study was shorter in comparison with the three ketoprofen doses evaluated. In contrast to the LPS challenge model, the temperature reducing effect of flunixin has been reported to last until one day after treatment in different natural infection BRD studies (Guzel et al., 2010; Lockwood et al., 2003; Thiry et al., 2014). The discrepancies might be explained by differences in inflammatory stimuli between an experimental LPS challenge and a natural infection (see below). Additionally, differences in the PK-PD profile between flunixin and ketoprofen might explain the observation although the ex vivo inhibitory effect on PGE_2 over a 24 h period in calves is similar for flunixin and ketoprofen (Landoni et al., 1995a), the $t_{1/2}$ of flunixin in calves is similar or even slightly longer than ketoprofen mono-substance (Kleinhenz et al., 2018), and the IC_{50} for inhibition of prostaglandin E_2 (PGE_2) of flunixin in calves (74 ng/ml) falls within the range reported for ketoprofen (42–99.9 ng/ml) (Lees, Giraudel, et al., 2004). More research is needed to clarify the observed difference between ketoprofen and flunixin.
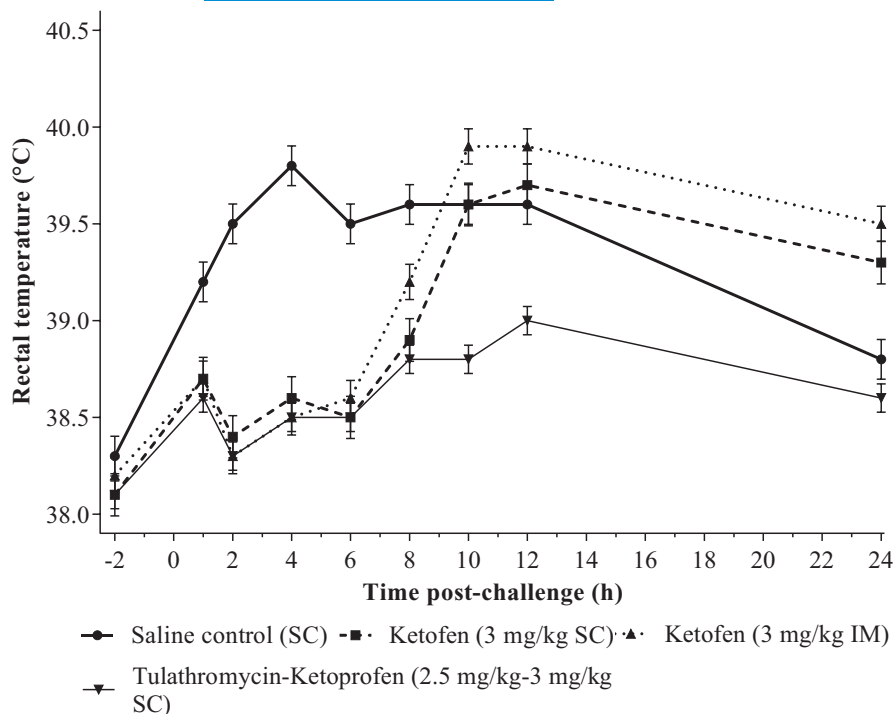
FIGURE 4 Rectal temperature (°C, LS means ± SEM) for the different treatment groups after lipopolysaccharide (LPS) challenge (time 0 h) and treatment in the second LPS challenge study (17 h time point pre-challenge not shown in this figure)

TABLE 5 Parameter estimates for the 2-compartment linear pharmacokinetic (PK) model with Weibull absorption

Parameter	Mono – SC[a]	Mono – IM[a]	Combo – SC[a]
Relative bioavailability	1	0.882	0.931
Weibull scale parameter, λ (h)	0.388	0.0541	6.73
Weibull shape parameter, β	0.801		
CL/F (L/h)	$19.4 \cdot (BW/150)^{1.10}$		
Q/F (L/h)	$3.34 \cdot (BW/150)^{1.10}$		
V_c/F (L)	$35.5 \cdot (BW/150)^{1.39}$		
V_p/F (L)	$17.4 \cdot (BW/150)^{1.39}$		
Residual error (% CV)	13.6		

Note: F in the denominators refers to the unknown absolute bioavailability.

Abbreviations: CL, systemic clearance; Q, distributional clearance; V_c, volume of central compartment; V_p, volume of peripheral compartment.
[a]Mono – SC = ketoprofen mono-substance administered subcutaneous, Mono – IM = ketoprofen mono-substance administered intramuscular, Combo – SC = ketoprofen within the combination product administered subcutaneous

All treated groups in the second LPS challenge study provided a significant improvement in control of pyrexia compared with saline-treated controls. Administration of ketoprofen via the subcutaneous and intramuscular route provided similar control of LPS-induced pyrexia from 1 h until 8 h post-challenge. Similarly, in the study of Plessers et al., (2016), the pyrexia controlling effect of ketoprofen as mono-substance lasted from 1 h to 6 h after LPS challenge in comparison with untreated controls although the inhibitory effect of ketoprofen on PGE$_2$ production persists up to 24 h after treatment (Landoni et al., 1995c). The difference between the molecular and clinical effect of ketoprofen may be explained by the fact that a high level of PGE$_2$ inhibition is needed to achieve a clinical effect (see further below). In addition to that, the duration of the inflammatory stimuli was different between the study of Landoni et al., (1995c) and the in vivo LPS challenge studies. The inhibitory effect on PGE$_2$ production was measured using tissue cages with induced inflammation at 0 h and 9 h (Landoni et al., 1995c) while the inhibitory effect of fever was measured in vivo with induced inflammation at 0 h by a single LPS bolus (studies in the current manuscript and study of Plessers et al., (2016)). A single LPS bolus increases rectal temperature for a short period of time. In both LPS challenge studies in the current manuscript, the rectal temperature of the saline-treated groups returned to normal between 12 and 24 h after challenge. This reduces the ability to detect a treatment effect once the impact of the LPS challenge has ceased. Despite this limitation, the temperature reducing effect of the combination product lasted longer (up to 12 h) in comparison with the mono-substance products due to the prolonged ketoprofen exposure in animals treated with the combination product. The PK profile of ketoprofen in the combination product is characterized by a longer $t_{1/2}$ and lower C_{max} in comparison with the mono-substance ketoprofen products. The altered PK profile results in an increased AUC for ketoprofen in the combination product. The changed PK profile of ketoprofen indicates that the prolonged exposure is due to altered absorption of ketoprofen (longer time to 63.2% absorbed, see Table 5) in the combination product resulting in flip–flop pharmacokinetics (Toutain & Bousquet-Melou, 2004). The cause of the prolonged ketoprofen absorption in the combination product is unclear. The combination product is administered as a solution and gross examination of the injection site did not reveal any visible drug precipitate 1-h post-injection (Zoetis internal data).

FIGURE 5 Fit of the 2-compartment pharmacokinetic (PK) model with Weibull absorption to the mean ketoprofen plasma concentration (±1 sd). Panels on the top row are based on the PK data from the first lipopolysaccharide (LPS) challenge study. Panels on the bottom row are based on the PK data from the PK study
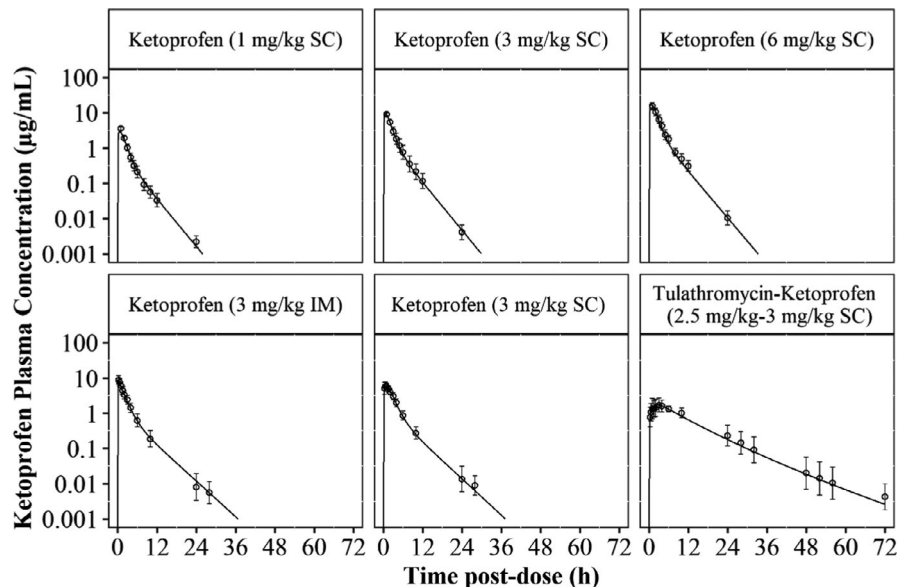

TABLE 6 Parameter estimates for the indirect response pharmacokinetic-pharmacodynamic (PK-PD) model for the treatment response relative to placebo

Parameter	Estimate
k_{out} (h^{-1})	0.744
IC$_{50}$ (µg/ml)	0.796
I$_{max}$ (proportion)	0.0337
gamma	2.04
Residual standard deviation (°C)	0.27

Note: k_{out} = first-order rate constant for loss of the response. IC$_{50}$ = the plasma concentration that produces half-maximal effect. I$_{max}$ = proportional maximum inhibitory effect. gamma = parameter controlling the slope and sigmodicity of the inhibition curve.

Due to the altered ketoprofen PK profile in the combination product in comparison with the ketoprofen mono-substance products, it was unclear if a 3 mg/kg dose of ketoprofen would be the optimal dose when co-formulated with tulathromycin. To avoid the conduct of large animal studies, a PK-PD model of the antipyretic effect of ketoprofen was used to evaluate doses of 1, 3, and 6 mg/kg of ketoprofen in the combination product. At 1 mg/kg, there was a maximal effect of approximately 0.5°C which represents 36% of the maximal ketoprofen effect. The additional efficacy gained from increasing the dose from 3 to 6 mg/kg appeared to be limited; the maximal effect for 3 and 6 mg/kg represents 81% and 93%, respectively, of the maximal ketoprofen effect. At the maximum efficacy point (suppression of rectal temperature increase by 1.05°C at 4.7 h for a 3 mg/kg dose; suppression of rectal temperature increase by 1.20°C at 5.7 h for a 6 mg/kg dose), the difference between a dose of 3 and 6 mg/kg was 0.15°C. This difference is less than one-half standard deviation of the pre-challenge rectal temperatures of either LPS challenge study (std dev = 0.39–0.42°C). Therefore, the 3 mg/kg dose was considered an appropriate dose.

The model parameters estimated from the PK-PD model (Table 6) indicate that the maximal effect of ketoprofen was a suppression of rectal temperature increase by 1.3°C following the LPS challenge. At 3 mg/kg, this suppression (1.05°C) approaches 81% of this maximal level. The rate of loss of effect is determined by the k_{out} parameter which was estimated to be 0.744/h, which corresponds to a half-life of 0.93 h. For the indirect response model, the k_{in} and k_{out} parameters account for the production and loss of the response, in this case, because the temperature increases are due to the LPS challenge these parameters are driven by the nature of that challenge and are unlikely to have a physiological interpretation. The IC$_{50}$ was estimated to be 0.796 µg/ml indicating that to achieve a half-maximal rectal temperature response a plasma concentration of 0.796 µg/ml is required. The 3 mg/kg dose in the combination product provides approximately 10 h above the IC$_{50}$; this is 4 additional hours above the IC$_{50}$ when compared the mono-substance products.

The ketoprofen IC$_{50}$ for inhibition of prostaglandin E$_2$ (PGE$_2$) is a commonly used marker for COX-2 activity. The IC$_{50}$ for inhibition of prostaglandin E$_2$ (PGE$_2$) of ketoprofen based on ex vivo inflamed exudates in cattle has been reported to be 42–99.9 ng/ml (Landoni et al., 1995c; Lepist & Jusko, 2004). Based on the PK-PD model, the IC$_{50}$ for the in vivo inhibition of LPS-induced pyrexia of ketoprofen was 0.796 µg/ml. The large difference between ex vivo and in vivo IC$_{50}$ values can be explained by the fact that a high level of PGE$_2$ inhibition is needed to achieve a clinical effect (Lees, Landoni, et al., 2004; Papich & Messenger, 2015). Typically, IC$_{80}$ values of COX-2 inhibition are considered good predictors of clinical efficacy (Lees, Landoni, et al., 2004; Papich & Messenger, 2015). In the study of Landoni et al., (1995c), a 3 mg/kg ketoprofen dose (mono-substance) inhibited PGE$_2$ by more than 80% compared to placebo treated animals from 6 until 12 h after treatment. This inhibitory effect decreased to approximately 70% at 24 h after treatment. In the LPS challenge studies reported in the present manuscript, administration of 3 mg/kg ketoprofen (mono-substance either SC or
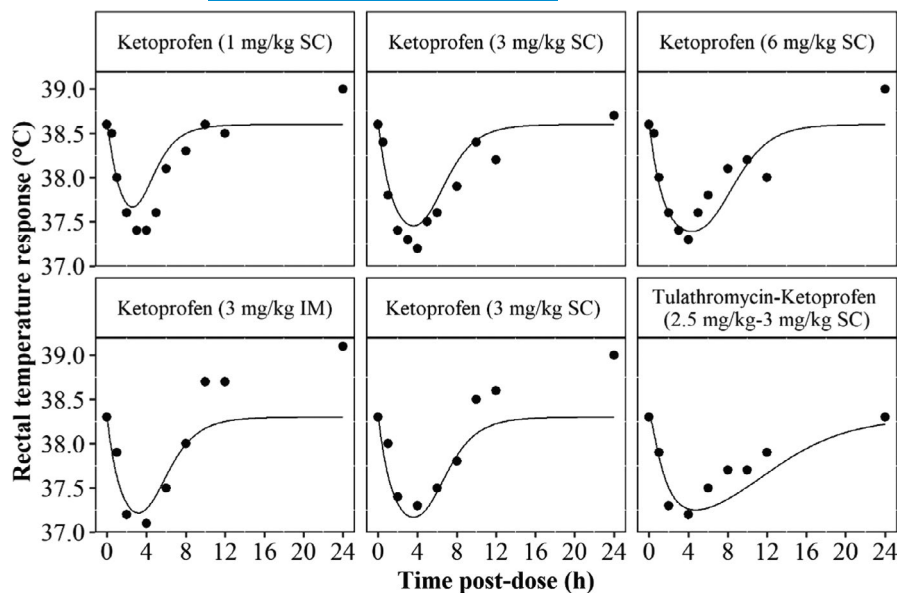
FIGURE 6 Fit of the indirect response model to the rectal temperature response relative to placebo. Panels on the top row are based on the pharmacokinetic-pharmacodynamic (PK-PD) data from the first lipopolysaccharide (LPS) challenge study. Panels on the bottom row are based on the PK data from the PK study and the PD data from the second LPS challenge study
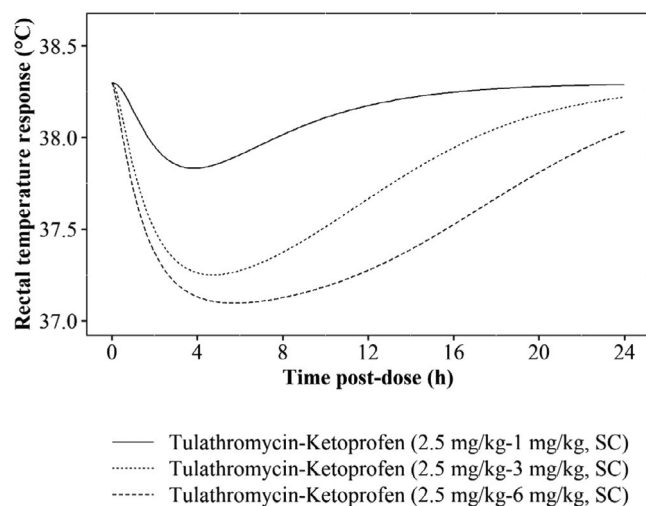

FIGURE 7 Simulation of the pharmacokinetic-pharmacodynamic (PK-PD) model for various doses (1, 3 and 6 mg/kg) of ketoprofen in the tulathromycin–ketoprofen combination product

IM) provided control of LPS-induced pyrexia from 1 until 8 h post-challenge. Taking the differences between the studies into account, the duration of the molecular effect (80% inhibition of PGE_2) seems to be in line with the duration of the clinical effect (reduction of pyrexia) of ketoprofen mono-substance products. Although the combination formulation at the 3 mg/kg ketoprofen dose had a reduced C_{max} for ketoprofen, the C_{max} (2.06 µg/ml) still exceeded the expected efficacious threshold for ex vivo inhibition of COX-2 and in vivo inhibition of LPS-induced pyrexia. Based on the PK models, the time above both values was longer for the combination product than for either SC or IM mono-substance products. The first time point where efficacy was lost, based on IC_{50} values for the in vivo inhibition of LPS-induced pyrexia, for the mono-substance products was at 10 h post-challenge, the plasma concentrations at that point were 0.20 and 0.25 µg/ml for IM and SC ketoprofen doses, respectively, but for the combination product, the plasma levels were

substantially higher with 0.82 µg/ml at 10 h and 0.65 µg/ml at 12 h. The altered ketoprofen PK profile explains the longer duration in activity of the combination product as observed in the second LPS challenge study.

Lipopolysaccharide challenge models are suitable to evaluate properties and doses of anti-inflammatory drugs (Plessers, Wyns, et al., 2015). However, an experimental LPS challenge model is not a complete representation of a natural infection (Remick & Ward, 2005). Ultimately, the selected dose should be carefully evaluated in field studies in natural infected animals. The effect of the combination product was evaluated in comparison with tulathromycin in a natural BRD infection field study (De Koster, under review). The results clearly demonstrated that the combination product has a fast and long anti-pyretic effect in cattle naturally infected with BRD, starting 1 h after treatment and continuing up to 24 h after treatment (De Koster, under review). In contrast, the effect of ketoprofen in the fixed combination product lasted for 12 h in the LPS challenge studies. As indicated above, differences between the LPS challenge study and field study might be explained by the fact that rectal temperature in the LPS challenge declined to normal values due to the short acting characteristic of a single LPS bolus. In a natural BRD field study, the nature of the LPS exposure is more prolonged over time and complicated by other factors (McGill & Sacco, 2020; Mosier, 2014).

5 | CONCLUSION

The PK profile of ketoprofen in a new fixed combination product containing tulathromycin and ketoprofen is characterized by a longer $t_{1/2}$, lower C_{max} and increased AUC in comparison with mono-substance ketoprofen products. The changed PK profile of ketoprofen is caused by a change in the absorption of ketoprofen in the combination product resulting in flip–flop pharmacokinetics. Due to the prolonged ketoprofen exposure associated with the altered PK

profile of ketoprofen in the combination product, the pyrexia reducing effect of the combination product lasted longer in comparison with ketoprofen mono-substance products. A PK-PD model of the anti-pyretic effect of ketoprofen indicated that a 3 mg/kg ketoprofen dose in the combination product provided optimal efficacy.

CONFLICT OF INTEREST

JDK, JB, JKT, and MRS are employees of Zoetis. RG was consulted by Zoetis for review of the PK-PD data.

AUTHOR CONTRIBUTIONS

All authors read and approved the final manuscript. JDK assisted in the interpretation of the data and wrote the manuscript. JKT performed statistical analyses and assisted in the interpretation of the data. JB initiated, designed, and coordinated the studies, performed the pharmacokinetic analysis, created the PK-PD model, and assisted in the interpretation of the data. RG provided input in the creation of the PK-PD model and provided intellectual input to the interpretation of the data. MRS assisted in drafting the manuscript and provided intellectual input to the interpretation of the data.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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