Pharmacokinetics (PK), pharmacodynamics (PD), and PK-PD integration of ceftiofur after a single intravenous, subcutaneous and subcutaneous-LA administration in lactating goats

Emilio Fernández-Varón1*, Carlos Cárceles-García1, Juan Manuel Serrano-Rodríguez2 and Carlos M. Cárceles-Rodríguez1

Abstract

Background: Bacterial pneumonia in goats is usually caused by Mannheimia haemolytica and Pasteurella multocida. Another important infection disease in lactating goats is intramammary infection producing mastitis, usually associated with coagulase-negative Staphylococcus spp. However, treatment of bacterial pneumonia in goats not affected by mastitis problems should be restricted to antimicrobials with scant penetration to milk in order to avoid long withdrawal times. Ceftiofur is a third-generation cephalosporin antimicrobial with activity against various gram-positive and gram-negative, aerobic and anaerobic bacteria encountered by domestic animals. The objectives of the present study were to establish the serum concentration–time profile for ceftiofur in lactating goats after intravenous, subcutaneous and a SC-long-acting ceftiofur formulation; to determine ceftiofur penetration into milk; to determine in vitro and ex vivo activity of ceftiofur establishing MIC, MBC, MPC and time-kill profiles against field strains of M. haemolytica and finally to calculate the main surrogate markers of efficacy.

Results: The pharmacokinetics studies revealed an optimal PK properties for the SC-LA formulation tested. Ceftiofur was well absorbed following SC and SC-LA administration, with absolute bioavailabilities (F) of 85.16 and 84.43 %, respectively. After ceftiofur analysis from milk samples, no concentrations were found at any sampling time. The MIC, MBC and MPC data of ceftiofur against five M. haemolytica strains isolated from goats affected by pneumonia were tested showing excellent sensitivity of ceftiofur against this pathogen. For PK-PD analysis, ratios were calculated suggesting a high level of bacterial kill against the five strains of M. haemolytica tested.

Conclusions: The systemic ceftiofur exposure achieved in lactating goats following IV, SC and especially with the SC-LA administration is consistent with the predicted PK-PD ratios needed for a positive therapeutic outcome for M. haemolytica. Subcutaneous administration of the long-acting formulation showed safety and tolerance for all the animals used. Ceftiofur concentrations exceeded the MIC and MBC for up to 72 h and MPC for up 32 h in serum. Thus, this drug could be effective in treating infectious diseases of goats caused by M. haemolytica at a dose of 6 mg/kg with the SC-LA formulation.

Keywords: Ceftiofur, Pharmacokinetic/Pharmacodynamic, Long-acting formulation, Lactating goats

* Correspondence: emiliovf@um.es
1Department of Pharmacology, Faculty of Veterinary Medicine, University of Murcia, Campus de Espinardo, 30.071, Murcia, Spain

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Background

Ceftiofur is a third-generation cephalosporin antimicrobial with activity against various gram-positive and gram-negative, aerobic and anaerobic bacteria encountered by domestic animals [1]. As with all cephalosporins, it is considered bactericidal because it inhibits bacterial cell wall synthesis and its pharmacokinetic and pharmacodynamic (PK/PD) parameter most associated with efficacy is the time above a threshold concentration, typically the MIC [2].

Over the past years, the food and milk that goats provide have become more popular in Europe and in the United States [3]. Bacterial pneumonia are frequent in this species, and the most common bacterial isolates from lungs of goats affected by pneumonia are Pasteurella multocida and Mannheimia haemolytica [3, 4]. Another important infection disease in lactating goats is intramammary infection producing mastitis, usually associated with coagulase-negative Staphylococcus spp. However, treatment of bacterial pneumonia in goats not affected by mastitis problems should be restricted to antimicrobials with scant penetration to milk in order to avoid long withdrawal times. Other antimicrobials agents that could be used in pneumonia are fluoroquinolones and macrolides that have a wide milk penetration.

Ceftiofur has been synthesized in various salt forms. The sodium and hydrochloride salts were first developed and approved worldwide for treatment of respiratory disease in beef and dairy cattle. Additional indications were also approved in swine, goats, sheep, horses, day-old chickens, day-old turkey poults and dogs [5]. The third salt approved was a ceftiofur free acid form in a sterile oil formulation (CCFA) showing excellent sustained-release properties. This oily, non-aqueous suspension of CCFA has good efficacy and has been studied in different animal species like horses [6], cattle [5], lactating and non-lactating goats [3]. However, issues with residues, inflammatory reactions and pain at the injection site have been reported.

In the present study, we have assayed a subcutaneous, aqueous, polymeric sustained-release formulation with the sodium salt. This subcutaneous long-acting formulation has been made with polymer P407. P407 is a block of copolymers contains 7 % polyoxyethylene units and 30 % polyoxypropylene units. This polymer has some advantages as its low toxicity, excellent compatibility with other chemicals and a high solubilizing capacity for different drugs. P407 shows temperature-dependent gelation (gel consistency at 37 °C and liquid consistency at 4 °C) [7]. The P407 gel has been evaluated for the delivery of biologically active proteins such urease and interleukin-2 [8, 9] and antibiotics as ceftiofur [7], doxycycline [10], moxifloxacin [11] and difloxacin [12].

The objectives of the present study were: (1) to establish the serum concentration–time profile and to derive pharmacokinetics data for ceftiofur in lactating goats after intravenous (IV), subcutaneous (SC) and a subcutaneous long-acting (SC-LA) ceftiofur formulation; (2) to determine the rate and extent of ceftiofur penetration into and elimination from milk after IV, SC and SC-LA administration of ceftiofur; (3) to determine in vitro activity of ceftiofur establishing MIC and MBC in serum and Mueller Hinton broth, and MPC of ceftiofur for five strains of Mannheimia haemolytica isolated from caprine (4) to determine ex vivo time-kill profiles of ceftiofur against the five strains of M. haemolytica in both in serum and Mueller-Hinton broth (5) to calculate the surrogate markers of efficacy against Mannheimia haemolytica strains isolated from goats affected by pneumonia.

Methods

Animals

Six clinically healthy Murciano-Granadina female lactating goats weighing between 40.5 and 56 kg and aged from 2.5 to 3.5 years from the Caprine Farm of the University of Murcia were used. The animals were housed and fed an antibiotic-free diet for at least 30 days preceding the study. For each treatment period of the cross-over study, they were observed daily for general health, and clinical observations were made prior to injection and at 2, 10 and 24 h post-injection. Alfalfa hay and water was provided ad libitum together with a drug-free concentrate. The study was approved by the Bioethics Committee of the University of Murcia.

Experimental design

A cross-over design (2 × 2 × 2) was used in three phases. Each animal received either a single IV or SC injection of ceftiofur sodium (Excenel® 4 g, Pfizer, Madrid, Spain) at a dose of 2.2 mg/kg or a SC-LA administration of 6.6 mg/kg with at least a 15-day washout period.

For the IV administration, the solution was injected into the left jugular vein and blood samples (4 mL) were collected from the contralateral jugular vein. SC and SC-LA injections were administered under the skin of the back at a single location in the thoraco-lumbar region lateral of the mid-line. Blood samples were collected at 0 (pre-treatment), 0.083, 0.167, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96 and 120 h post-injection. Samples were centrifuged at 1500 g for 15 min and the serum taken and stored at −90 °C until assayed.

Milk samples for analysis were collected from each goat after complete evacuation of the udder by manual stripping of each gland immediately before dosing on the day of treatment administration (time 0) and at 1, 2, 4, 6, 8, 10, 12, 24, 32, 48, 72, 96 and 120 h after
administration. After shaking the milk to homogenize, a 4–5 mL sample was collected and stored at −90 °C until assayed.

**Gel preparation**

Gel was prepared on a weight basis using the cold method [13]. Concentrations of P407 and ceftiofur reported here are expressed as weight percentage (% wt/wt). An amount of P407 sufficient to yield 25 % and Carboxymethylcellulose sodium to yield 2 % gel was slowly added at 4 °C and ceftiofur sodium sufficient to yield a 20 % concentration was dissolved in the cold solution.

**Analytical method**

Serum and milk samples were analyzed for concentrations of ceftiofur, desfuroylceftiofur, and related metabolites by reduction and derivatization to desfuroylcefotaxime (DCA) [14]. Ceftiofur and desfuroylcefotaxime-related metabolites were extracted from serum and milk following a reduction step through the addition of 1,4-dithioerythritol solution (20 mg/mL in 0.1 M ammonium acetate, pH = 8.9). An internal standard consisting of a cefotaxime solution (200 μg/mL) was incorporated in this step. Following a 30-min incubation step at 50 °C to fully reduce the thioester bond in ceftiofur and desfuroylcefotaxime-related metabolites, the resulting desfuroylceftiofur was captured on a C18 solid-phase extraction columns (Oasis HLB SPE cartridges, Waters, Barcelona, Spain) and further derivatized with iodoacetamide to create desfuroylacetamide (DCA) [14]. DCA was removed from the column with 30:70 acetonitrile: 0.01 M ammonium acetate with 0.1 % trifluoroacetic acid providing a final injection equal to isocratic HPLC conditions (15 % acetonitrile: 85 % 0.01 M ammonium acetate (0.1 % trifluoroacetic acid)). The HPLC separation was performed using a reverse-phase Kinetex^™^ PFP C18 column (250 × 4.6 mm; 5 μm) with an injection volume of 100 μL. Ultraviolet detector was set at 240 nm.

**Method validation**

Quality controls were prepared from a pool of blank goat serum or milk spiked with seven concentrations of ceftiofur between 0.10 and 10 μg/mL. Serum and milk aliquots were stored at −90 °C until assay. Aliquots of quality controls were extracted as above and 100 μL was injected into the chromatographic system. Standard curves were obtained by unweighted linear regression of ceftiofur and cefotaxime peak areas versus known concentrations. Each point was established from an average of five determinations. Correlations coefficients (r) were >0.99 for calibration curves. The percentage recovery was determined by comparing the peak areas of serum and milk blank samples spiked with different amounts of drug and treated as any samples, with the peak areas of the same standards prepared in phosphate buffer. Each point was established from an average of five determinations. The mean percentage recoveries of ceftiofur from serum and milk were 84.79 and 88.16 %, respectively. The assay precision (R.S.D.) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. Serum intra-day precision was estimated from six replicates of three standard samples used for calibration curves (R.S.D. < 5.83 %). Milk intra-day precision was R.S.D. < 10.2 %. Inter-day precision was estimated from the analysis of standard samples (serum or milk) on three separate days. Serum inter-day obtained a R.S.D. < 4.91 %. Milk inter-day assay obtained a R.S.D. < 6.54 %. The limit of quantification (LOQ) and the limit of detection (LOD) for was 0.1 μg/mL for serum and milk.

**Bacterial strains, MIC, MBC and MPC determination**

Five field strains of *M. haemolytica* isolated from goats affected by pneumonia in Spain were used. The strains were stored at −80 °C in a nutrient broth enriched with 15 % glycerol until assayed. The MIC of ceftiofur was determined in goat serum and Mueller-Hinton broth (MHB, Fluka analytical, Madrid, Spain) using the microdilution method recommended by CLSI [15]. The assay was performed in U-bottomed, 96-well, custom-designed microtiter plates. After overnight incubation at 37 °C on tryptone soya blood agar plates, bacterial suspensions equal to a 0.5 McFarland standard were further diluted and 10 μL were added to the plates to achieve a final inoculum of 5 × 10^5 CFU/mL. The plates contained antimicrobial dilutions ranging from 0.03 to 128 μg/mL in 90 μL/well of goat serum or MHB, the final volume was 100 μL. Assays were incubated at 37 °C and observed after 24 h. The MIC was taken as the lowest drug concentration that inhibited visible growth. MBC was established by plate count as the concentration of antibacterial to reduce a 3log_{10} (99.9 % killing) the initial inoculum, in accordance with CLSI guidelines [15].

The MPC was measured by agar dilution using a method previously described [16]. Briefly, the content of overnight cultures of each strain of *M. haemolytica* (5 plate per isolate) in TSBA was transferred to 100 mL of MHB and incubated overnight at 37 °C with shaking at 200 rpm. The next day, bacterial suspensions were estimated to have concentrations close to 3·10^8 CFU/mL by turbidity measurements. Then, cultures were concentrated by centrifugation at 5000 g for 30 min at 5 °C and re-suspended in 3 mL of fresh MHB. Aliquots of 200 μL containing 10^10 CFU were inoculated in TSBA plates previously prepared with ceftiofur over a range from 0.03 to 128 μg/mL. Plates were incubated for 48 h at 37 °C and screened visually each 24 h for growth. The
MPC was recorder as the lowest concentration that prevented the growth of colonies.

For MIC, MBC and MPC assays, a non-inoculated plate was included as a negative control, and an inoculated plate without drug as a positive control. Also, S. aureus ATCC 29213 and E. coli ATCC 25922 strains were used as controls. All determinations were performed in duplicate, and the geometric mean was calculated.

In vitro antimicrobial growth (time-kill) curves

Time kill curves were obtained in a second trial after MIC, MBC and MPC determination. In vitro and ex vivo activity of ceftiofur against M. haemolytica was obtained using a method previously described [17]. Eight to 10 colonies from overnight cultures in TSBA of each strain were used to inoculate 9 mL of MHB and incubated overnight at 37 °C with shaking at 200 rpm.

For in vitro assays, 480 μL of MHB or goat serum were spiked with 10 μL of concentrated solutions of ceftiofur. The final samples contained antimicrobial concentrations at 0 (control), 0.25, 0.5, 1, 2, 4, 8, and 16 multiples of MIC previously obtained in each fluid. A total of 10 μL of stationary-phase bacterial culture was added to give a final concentration of approximately 5 × 10^6 CFU/ml. Aliquots of 25 μL were sampled from each culture at 0, 1, 2, 4, 8 and 24 h. Counts were determined by serial dilution in saline and spread on TSBA agar plates [18]. For ex vivo testing, serum samples from goat which had received ceftiofur IV, SC or SC-LA at 2.2 or 6.6 mg/kg respectively, were collected at 0, 1, 2, 4, 12, 24, 32, 48, and 72 h. Concentrations of ceftiofur in these samples were previously determined with the HPLC method described in the above section. Then, a total of 10 μL of stationary-phase bacterial culture was added, and aliquots of 25 μL were sampled from each culture at 0, 1, 2, 4, 8 and 24 h using the same methodology described for in vitro time kill curves. Counts were determined by serial dilution in saline and spread on TSBA agar plates. The lower limit of detection was 40 CFU/mL for both in vivo and ex vivo assays. Time kill curves were performed at 37 °C with shaking at 200 r.p.m.

Pharmacokinetic analysis

The serum ceftiofur time-concentration data were analysed by non-compartment methods using WinNonLin 5.2 (Pharsight Corp, Mountain View, Calif). The area under the concentration-time Curve (AUC) was calculated using the linear trapezoidal rule with extrapolation to time infinity. Mean Residence Time was calculated as MRT = AUMC/AUC. Mean absorption times were calculated as MAT = MRT_{IV} - MRT_{SC, SC-LA}. The systemic clearance was estimated as Cl = Dose/AUC. The apparent volume of distribution at steady state were calculated as V_{ss} = (Dose · AUMC)/AUC^2. The apparent volume of distribution (area method) was calculated as V_{a} = Dose/(AUC · λ_{a}). Bioavailability (F) was calculated by the method of corresponding areas:

\[ F(\%) = \left( \frac{\text{AUC}_{\text{SC, SC-LA}} \cdot \text{Dose}_{\text{IV}}}{\text{AUC}_{\text{IV}} \cdot \text{Dose}_{\text{SC, SC-LA}}} \right) \times 100 \]

Statistical analysis

Descriptive statistical parameters as mean, standard deviation and coefficient of variation were calculated. Harmonic means were calculated for the half-lives of elimination. A Kruskal-Wallis analysis of variance, followed by a Dunn multiple comparison test if applicable was used to test parameters for significant differences between IV, SC and SC-LA administration. Dose-dependent (AUC and C_{max}) parameters were corrected with dose to test for statistical differences. The level of significance was P ≤ 0.05. The statistical software used was SPSS (IBM SPSS Statistics, Version 19.0. Armonk, NY: IBM Corp).

Results

The mean (±SD) serum concentrations of ceftiofur following IV, SC and SC-LA administration are plotted in Fig. 1. The estimates of the non-compartmental pharmacokinetics parameters following IV, SC and SC-LA administration are summarized in Table 1.

Clinical examination of all goats after each phase of the trial did not reveal any abnormalities. Local or systemic adverse reactions were not observed neither during nor after IV, SC and SC-LA administration, respectively.

The ceftiofur half-life (t_{1/2,α}) for IV, SC and SC-LA routes was 4.21, 5.10 and 41.12 h. Clearance value after IV dosing was 0.04 ± 0.01 L/kg·h. After SC and SC-LA administration, the absolute bioavailability was mean (±SD) 85.16 ± 10.24 % and 84.43 ± 7.40 %, respectively. The C_{max} was 6.25 ± 0.85 μg/mL (SC) and 4.26 ± 0.68 μg/mL (SC-LA) with a T_{max} of 0.91 h (SC) and 4.66 h (SC-LA). There were no significant differences between values calculated for C_{max}, AUC_{0-∞} and AUC_{0-24} for both subcutaneous routes of administration (dose corrected for comparison). However, significant differences (P < 0.05) were found between SC and SC-LA formulations for λ_{2}, t_{1/2,2x}, MRT, MAT, K_{a} and T_{max} as expected.

After ceftiofur analysis from milk samples, no concentrations were found at any sampling time.

The MIC, MBC and MPC values were obtained from strains of M. haemolytica isolated from goats affected by pneumonia. Ceftriaxone showed excellent in vitro activity, the surrogate markers were calculated and are shown in Table 2. Time-kill curves in serum and Mueller-Hinton
broth are shown in Fig. 2. Time-kill curves in goat serum after SC and SC-LA administration of ceftiofur are shown in Fig. 3.

**Discussion**

The pharmacokinetics of ceftiofur sodium has been studied in goats [19], sheep [20], camels [21], llamas [22], deers [23], horses [6], fowls [14] and pigs [24]. However, there are limited data available in lactating animals and especially in small ruminants like lactating goats. Besides, to our knowledge there is no information about ceftiofur pharmacokinetics and pharmacodynamics integration data of the main pathogens involve in pneumonia in goats (Pasteurella multocida and Mannheimia haemolytica) and studies with long-acting formulations are very limited [7, 11, 12]. Some years after ceftiofur sodium development and commercial availability, a new sustained-release ceftiofur salt was developed as crystalline-free acid

**Table 1** Pharmacokinetic parameters (mean ± S.D.) of ceftiofur in lactating goats after IV and SC administration at a dose of 2.2 mg/kg bodyweight, and after SC-LA administration at a dose of 6.6 mg/kg bodyweight (n = 6)

| Treatment       | Intravenous | SC      | SC-LA   |
|-----------------|-------------|---------|---------|
| Parameters      |             |         |         |
| λz (1/h)        | 0.16 ± 0.07 | 0.14 ± 0.06 | 0.02 ± 0.01<sup>a, b</sup> |
| t½ λz (h)       | 4.21        | 5.10    | 41.12<sup>b</sup> |
| Vz (L/kg)       | 0.18 ± 0.05 | –       | –       |
| Vss (L/kg)      | –           | –       | –       |
| AUC<sub>0-24</sub> (μg · h/mL) | 45.51 ± 6.80 | 38.17 ± 6.97 | 85.13 ± 7.53<sup>a</sup> |
| AUC<sub>0-∞</sub> (μg · h/mL) | 47.06 ± 6.84 | 39.88 ± 6.26 | 117.12 ± 11.54<sup>a</sup> |
| MRT (h)         | 4.27 ± 0.98 | 6.29 ± 0.35<sup>a</sup> | 25.11 ± 4.35<sup>a, b</sup> |
| Cl (L/h · kg)   | 0.04 ± 0.01 | –       | –       |
| T<sub>max</sub> (h) | –           | –       | –       |
| Ka (1/h)        | –           | 0.53 ± 0.49 | 0.03 ± 0.01<sup>b</sup> |
| C<sub>max</sub> (μg/L) | –   | 6.25 ± 0.85 | 7.77 ± 1.26 |
| F (%)           | –           | 85.16 ± 10.24 | 84.43 ± 7.40 |

<sup>a</sup> Significantly different from IV (P < 0.05). <sup>b</sup> Significantly different from SC (P < 0.05). <sup>1</sup> Harmonic Mean

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**Fig. 1** Semilogarithmic plot of serum concentrations (mean ± SD) of ceftiofur and desfuroylceftiofur-related metabolites after IV administration (2.2 mg/kg) (IV; –●–), SC administration (2.2 mg/kg) (SC; –○–), and SC-LA administration (6.6 mg/kg) (SC-LA; –□–) in goats (n = 6)
The long-acting formulation has been studied in different animal species as horses [6], cattle [5], lactating and non-lactating goats [3]. Importance of development of sustained-release formulations in veterinary medicine and especially in food animal species has been increasing last years. The advantages of long-acting formulations of ceftiofur (and other drugs) in lactating goats include less quantity of drug used (decreasing collateral effects and accumulation in long-term treatments), increased treatment efficacy (less fluctuations of the stationary concentrations and much longer release times) and a reduction in handling (stress of the animals and veterinary costs are decreased). On the other hand, it is necessary to consider drug safety, efficacy and residues at the site of infection as serious issues with these formulations. Although most studies have concluded that ceftiofur free-acid in pigs and cattle was well tolerated systemically, frequent adverse effects have been reported at the injection sites with the CCFA formulation. Other formulations have been studied like liposomes in cows [25].

In the present study an aqueous polymeric sustained-release formulation has been developed for subcutaneous administration. After IV administration, the half-life ($t_{1/2}$) was slightly higher than that reported in lactating goats after IV dosing of ceftiofur sodium ($t_{1/2} = 3.88$ h) [19], and clearance ($Cl = 0.04$ L/h·kg) was lower than the data reported by the same authors (0.08 L/h·kg). The volume of distribution ($0.31$ and $0.18$ L/kg calculated by the area method ($V_z$) and at steady-state ($V_{ss}$), respectively, suggests limited penetration through biological membranes, and these values indicate that ceftiofur is widely distributed into the extravascular tissues. In other studies, larger but also limited volume of distribution $V_{ss}$ was described for ceftiofur sodium in lactating (0.31 L/kg) and non-lactating (0.25 L/kg) goats [19]. Elimination half-life ($t_{1/2\alpha}$) of the SC-LA formulation (41.12 h) was far longer than that for the SC administration (5.1 h) showing prolonged permanence.

### Table 2

| Parameter | IV | SC | SC-LA |
|-----------|----|----|------|
| Mean MIC ($M. haemolytica$) | 0.0436 μg/mL | | |
| $C_{max}/MIC$ | $–$ | $130.53 \pm 28.65$ | $145.47 \pm 22.55$ |
| $AUC_{0-\infty}/MIC$ (h) | $1079.16 \pm 157$ | $914.77 \pm 143$ | $2686.34 \pm 264$ |
| $AUC_{24}/MIC$ (h) | $1043.80 \pm 155$ | $875.38 \pm 160$ | $1952.44 \pm 173$ |
| $T > MIC$ (h) | $24$ h | $24$ h | $72$ h |
| Mean MBC ($M. haemolytica$) | 0.10 μg/mL | | |
| $C_{max}/MBC$ | $–$ | $56.91 \pm 12.49$ | $63.42 \pm 9.83$ |
| $AUC_{0-\infty}/MBC$ (h) | $470.51 \pm 68.46$ | $398.84 \pm 62.60$ | $1171.24 \pm 115.4$ |
| $AUC_{24}/MBC$ (h) | $455.1 \pm 67.98$ | $381.66 \pm 69.78$ | $851.27 \pm 75.36$ |
| $T > MBC$ (h) | $24$ h | $24$ h | $72$ h |
| Mean MPC ($M. haemolytica$) | 0.55 μg/mL | | |
| $C_{max}/MPC$ | $–$ | $10.34 \pm 2.27$ | $11.53 \pm 1.79$ |
| $AUC_{0-\infty}/MPC$ (h) | $85.54 \pm 12.36$ | $72.51 \pm 11.38$ | $212.95 \pm 20.98$ |
| $AUC_{24}/MPC$ (h) | $82.74 \pm 12.36$ | $69.39 \pm 12.69$ | $154.77 \pm 13.70$ |
| $T > MPC$ (h) | $12$ h | $12$ h | $32$ h |

**Fig. 2** In vitro antibacterial activity of ceftiofur in MHB (a) and serum (b) of goats against *M. haemolytica* expressed as mean values ($n = 5$). SD values are excluded for clarity.
of the drug in serum with this long-acting formulation. Cef-
tiofur was well absorbed following SC and SC-LA adminis-
tration, with absolute bioavailabilities (F) of 85.16 and
84.43 %, respectively. MAT was 2.02 h for the SC adminis-
tration and ten-fold longer for the SC-LA formulation
(20.83 h). Absorption rate constant (K_a) was 0.53 h
−1 for the SC administration and 0.03 h
−1 after SC-LA. These ab-
sorption data reflect that the polymeric formulation has re-
duced considerably the rate of absorption affecting the
elimination rate where the SC-LA formulation showed a
significantly longer elimination half-life respect to the con-
ventional SC formulation.

After ceftiofur analysis from milk samples, no concen-
trations were found at any sampling time. This result is
in agreement with other studies in goats [3] and cows
[26]. Ceftiofur sodium has a pK_a value of 3.7. Ceftiofur
sodium in the blood stream (pH 7.4) would act as a
weak acid with and insufficient lipid-soluble properties
at this pH to penetrate milk, which can also explain lim-
ited volume of distribution obtained.

Pharmacodynamics of ceftiofur against M. haemoly-
tica in MHB showed in vitro maximum growths from
3.5°10^9 to 7°10^9 CFU/mL obtained at 24 h without
drug. Ceftiofur concentrations multiples to 1 × MIC
produced a 0log_{10} reduction in count over 24 h (bacteriostatic effect), whereas concentrations less than
1 × MIC produced no growth inhibition (Fig. 2a). A 3-4log_{10} reduction of count was achieved after 24 h from
2 × MIC (bactericidal effect). After in vitro time kill curves in serum, maximum growths from 4.4°10^9
to 8.5°10^9 CFU/mL were obtained at 24 h without
drug. Concentrations less than or equal to 2 × MIC
produced no growth inhibition with a 0-2log_{10} reduc-
tion in count over 24 h. A 3-4log_{10} reduction was
achieved at concentrations similar or higher than 4 ×
MIC after 24 h of incubation, and a regrowth was not
observed (Fig. 2b).

Ex vivo time kill curves showed antibacterial activity of
ceftiofur against M. haemolytica in serum of lactating
goats administered as a conventional SC and SC-LA for-
mulation. The time points selected for the study with
serum samples were of 0 (control), 1, 2, 4, 12, 24, 32, 48
and 72 h after drug administration. Maximum growths
closed to 5°10^9 CFU/mL were obtained at 24 h without
drug. For SC time kill curve (Fig. 3a), a 3-4log_{10} reduc-
tion after 24 h incubation was achieved with ceftiofur
samples obtained from 1 to 24 h. A 2 log_{10} reduction
was observed with at samples measured at 32 h. How-
ever serum samples from 48 to 72 h produced no
growth inhibition, whereas these time points achieved a
3log_{10} reduction after 24 h of incubation with the SC-
LA formulation. Moreover, samples between 1 and 32 h
produced an elimination of organisms from 8 to 24 h of
incubation with a 4-5log_{10} reduction. These results
could be explained by the ceftiofur concentrations
achieved with the SC-LA formulation.

The most important factor determining the efficacy of
β-lactam antimicrobials such as ceftiofur is the amount
of time that serum concentrations exceed the MIC of a
given pathogen [27, 28]. However, this approach, almost
unanimous in literature, is been questioned in some
cases. In a recent PK/PD study of a depot formulation of
amoxicillin in calves against bovine respiratory pa-
thogens, Mannheimia haemolytica and Pasteurella
multocida, it was stated that for sustained-release for-
mulations a concentration-dependent action would bet-
ter fit according to the time-kill data obtained [29].
Similar results were found in sheep against Mannheimia
haemolytica and Pasteurella multocida isolates for
amoxicillin [30]. Additionally, it has been reported that
for drugs like the β-lactams, where efficacy has been
found to be correlated to the T > MIC, the best PK/PD
index shifts towards AUC/MIC dependence as half-life
increases [31]. In the present study, in vitro and ex vivo
time-kill curves against *Mannheimia haemolytica* also suggest a possible concentration-dependent action. Therefore, the modelling of AUC/MIC data as surrogate for/predictor of efficacy is justified at least for these respiratory tract pathogens [32, 33]. The longer half-life obtained in the present study for the SC-LA formulation (41.12 h) regard to the SC (5.1 h) reinforce this approach for PK/PD integration of our data. Thus, PK/PD integration was calculated for the five strains of *M. haemolytica* using the three commonly used surrogates markers of efficacy, AUC/MIC, C\(_{\text{max}}$/MIC and T > MIC, required to inhibit growth.

The MIC, MBC and MPC data of ceftiofur against five *M. haemolytica* strains isolated from goats affected by pneumonia were tested. The MIC values in this study showed good sensitivity of ceftiofur against this pathogen. MIC, MBC and MPC are values tested in in vitro conditions, and it is known influence of matrix when MIC and MBC values are calculated (MHB and serum) mainly by effect of drug protein binding that should correspond to lower MIC and MBC values in MHB regard to serum values. In our study no differences were found in MIC values between both matrices. However in the case of MBC values calculated both in MHB and serum, differences were found in two strains (two-fold) between MHB and serum which is in agreement with the lower total protein concentrations present in MHB respect to serum [34].

On the other hand, although MIC values are the standard to calculate the three most important surrogate markers of efficacy C\(_{\text{max}}$/MIC, AUC\(_{0-24}$/MIC ratios and T > MIC to get an optimal dosage regimen, MIC values are considered less appropriate in preventing the emergence of resistant strains. The use of MPC values defined as the lowest drug concentration that prevents the growth of the least susceptible first-step resistant mutants have been considered to calculated AUC\(_{24}$/MPC ratios and the mutant selection window (MSW) that serve as an indicator of drug exposure that prevents the selection of drug-resistant mutant [35, 36]. In the present study, AUC\(_{24}$/MPC ratio and T > MPC were calculated and for the SC-LA formulation values of 154 h and 32 h were obtained, respectively, showing the good profile of the long-acting formulation to achieve both clinical efficacy and prevention of emergence of resistant strains. Besides, the MIC and MPC are also useful to define the bounds of the mutant selection window (MSW), a range of antibacterial concentrations in which drug least susceptible cells could be selected most frequently. MSW in this study range from 0.0436 and 0.55 μg/mL according to the mean MIC and MPC values. Concentrations of ceftiofur with the LA formulation exceed the MPC for the first 48 h which suggest that susceptible strains of *M. haemolytica* in vivo could be eradicated. However, it is necessary to be cautious with these results and to consider factors like in vitro conditions of MPC calculation, lack of studies, scores of the clinical symptoms in affected animals and the low number of strains tested in this study.

For PK-PD analysis, the MIC data of ceftiofur against *M. haemolytica* strains isolated from goats affected by pneumonia were tested resulting a mean MIC of 0.0436 μg/mL. The C\(_{\text{max}}$/MIC, AUC\(_{24}$/MIC, AUC\(_{0-\infty}$/MIC ratios and T > MIC were calculated (Table 2). The four ratios used suggest a high level of bacterial kill against the five strains of *M. haemolytica* tested. For the SC-LA formulation AUC\(_{24}$/MIC, even AUC\(_{0-\infty}$/MIC exceed widely 125 h, and C\(_{\text{max}}$/MIC exceed 10 as the ratios usually considered to have clinical efficacy. T > MIC was greater than 72 h for the SC-LA formulation. Although these ratios should be integrated with MIC distribution values from field isolates [29], these data show a high clinical efficacy against this pathogen and possibly against other pathogens in goats.

### Conclusions

The systemic ceftiofur exposure achieved in lactating goats following IV, SC and especially with the SC-LA administration is consistent with the predicted PK-PD ratios needed for a positive therapeutic outcome for *M. haemolytica*. Subcutaneous administration of the long-acting formulation showed safety and tolerance for all the animals used. Milk penetration of ceftiofur was nonexistent in all animals at least at the limit of detection of our study, which is a clear advantage in lactating animals affected by pneumonia avoiding withdrawal periods. This study has shown that ceftiofur concentrations exceeded the MIC and MBC for up to 72 h and MPC for up 32 h in serum. Thus, this drug could be effective in treating infectious diseases of goats caused by *M. haemolytica* at a dose of 6 mg/kg with the SC-LA formulation. However, further studies in affected animals taking into account other factors as scores of the clinical symptoms, to optimize and to know the optimal ratios of the surrogate markers of efficacy.

### Additional file

**Additional file 1:** Plasma Cefiofur concentrations and time kill curves data (XLSX 15 kb)

### Abbreviations

- HPLC: High performance liquid chromatography
- IV: Intravenous
- MPC: Minimum bactericidal concentrations
- MIC: Minimum inhibitory concentrations
- MSW: Mutant prevention concentrations
- PK-PD: Pharmacokinetics-pharmacodynamics
- SC: Subcutaneous
- LA: Subcutaneous-Long acting

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**Availability of data and material**
The data sets supporting the results of this article are included within the article and its supplementary file (Additional file 1).

**Authors’ contributions**
EF-V and CC-R were responsible for the study design, coordination, animal phase, data analysis and were involved in the elaboration of the manuscript. CC-G participated in design and elaboration of the LA formulations, micro-phase, data analysis and were involved in the elaboration of the manuscript. All authors read and approved the final manuscript.

**Competing interests**
The authors declare that they have no competing interests.

**Consent for publication**
Not applicable.

**Ethics approval and consent to participate**
The study was approved by the Bioethics Committee of the University of Murcia.

**Author details**
1. Department of Pharmacology, Faculty of Veterinary Medicine, University of Murcia, Campus de Espinardo, 30101, Murcia, Spain. 2. Department of Pharmacology, Toxicology and Legal and Forensic Medicine, Faculty of Veterinary Medicine, University of Cordoba, Campus de Rabanales, 14071, Córdoba, Spain.

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**References**
1. Hope KL, Tell LA, Byrne BA, Murray S, Wetzlich SE, Ware LH, et al. Pharmacokinetics of a single intramuscular injection of ceftiofur crystalline-free acid in American black ducks (Anas rubripes). J Am Vet Res. 2012;73(5):620–7.

2. Sadar MJ, Hawkins MG, Byrne BA, Cartoceti AN, Keel K, Drazenovich TL, et al. Pharmacokinetics of a single intramuscular injection of ceftiofur crystalline-free acid in red-tailed hawks (Buteo jamaicensis). Am J Vet Res. 2015;76(12):1077–84.

3. Doré E, Angelos IA, Rowe JD, Carlson JL, Wetzlich SE, Kieu HT, et al. Pharmacokinetics of ceftiofur crystalline-free acid after single subcutaneous administration in lactating and non lactating domestic goats (Capra aegagrus hircus). J Vet Pharmacol Ther. 2011;34(1):25–30.

4. Berge AC, Sischo WM, Craigmill AL. Antimicrobial susceptibility patterns of respiratory tract pathogens from sheep and goats. J Am Vet Med Assoc. 2006;229(8):1279–81.

5. Hibbard B, Robb EJ, Chester Jr ST, Dame KJ, Boucher JF, Alaniz GR. Dose determination and confirmation of a long-acting formulation of ceftiofur (ceftiofur crystalline free acid) administered subcutaneously for the treatment of bovine respiratory disease. J Vet Pharmacol Ther. 2002;25(3):175–80.

6. Collard WT, Cox SR, Lesman SP, Grover GS, Boucher JF, Halbert JW, et al. Pharmacokinetics of ceftiofur crystalline-free acid sterile suspension in the equine. J Vet Pharmacol Ther. 2011;34(5):476–81.

7. Zhang L, Parsons DL, Navare C, Kompella UB. Development and in Vitro evaluation of sustained release Poloxamer 407 (P407) gel formulations of ceftiofur. J Control Release. 2002;85(1–3):73–81.

8. Johnston TP, Punjabi MA, Froelich CJ. Sustained delivery of interleukin-2 from a poloxamer 407 gel matrix following intraperitoneal injection in mice. Pharm Res. 1992(9):425–34.

9. Pec EA, Wout Z, Johnston TP. Biological activity of urease formulated in poloxamer 407 after intraperitoneal injection in the rat. J Pharm Sci. 1996;81:626–30.

10. Gutierrez L, Ocampo L, Espinosa F, Sumano H. Pharmacokinetics of an injectable long-acting parenteral formulation of doxycycline hyclate in pigs. J Vet Pharmacol Ther. 2014;37(1):83–9.

11. Cácceres CM, Serano JM, Marín P, Escudero E, Fernández-Varón E. Pharmacokinetics of moxifloxacin in rabbits after intravenous, subcutaneous and a long-acting poloxamer 407 gel formulation administration. J Vet Med A Physiol Pathol Clin Med. 2006;53(6):300–4.

12. Marín P, Escudero E, Fernández-Varón E, Ramírez MJ, Cácceres CM. Pharmacokinetics and milk penetration of difloxacin after a long-acting formulation for subcutaneous administration to lactating goats. J Dairy Sci. 2010;93(7):3056–64.

13. Schmolka IR. Artificial skin. I. Preparation and properties of pluronic F-127 gels for treatment of burns. J Biom Mater Res. 1972(6):571–82.

14. Meyer S, Giguère S, Zielinski RL, Grover GS, Brown SA. Pharmacokinetics of intravenous cefotiofur sodium and concentration in body fluids of foals. J Vet Pharmacol Ther. 2009;32(4):309–16.

15. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobials Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. 2012.

16. Blouin J, Borso S, Blondeau LD, Blondeau BJ, Heje SE. Comparative minimum inhibitory and mutant prevention drug concentrations of enrofloxacin, ceftiofur, florfenicol, tilmicosin and tulathromycin against bovine clinical isolates of Mannheimia haemolytica. Vet Microbiol. 2012;160(1–2):85–90.

17. Ablaibadi FS, Landoni MF, Lees P, Pharmacokinetics (PK), pharmacodynamics (PD), and PK-PD integration of danofloxacin in sheep biological fluids. Antimicrob Agents Chemother. 2003;47(7):2626–35.

18. Clinical and Laboratory Standards Institute (CLSI). Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline. CLSI document 26-A. 1999.

19. Courtin F, Craigmill AL, Wetzlich SE, Gustafson CR, Amitd TS. Pharmacokinetics of ceftiofur and metabolites after single intravenous and intramuscular administration and multiple intramuscular administrations of cefotiofur sodium to dairy goats. J Vet Pharmacol Ther. 1997;20(3):368–73.

20. Craigmill AL, Brown SA, Wetzlich SE, Gustafson CR, Amitd TS. Pharmacokinetics of ceftiofur and metabolites after single intravenous and intramuscular administration and multiple intramuscular administrations of cefotiofur sodium to sheep. J Vet Pharmacol Ther. 1997;20(2):139–44.

21. Goudah A. Pharmacokinetics of ceftiofur after single intravenous and intramuscular administration in camels (Cameus dromedarius). J Vet Pharmacol Ther. 2007;30(4):371–4.

22. Drew ML, Johnson L, Pugh D, Navare CB, Taylor IT, Craigmill AL. Pharmacokinetics of ceftiofur in llamas and alpacas. J Vet Pharmacol Ther. 2004;27(1):13–20.

23. Drew ML, Waldrup K, Kreeger T, Craigmill AL, Wetzlich SE, Mackintosh C. Pharmacokinetics of ceftiofur in red deer (Cervus elaphus). J Vet Pharmacol Ther. 2004;27(1):7–11.

24. Brown SA, Hanson BJ, Mignot A, Milliéroux L, Hamlow PJ, Hubbard VL, et al. Comparison of plasma pharmacokinetics and bioavailability of ceftiofur sodium and ceftiofur hydrochloride in pigs after a single intramuscular injection. J Vet Pharmacol Ther. 1999;22(1):35–40.

25. Liu S, Guo D, Guo Y, Zhou W. Preparation and pharmacokinetics of ceftiofur Sodium liposomes in cows. J Vet Pharmacol Ther. 2011;34(1):35–41.

26. Hornish RE, Prough MJ. Determination of ceftiofur and desfuroylceftiofur related residues in milk of lactating dairy cattle receiving SC injections of high in vitro release rate of CFFA-SS (200 mg/ml) in the base of the ear and middle of the ear at 66 mg/kg bodyweight. Freedom Inform Sum. 2006.Suppl.NADA 141–209.27–28.

27. Turnidge JD. The pharmacodynamics of beta-lactams. Clin Infect Dis. 1998;27(1):10–22.

28. Toutain PL, del Castillo JR, Bouquet-Melou A. The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. Res Vet Sci. 2002;73(2):105–14.

29. Lees P, Pelligand L, Illambas J, Potter T, Lacroix M, Pycroft A, et al. Pharmacokinetic/pharmacodynamic integration and modelling of...
amoxicillin for the Calf pathogens *Mannheimia haemolytica* and *Pasteurella multocida*. J Vet Pharmacol Ther. 2015;38(5):457–70.

30. Delis GA, Koutsoviti-Papadopoulou M, Siarkou V, Kounenis G, Batzias GC. Pharmacodynamics of amoxicillin against *Mannheimia haemolytica* and *Pasteurella multocida* and pharmacokinetic/pharmacodynamic (PK/PD) correlation in sheep. Res Vet Sci. 2010;89(3):418–25.

31. Nielsen EI, Frisberg LE. Pharmacokinetic-pharmacodynamic modeling of Antibacterial drugs. Pharmacol Rev. 2013;65(3):1053–90.

32. Thomas JK, Forrest A, Bhavnani SM, Hyatt JM, Cheng A, Ballow CH, et al. Pharmacodynamic evaluation of factors associated with the development of Bacterial resistance in acutely illpatients during therapy. Antimicrob Agents Chemother. 1998;42(3):521–7.

33. Schentag JJ. Clinical pharmacology of the fluoroquinolones: studies in human dynamic/kinetic models. Clin Infect Dis. 2000;31 Suppl 2:S40–4.

34. Brentnall C, Cheng Z, McKellar QA, Lees P. Pharmacodynamics of oxytetracycline administered alone and in combination with carprofen in calves. Vet Rec. 2012;171(11):273.

35. Sidhu PK, Landoni MF, Aliabadi FS, Lees P. PK-PD integration and modeling of marbofloxacin in sheep. Res Vet Sci. 2010;88(1):134–41.

36. Zhang B, Gu X, Li Y, Li X, Gu M, Zhang N, Shen X, Ding H. In vivo evaluation of mutant selection window of cefquinome against *Escherichia coli* in piglet tissue-cage model. BMC Vet Res. 2014;10:297.