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Abstract

The objective of this investigation was to evaluate the pharmacokinetic parameters of tulathromycin in plasma and semen of beef bulls after administering a single sc dose at two different sites in the neck. Four Simmental bulls with excellent temperament received a comprehensive physical exam that included breeding soundness examination. In addition, blood was collected and analyzed for CBC and chemical panel in order to rule out any subclinical liver or kidney disease. All bulls were diagnosed as healthy and satisfactory potential breeders. The mean plasma levels of tulathromycin for the two neck sites of sc administration were not different between posterior aspect of the ear where it attaches to the head (RP; regio parotidea; 77.9 ± 43.3 ng/mL; X ± SD) and to the middle of the neck (RC; regio collis lateralis; 73.7 ± 39.7 ng/mL; P=0.84). The mean seminal plasma levels of tulathromycin after administration in the RP was 608 ± 374 ng/mL and for RC was 867 ± 599 ng/mL without differences between both sites (P=0.29). The mean level of tulathromycin in plasma was 75.8 ± 40.2 ng/mL, which was lower than mean seminal plasma levels of 781 ± 482 ng/mL (P=0.001). The plasma peak tulathromycin concentration (C_{max}) was 160 ± 27 ng/mL at 21 ± 6 h (T_{max}) post-administration. The seminal plasma C_{max} was 1,539 ± 44.4 ng/mL at 33.00 ± 18.00 h (T_{max}) post-administration. The C_{max} between plasma and seminal plasma were different (P=0.008) without any differences in T_{max} between plasma and seminal plasma (P=0.35). The terminal half-life for plasma tulathromycin (81.4 ± 27.6 h) showed a tendency to be shorter than in seminal plasma (114.7 ± 21.7; P=0.10). The plasma area under the curve concentration time from the first to the last sample ($AUC_{0–last}$) was 15,440 ± 1,717 ng/mL/h, which was significatively smaller compared with 171,071 ± 58,556

ng/mL/h for seminal plasma AUC_{0-last} (P=0.01). The plasma means residence time from the first to the last sample (MRT_{0-last}) was 89.3 ± 5.1 h and it was shorter than for seminal plasma of 96.6 ± 5.0 h (P=0.05). From the present investigation, it was concluded that tulathromycin is a suitable antibiotic based in its pharmacokinetic properties that could be used for treatment of bull genital infections when its application is indicated.

Keywords: Bull, tulathromycin, pharmacokinetics, plasma, semen

1. Introduction

The use of antibiotics is required in many reproductive clinical conditions of bulls [1–3]. One of the most common reproductive diseases in young and old bulls is seminal adenitis syndrome [3,4]. One of the recommendations to treat this disorder is the administration of either local or systemic antibiotics [3,5,6]. Antibiotic selection for this clinical condition and other genital infections (orchitis, epididymitis) is based on personal experience, anecdotal, extrapolation from other species, or on the results of microbiological culture and sensitivity tests. The chosen antibiotic needs to be used at the correct dose, route and frequency for an acceptable period (antibiotic stewardship) [7]. Furthermore, a judicious use of antibiotics remains critical for minimizing risk of microbial resistance. Unfortunately, information on antibiotic levels in the bull's genital tract or in semen is not available. Hence, new information on this subject is paramount not only to design an appropriate treatment regimen and preclude the uses of

antibiotics that cannot be effective, but also to avoid their unnecessary use. One of the recommendations to reduce medication errors and harm is to use the "five rights"—the right patient, the right drug, the right dose, the right route, and the right time [8].

Most information about the pharmacokinetic of antibiotics in the male genital tract is derived from human and dog models [9–11]. However, the anatomy and physiology of these two species are different from ruminants [12]. As a result, extrapolation of the information from such different species should only be done when no other data is available.

Tulathromycin is a macrolide triamilide antibiotic that has been approved for use in the treating and preventing respiratory diseases in cattle, swine and other animals [13,14], infectious bovine keratoconjunctivitis and interdigital necrobacillosis [14]. Like other macrolides, it binds to the 50S subunit of bacterial ribosomes and inhibits protein synthesis, leading to inhibition of cell division and cell death. Tulathromycin's spectrum of activity includes Gram-negative, Gram-positive, and Mycoplasma microorganisms [13,15], and it exhibits a mixed bacteriostatic and bactericidal concentration [13]. The minimum bactericidal concentration (MBC) was found to be the same as the minimum inhibitory concentration for 70% of M. haemolytica and Pasteurella multocida isolated [13]. In cattle, this antibiotic presents unique pharmacokinetic characteristics such as rapid absorption from the injection site, extensive tissue and high-volume distribution, elevated and sustained drug concentration in the lungs, and slow elimination [13]. Studies have shown that the level of tulathromycin in plasma did not correlate with the therapeutic level in tissues of the respiratory system [13,15]. On the other hand, when tulathromycin was administered parenterally, the concentrations in the synovial fluid were higher

and persisted longer than in plasma [16]. Moreover, treatment with tulathromycin resulted in clearance of Leptospira hardjo-bovis organisms from the urine and kidney tissue of all positive heifers [17].

The pharmacokinetic of tulathromycin in the bull's genital tract or semen has not been investigated. Due to their known above-mentioned characteristics, this drug is the prime candidate for further investigation in semen. The availability of an antibiotic with long-acting effects would limit the frequency of administration and animal handling with the consequent reduction in animal stress while also improving compliance. Research on the pharmacokinetic parameters of a second site of injection in the neck is not only valuable "per se" but also for practical reasons. Bulls are heavy animals that require high volume doses of medications. In the case of tulathromycin, it is recommended to inject not more than 10 ml per injection site with a distance not less than 10 cm between administration places. Therefore, tulathromycin administration will require two or more sites of administration.

The objective of this investigation was to evaluate the pharmacokinetic of tulathromycin in plasma and semen in beef bulls by administering a single sc at two different sites.

2. Material and methods

2.1. Animals

Six Simmental bulls with excellent temperament and healthy appearance were selected for the study. Each one had a comprehensive physical examination including breeding soundness

101 examination according to the guidelines by Society for Theriogenology [18]. In addition, blood
102 was collected from the tail vessels and analyzed for CBC and chemical panel in order to rule out
103 any subclinical liver or kidney disease. None of these showed any abnormalities. All bulls were
104 diagnosed as healthy and satisfactory potential breeders. Four of these bulls were randomly
105 selected for this investigation. The age of the bulls was 15 ± 0. 2 mo (range: 15–16). The weight
106 was 639.3 ± 32.9 kg (604–681 kg). The body condition score was 6.1 ± 0.5 (5.5–6.50) [19]. The
107 bulls were maintained in individual pens and received a ration of corn silage, mixed hay, and
108 alfalfa with water ad libitum. In addition, each bull received 2.5 kg of pellet concentrate once a
109 day containing 14% crude protein.

110 2.2. Experimental design

111 These bulls had no history of tulathromycin administration. Each bull received a single sc
112 dose of tulathromycin (Draxxin, Zoetis Italy, Rome) at the dose of 2.5 mg/kg of body weight
113 (day 0 time 0). Two of the bulls received the dose posterior aspect of the left ear where it
114 attaches to the head (RP; regio parotidea sinister) and two in the middle of the left side of the
115 neck (RC; regio collis lateralis sinister)[20]. The order of sample collection was blood and
116 semen, collected at 0, 12, 24, 48, 72, 96, 144, 192, and 240 h after tulathromycin administration.
117 Blood was collected from the tail vessels using vacuum tubes containing lithium heparin (10
118 mL). Semen was collected from each bull by electroejaculation by using an electro-ejaculator in
119 automatic mode; the same set-up was used for all the bulls (Pulsator V, Lane Manufacturing,
120 Denver, CO, USA) using a two-electrode rectal probe of 60 mm diameter. All the samples were
121 immediately refrigerated, then centrifuged at 600 g for 30 minutes, processed within the first h,

and stored at -80° C. Procedures used in this investigation were approved by the Committee for Animal Welfare, University of Bologna (Prot. n.0005783).

2.3. Tulathromycin analysis

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to measure tulathromycin concentrations in bull plasma and seminal plasma, with an approach similar to the technique described by Zhou et al. [21]. Two hundred µL of thawed sample was placed in a microcentrifuge tube, then 180 µL of acetonitrile and 20 µL of internal standard tulathromycin-d7 (Toronto Research Chemicals, North York, ON, Canada) in acetonitrile were added. The tube was agitated in a vortex mixer for 30 sec, centrifuged at 21,000 ×g for 10 min at 4 °C and the supernatant was filtered through a 0.22 µm nylon syringe filter. A 100 µL aliquot of the purified sample was diluted in a vial with an equal amount of 0.1% formic acid aqueous solution, and, finally, 10 µL from each vial was injected in the LC-MS/MS system.

The apparatus consisted of a Waters Acquity UHPLC binary pump (Waters, Milford, MA, USA) and thermostated autosampler, kept at 20 °C. Chromatographic separation was obtained with a Waters Acquity BEH C18 (50 × 2.1 mm, 1.7 µm) column (Waters, Milford, MA, USA), maintained at 40 °C to lower system backpressure. The mobile phase was a mixture of 0.1% formic acid in water (A) and acetonitrile (B) flowing at 0.3 mL/min during a 5 min run: its composition changed from 90% to 50% A in the first 2 min, then was kept at 50% A for 1.75 min, brought back to 90% A in 0.5 min and finally kept at 90% A for 0.75 min to allow column equilibration. The detector was a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters, Milford,

MA, USA), equipped with an electrospray ionization source (ESI), with capillary voltage set at +3.0 kV, source temperature at 120 °C and desolvation temperature at 400 °C. Desolvation and cone gas flow were 600 and 100 L/h, respectively, and argon was used as collision gas. The retention time was 1.23 min for both tulathromycin and tulathromycin-d7. The instrument operated in MRM mode, monitoring the 403.7>576.9 m/z (quantification) and 403.7>229.9 m/z (confirmation) transitions for tulathromycin and the 407.3>236.9 m/z transition for the internal standard. Data acquisition and processing were carried out with MassLynx 4.1 software (Waters, Milford, MA, USA).

Aliquots (200 µL) of each matrix were fortified with tulathromycin (Toronto Research Chemicals, North York, ON, Canada) at different concentrations to obtain matrix-matched calibration curves at suitable ranges (10 -1000 ng/mL) for plasma and 50-5000 ng/mL for seminal plasma and quality control (QC) samples at three different levels for each day of the analysis. Tulathromycin/internal standard peak area ratios were plotted against the correspondent concentrations and a linear least square regression model was applied; the good linearity of the method was proved by the correlation coefficient (R2) always ≥0.99 and all the calibration standards within ±15% of the nominal value. The lower limit of quantification (LLOQ), that is, the lowest tested concentration of tulathromycin showing a signal/noise ratio ≥10, was 10 ng/mL for plasma and 20 ng/mL for seminal plasma. Accuracy and precision, intended as measured value-expected concentration relative difference and coefficient of variation (CV%), respectively, were always within ±15% at all QC concentration and all the three matrices.

2.4. Pharmacokinetic parameters.

Noncompartmental analysis was used to estimate the pharmacokinetic parameters in plasma and seminal plasma for each individual animal. Standard software, PK-Solver add-in for Excel [22] was used to estimate the pharmacokinetic parameters. The following variables were calculated for plasma and seminal plasma of each animal: time of peak drug concentration (T_{max}), peak drug concentration (C_{max}), apparent elimination half-life (t1/2), calculated as ln (2)/λz, λz being the first order rate constant associated with the terminal portion of the time-concentration curve as estimated by linear regression of time versus log concentration, area under the time-concentration curve from time zero to the last observed concentration ($AUC_{0\text{-last}}$), calculated by the linear trapezoidal rule, area under the time-concentration curve from time zero extrapolated to infinity ($AUC_{0\text{-inf}}$), calculated by adding the last observed concentration divided by λz to the $AUC_{0\text{-last}}$), area under the moment curve from time zero to last observed concentration ($AUMC_{0\text{-last}}$), area under the moment curve from time zero extrapolated to infinity ($AUMC_{0\text{-inf}}$), mean resident time estimated using time zero to last observed concentrations ($MRT_{0\text{-last}}$, calculated as $AUMC_{0\text{-last}}/AUC_{0\text{-last}}$), and mean residence time estimated using time zero to infinity ($MRT_{0\text{-inf}}$, calculated as $AUMC_{0\text{-inf}}/AUC_{0\text{-inf}}$)

2.5. Statistical Analysis.

Statistical software [23] was used to determine parameters such as mean, standard deviation, and range. Student "t" test for paired samples was used. In addition, a software program (PK-Solver) for pharmacokinetic parameters as previously mentioned was used [22]. An alpha error of 5% was used to accept the alternative hypothesis.

185

186 3. Results

187 All bulls remained clinically healthy throughout the study period.

188 Mean plasma levels of tulathromycin for the two sc neck injection sites were not different
189 between RP (77.9 ± 43.3 ng/mL) and the RC (73.7 ± 39.7 ng/mL; P=0.84). Mean seminal plasma
190 levels of tulathromycin after administration on the RP were 608 ± 374 ng/mL and 867 ± 599
191 ng/mL for RC without differences between both sites (P=0.29). Because no significant
192 differences were noticed at the two sites of administration, the means for plasma and seminal
193 plasma were combined. The mean level of tulathromycin in plasma was 75.8 ± 40.2 ng/mL
194 which was lower than mean seminal plasma level of 781 ± 482 ng/mL (P=0.001). The ratio for
195 mean plasma/seminal plasma of tulathromycin levels was 10.3. Mean (± SD) plasma and seminal
196 plasma of tulathromycin concentration (ng/mL) throughout the investigation period is presented
197 in Fig 1.

198 All pharmacokinetic parameters calculated for non-compartmental analysis of plasma and
199 seminal plasma are presented in table 1. The plasma C_{max} was 160 ± 27 ng/mL at 21 ± 6 h (T_{max})
200 after administration. The seminal plasma C_{max} was 1,539 ± 444 ng/mL at 33.00 ± 18.00 h (T_{max})
201 after administration. The C_{max} between plasma and seminal plasma was different (P=0.008)
202 without any differences in T_{max} between plasma and seminal plasma (P=0.35). The ratio C_{max}
203 between plasma/seminal plasma was 9.6. The terminal half-life for plasma (81.4 ± 27.6 h)
204 showed a tendency to be shorter than in seminal plasma (114.7 ± 21.7; P=0.10). The plasma for
205 AUC_{0-last} was 15,440 ± 1,717 ng/mL/h, significantly smaller compared to 171,071 ± 58,556

206 ng/mL/h for seminal plasma AUC_{0-last} (P=0.01). The ratio AUC_{0-last} plasma/seminal plasma of
207 tulathromycin levels was 11.9. The plasma means residence time from the first to the last sample
208 (MRT_{0-last}) was 89.3 ± 5.1 h and it was shorter than for seminal plasma of 96.6 ± 5.0 h (P=0.05).

209

210 4. Discussion

211 No side effects at the dose used such as hypersalivation, head shaking, pawing the ground
212 or decreased feed intake as previous reported were observed [14,24]. Only a mild swelling at the
213 site of injection was detected, especially at the RP, which disappeared in 5 days. The two neck
214 locations of administration did not present any difference either in plasma or seminal plasma
215 concentrations of tulathromycin; therefore, this could be considered an extra benefit in which an
216 additional site of administration could be used without affecting the beef quality assurance. Bulls
217 are big animals that require a high volume dose. It is recommended not more than 10 ml per
218 injection site a distance not less than 10 cm between administration places. Therefore, bull
219 treatment will require two or more injections sites of tulathromcyin.

220 In cattle, the parenteral administration of tulathromycin at label dose (2.5 mg/kg) was
221 characterized by rapid rate of absorption, early maximal plasma concentrations, extensive
222 distribution, and slow elimination [13,15,25,26]. In plasma, tulathromycin has a long terminal
223 half-life, ranging across studies from 64 h [26], 90 h [13,15], 110 h [25], 112 h [27], and up to
224 189 h [28]. In the current study, a terminal half-life of 81.4 ± 27. 6 h (range: 71–96 h) was
225 obtained in agreement with some of aforementioned reports.

226 The plasma C_{max} obtained was 160 ng/mL (range 121–180 ng/mL) a low value compared
227 with former findings which reported 277 ng/mL [26], 300 ng/mL [25], 500 ng [13,15], 718
228 ng/mL [28]. However, the current evaluation agrees with two recent reports using 10 mo
229 Holstein steers and bisons in which levels of 154 ng/mL [27] and 195 ng/mL [29], respectively
230 were reported. One possible explanation for this difference with those studies could be that the
231 first blood sample collection was performed 12 h after tulathromycin administration; therefore,
232 due to rapid rate of absorption and quick systemic distribution, the plasma concentration of
233 tulathromycin was already in a descending phase. This is supported by two reasons. First, in
234 those studies the first the T_{max}, time of C_{max}, was obtained at 0.25 h [29], < 1 h [13], 0.7 h [26], 1
235 h [28], 1.8 h [15], or 3 h [26]; second, when the present values from 24 to 240 h were compared
236 with the results obtained by Nowakowski et al. [15] or Evans [13], similar profiles were
237 obtained. These, therefore, supported and confirmed the current plasma outcomes.

238 The plasma $AUC_{0\text{-last}}$ in the present study was 18,382 ng/mL/h in conformity with the
239 results of 17,885 ng/mL/ h by Rivera et al. [28] and 16,700 ng/mL/h by Evans [13] but higher
240 than previous stated by other investigators [15,25-27]. The MRT for plasma was 134.3 h agreed
241 with 146 h reported by Nowakowski [15] and it was in between results from other two studies of
242 65 h [27] and 171.5 h [29]. Differences in plasma pharmacokinetic parameters compared with
243 previous investigations were detected; they were, however, within the normal range. Therefore,
244 the present outcomes permit to be confident that not only the plasma analysis was appropriate,
245 but it also supported the seminal plasma results.

246 The efficacy of any antimicrobial is determined by both its pharmacokinetic and
247 pharmacodynamic properties. Antibiotics have been classified in two major groups—those with
248 bacteriostatic antimicrobial action that exhibit time-dependent killing action or those with
249 bactericidal antimicrobial action that behave with either time-dependent or concentration-
250 dependent killing [15]. Tulathromycin has shown to have bacteriostatic antimicrobial action and
251 also bactericidal antimicrobial time-dependent action [13] with a bioavailability after parenteral
252 administration more than 85% for cattle and swine [13,30,31]. Antimicrobial having time-
253 dependent action is associated to the exposure to pathogens to an appropriate amount of time.
254 Therefore, concentration of antibiotic above the minimum inhibitory concentration (MIC) of
255 each specific pathogen is one accepted method of evaluation [13]. In a recent report, the AUC
256 above the minimum inhibitory concentration (MIC) for a specific microorganism (AUC/MIC)
257 was considered the primary pharmacokinetic/pharmacodynamics predictor for tulathromycin
258 clinical effectiveness [32]. In vitro studies of tulathromycin in the bacteriostatic and bactericidal
259 activity were both affected by pH, carbon dioxide, and serum, which have a possible significant
260 relevance in vivo [13]. Unfortunately, correlation between in vitro susceptibility test and clinical
261 effectiveness is undetermined for certain clinical conditions.

262 The ejaculate consists of spermatozoa suspended in a fluid medium called seminal
263 plasma (SP). The components of SP are produced from rete testis, epididymis, and accessory sex
264 glands (AG) of the male reproductive tract [12,33,34]. In the bull, the AG are seminal glands
265 (vesicles), prostate (compact and disseminate), and Cowper glands that contribute to the major
266 portion of SP at ejaculation [12,35]. The spermatozoa present in the ejaculate collected either by

267 artificial vagina or electroejaculation come from the tail of epididymis and ampulla [12.33-35].
268 Therefore, the presence of tulathromycin in the seminal plasma could be considered a strong
269 indication that the antibiotic was released from the tail of epididymis, and/or accessory sexual
270 glands. In multiple previous independent investigations, high and extended concentrations of
271 Tulathromycin in lung tissue feature have been reported. Lung concentrations were many times
272 higher than plasma concentration with lung plasma area under the concentration-time curve
273 ratios being more than 50 times with a long half-life values than plasma [13,26]. In vitro studies
274 show that tulathromycin accumulates in neutrophils and blood macrophages, pulmonary epithelia
275 lining cells from normal cattle [26,36]. In a recent study, the parenteral administration of
276 tulathromycin resulted in synovial fluid concentrations that were higher with a longer duration
277 that previous reported plasma values [16]. To the best of the authors' knowledge, this is the first
278 study that shows pharmacokinetic of tulathromycin in bull semen after a standard dose of this
279 antibiotic as recommended for cattle. The seminal plasma Cmax of tulathromycin was almost 10
280 times higher than in plasma with a tendency of longer half time compared with plasma.
281 Moreover, the seminal plasma $AUC_{0\text{-last}}$ was almost 14 times higher contrasted with $AUC_{0\text{-last}}$ in
282 plasma. Finally, both mean residency times ($MRT_{0\text{-last}}$ and $MRT_{0\text{-inf}}$) for seminal plasma were
283 extended compared with $MRT_{0\text{-last}}$ and $MRT_{0\text{-inf}}$ of plasma. In the case of seminal plasma, $MRT_{0\text{-inf}}$
284 was 43% longer than plasma $MRT_{0\text{-inf}}$. Therefore, it appears that tulathromycin elimination
285 from male's genital tract was slower, probably because of delayed exposure in the organs of
286 elimination, and this can be considered an advantage for male reproductive treatments. Based on
287 the present pharmacokinetic findings, the sc administration of tulathromycin at 2.5 mg/kg body
288 weight in bulls produced rapid absorption with higher levels in seminal plasma that continue

longer when contrasted with plasma levels. The present pharmacokinetic information will allow establishing an adequate dose regime of tulathromycin for bull genital infections.

From the present investigation, it was concluded that tulathromycin is a suitable antibiotic based on its pharmacokinetic properties that could be used for treatment of bull genital infections when its application is indicated.

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Competing interests

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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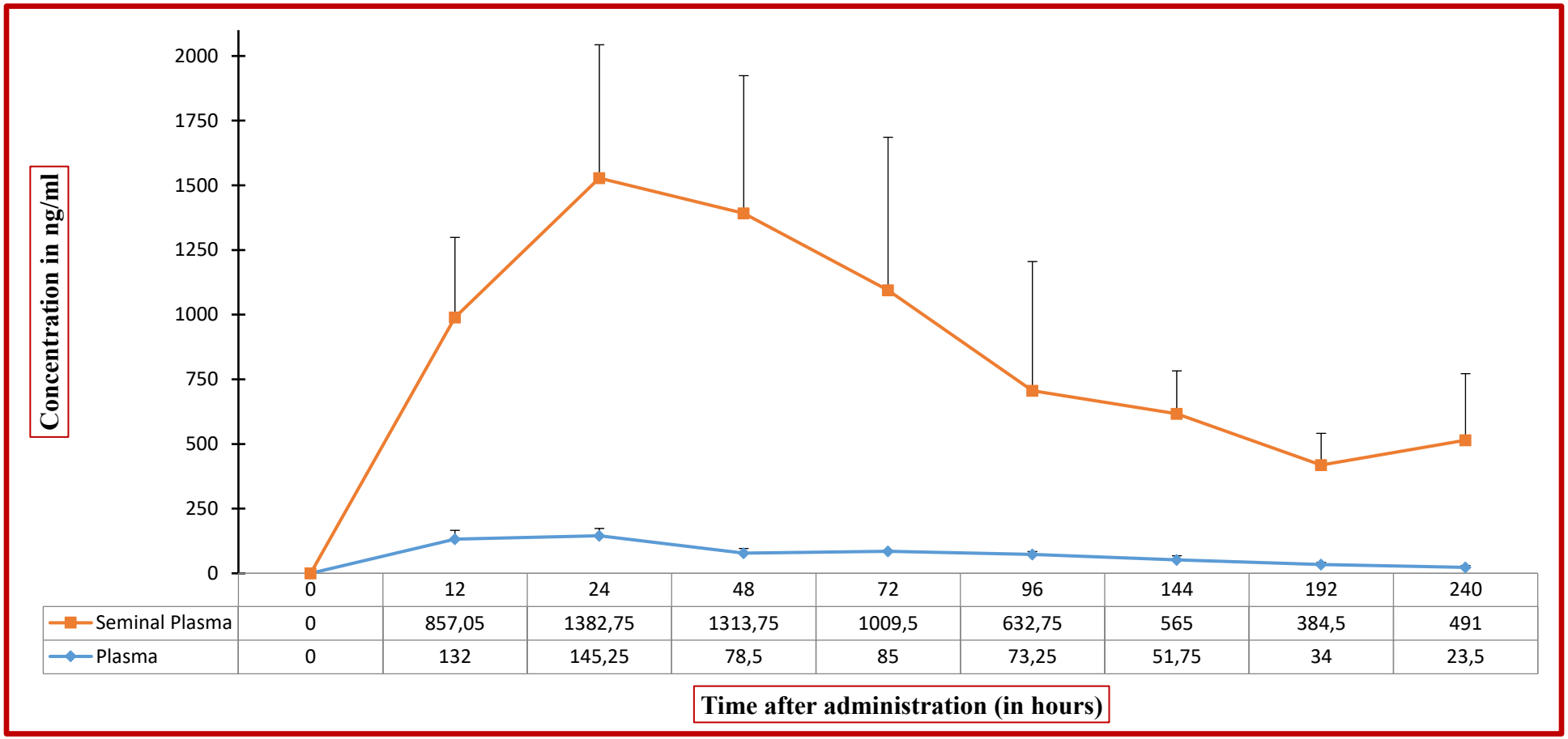
		Plasma			Seminal Plasma			
Parameter	**Unit**	**Mean**	**SD**		**Mean**	**SD**		**Probability**
Lambda z (λ_z)	1/h	0.009427	0.003654		0.006188	0.001002		0.16
t1/2	h	81.4	27.6		114.7	21.7		0.1
T_{max}	h	21	6		33	18		0.35
C_{max}	ng/mL	160	26.5		1,539	444.3		0.008
AUC $_{0\text{-last}}$	ng/mL*h	15,440	1,717		171,071	58,556		0.01
AUC $_{0\text{-inf}}$	ng/mL*h	18,382	11,729		247,892	89,099		0.01
AUMC $_{0\text{-last}}$	ng/mL*h^2	1,379,000	176,000		16,442,000	5,274,000		0.01
AUMC $_{0\text{-inf}}$	ng/mL*h^2	2,479,756	673633		47,130,701	15,873,682		0.01
MRT $_{0\text{-last}}$	h	89.3	5.1		96.5	5.0		0.05
MRT $_{0\text{-inf}}$	h	134.3	32.4		191.4	9.3		0.05

Table 1. Plasma and seminal plasma pharmacokinetics parameters of tulathromycin administered by sc route at 2.5 mg/kg.

λ_z being the first order rate constant associated with the terminal portion of the time-concentration curve; t1/2 : apparent elimination half-time calculated as ln(2)/ λ_z ; T_{max}: time of peak drug concentration; C_{max}: peak of drug concentration; $AUC_{0\text{-last}}$: area under the time-concentration curve from time zero to the last observed concentration; $AUC_{0\text{-inf}}$:area under the time-concentration curve from time zero extrapolated to infinity; $AUMC_{0\text{-last}}$: area under the moment curve from time zero extrapolated to last observed concentration; AUMC $_{0\text{-inf}}$: area under the moment curve from time zero extrapolated to infinity; MRT $_{0\text{-last}}$: Mean resident time calculated as AUMC $_{0\text{-last}}$ /AUC $_{0\text{-last}}$; $MRT_{0\text{-inf}}$: Mean resident time calculated as AUMC $_{0\text{-inf}}$ / AUC $_{0\text{-inf}}$.. Mean (± SD) pharmacokinetics parameters in plasma and seminal plasma calculated via noncompartmental analysis after sc administration.



	0	12	24	48	72	96	144	192	240
Seminal Plasma	0	857,05	1382,75	1313,75	1009,5	632,75	565	384,5	491
Plasma	0	132	145,25	78,5	85	73,25	51,75	34	23,5

Figure 1. Mean (± SD) plasma and seminal plasma of tulathromycin concentration (ng/mL) after single sc administration at 2.5 mg/kg in four Simmental bulls.