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Research Article

Comparative Pharmacokinetics and Bioequivalence of Two Oxyteracycline Preparations Following IM Administered in Egyptian Calves Post-Weaning

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ABSTRACT

A parallel designed study was conducted in 24 healthy calves of both sexes post weaning to investigate the bioequivalence of two long acting oxytetracycline OTC formulations. Plasma disposition kinetics were studied for spectropan LA compared to terramycin L.A, as reference standard, following intramuscular administration IM at a dose of 20 mg/kg b.wt. Validated high performance liquid chromatography method HPLC was used for determination of oxytetracycline in plasma with 99% recovery and 1.2% coefficient of variation. Scatterplot and Bland-Altman plot indicated the identical relationship between both products plasma OTC concentrations with average difference of 0.06 µg/mL. C_{max} values of 3.28±0.125 and 3.21±0.231 µg/mL were achieved at 3.67±0.534 and 3.66±0.450 h post-administration for both spectropan and terramycin, respectively. Plasma concentration sustained above 0.5 µg/mL for more than 72 h post injection. The hastened OTC elimination probably attributed to, the effect of increased water consumption post weaning, in addition to breed variation. Almost all estimated kinetic parameters; $C_{max,}$ Tmax, AUC_{0-96}, $AUC_{0-\infty}$, $AUMC_{0-\infty}$ and K_{el}, show no statistically significant difference between both formulations. Westlake's 90% confidence intervals of either untransformed or transformed log of AUC_{0-96}, $AUC_{0-\infty}$, C_{max} and Tmax, did fall within the 80-125% range for both tested formulations. According to, the guidelines implemented by the Committee for Veterinary Medicinal Products (CVMP) and the Food and Drug Administration (FDA), both studied formulations are bioequivalent.

Key words: Oxytetracycline, Bioequivalence, Pharmacokinetic, Weaning, Calves, HPLC

INTRODUCTION

The bioequivalence determination described by both food and drug administration (FDA) and committee for medicinal products for veterinary use (CVMP) guidelines as the state in which equivalent rate and extent of absorption will produce same plasma drug concentration time profiles, so, the same level of therapeutic or toxic effects (Mestorino *et al.*, 2016). Consequently, bioequivalence is important for the development of new pharmaceutical formulations (Vetchý *et al.*, 2007).

Over the counter oxytetracycline is a broad-spectrum antibiotic, which is active against aerobic Gram positive and Gram-negative bacteria as well as, Mycoplasma, Chlamydia and some protozoa (Craigmill *et al.*, 2000). OTC antibiotic treatments for respiratory and gastrointestinal infections are widely used in veterinary medicine (Riviere and Spoo, 1995). Long acting 20% oxytetracycline solutions eliminated the difficulties of daily OTC injections in cattle (Archimbault *et al.*, 1994). Following a single intramuscular injection IM of OTC LA formulation at a dose of 20 mg/kg, it could achieve a fast and high OTC plasma concentrations that persist in the systemic circulation for several days (Toutain and Raynaud, 1983; Michalova *et al.*, 2004 and Mestorino *et al.*, 2007). The pharmacokinetics of diverse OTC long acting formulations has been studied in various animal species including: Calves (Kumar and Malik, 1999 and Brentnall *et al.*, 2013), cattle (Meijer *et al.*, 1993 and Mestorino *et al.*, 2007), swine (Archimbault *et al.*, 1994; Banting AdL and Baggot, 1996 and Attaie *et al.*, 2015), sheep (Nouws *et al.*, 1990 and Yar *et al.*, 2000) and goats (Escudero *et al.*, 1996 and Attaie *et al.*, 2015).

The aim of the present study was to evaluate the plasma bioequivalence and comparative disposition of two long-acting-oxytetracycline (OTC-LA) 20% formulations,

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spectropan LA® as test preparation in comparison with terramycin®/L.A, as a reference preparation, following intramuscular administration in calves at 20 mg/kg dose.

MATERIALS AND METHODS

Drugs

Oxytetracycline was used in the form of two patent formulations. Terramycin®/L.A ® (Pfizer, Animal Health Division, Cairo, Egypt) was obtained as a sterile, clear, stable and ready to use, 200 mg/mL injectable solution of oxytetracycline in 25, 100 and 500 mL vials. Each mL of the later contains 200 mg of OTC in the form of oxytetracycline dihydrate in 2-pyrrolidone vehicle system. Terramycin®/L.A is specially formulated to give long acting effect following IM injection and used only in cattle, sheep, goats and swine. The second formulation was, spectropan 20% LA® (Pharma swede, Industrial Zone B3, 10th of Ramadan City, Egypt). It was obtained as a sterile, clear and ready to use injectable solution in 10, 20, 50 and 100 mL vials. Each mL of spectropan LA® formulation contain 200 mg OTC as an equivalent to oxytetracycline dihydrate 215.7 mg and is labeled only for the use in cattle, buffalo, sheep and goats.

Animals

The protocol of this study was approved by the Animal Care and Use Committee of Cairo University. Twenty-four clinically healthy calves (native breed, Egyption baladi, Menufi breed) of both sexes (12 male & 12 female) weighing 180-240 kg at post-weaning age (8-10 month), were purchased from a local cattle farm. The selected species is one of the largest popular species in Egypt and known to represent other cattle species, in which the product is intended to be used in veterinary therapeutics. The calves were placed in animal housing facilities at faculty of veterinary medicine, Cairo University, Egypt. Animals were acclimatized for at least 4 weeks before the start of the study to ensure they did not receive any medication during this period. The calves had free access to water and commercial diet ad libitum. Seven days before the start of the experiment, each group of calves was housed separately in a large stall. Only healthy calves were allocated to the study.

Experimental design

The calves were randomly divided into two groups; A and B of twelve animals each. Experimental animals in group A were treated with a commercially available reference product (terramycin/LA) by intramuscular route at a rate of 1mL per 10 kg body weight of the product (20 mg oxytetracycline/ kg b.wt.). On the other hand, experimental animals in group B were treated with tested formulation (spectropan 20% L.A) which is commercially available, with the same route and at the same dose as reference formulation. Intramuscular injections were conducted in the gluteal area where, injected volume did not exceed 4 mL/ injection site and followed by massaging. The health of all calves was monitored before and throughout the experiment by periodic physical examination for vital signs along with observing feed consumption and fecal characteristics. No clinical signs or any drug related adverse effects were recorded in any of the calves throughout the study.

Sampling

Blood samples (5 mL) were collected from each the right calve jugular vein immediately before and at intervals of 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 84, 96 and 120 hours post drug administration. Blood samples were collected into 6 mL heparinized centrifuge tubes (Becton Dickinson, Rutherford, NJ, USA) and were centrifuged at 2000 ***g*** for 20 min. The recovered 2 mL plasma samples were kept in labeled vials, transferred to the analytical lab in ice box and kept at –80°C until analyzed by HPLC within 30 days of their collection.

Analytical procedures

The extraction, Chromatographic conditions and analysis of oxytetracycline from spiked and experimental samples was carried out using minor modification of the previously proposed procedure by (Korchi *et al.*, in 2001). Chromatography class-VP Shimadzu low pressure gradient system equipped with Fluorescence and UV detector, oven, auto-sampler with computing software, crest ultrasonic bath, Metller, AG 204 four digits balance and C-18 silica based column particle size 5µm stainless steel (150X4.6) mm Hypersil C18 column were used. Oxalic acid, methanol and acetonitrile used during the extraction and chromatographic analysis of oxytetracycline were HPLC grade (Fisher chemicals HPLC, United States). The mobile phase of acetonitrile: methanol: 0.05M oxalic acid (13: 13: 74) was freshly prepared daily and was filtered through a 0.45 m HPLC filter under vacuum. The flow rate was adjusted for 1.0 mL/min, variable wavelength UV-visible detector was set at 365 nm and at temperature was set at ambient. The peak areas were considered to calculate the oxytetracycline concentrations in spiked and experimental plasma samples. Standard solution was prepared by dissolving and diluting of 50 mg standard OTC into 100 mL volumetric flask. Five mL from the above solution was then dilute to 50 mL using HPLC grade water and was considered as stock solution. Freshly prepared stock solution was then serially diluted and ultrasonicated with shaking to get final concentrations.

Validation procedure

The HPLC analytical method used for extraction, derivatization and quantification of OTC concentrations in calve plasma was validated using ICH guidelines. Six standard curves were performed at six distinct days to evaluate the linearity of each standard curve. Quality control samples were used to accept or reject the run as each analysis set included blank samples in addition to, standard samples which were run before and after the tested samples. The acceptance criteria for the run were depending on both blank and spiked standard additional sample. As for blank sample, it must have no peaks or interference at the stated retention time for each ingredient. As well as the standard spiked sample had to be within -20%; 1% of the spiked nominal value which was chosen to be in the mid of range. Sample analysis was completely controlled through the incorporation of spiked sample exactly with run samples at the same conditions and at the same time and this eliminates variation of extraction.

The limit of detection (LOD) and quantification (LOQ) was determined from spiked calve plasma samples with standard OTC at the following concentrations 0.25, 0.5, 1, 5 and 12.5 µg/mL of OTC. The later calculated as following: the limit of detection (LOD) = 3.3 × σ/S and the limit of quantification (LOQ) = 10 × σ/S, where σ = standard deviation (SD) of the response and S is the slope of the calibration curve (El Badawy *et al.,* 2015). The detection limit was 0.0137 µg/mL while, the limit of quantification was 0.0457 µg/mL. The calibration curve between peak areas and OTC concentrations in calve plasma were found to be linear between 0.25 and 12.5 with correlation coefficients of 0.996. The inter-assay coefficient of variation CV was calculated as: CV = (standard deviation) × 100/mean. The mean coefficient of variation and extraction recovery were calculated using standard spiked calve plasma samples at concentrations of 0.25, 1 and 12.5 µg/mL.

Pharmacokinetic and statistical analysis

A computerized curve-stripping program (WinNon lin, Pharsight) was used to analyze plasma concentration–time curves for each individual animal after IM administration of oxytetracycline formulations. The data from each calve were fitted for non-compartmental open model (Mestorino *et al.,* 2016). This program calculated non-compartmental parameters using the statistical moment theory (Gibaldi and Perrier, 1982 and Martinez, 1998). The elimination rate constant k_{el} was calculated as k_{el} = 1/MRT. The apparent elimination half-life ($T_{1/2el}$) calculated as ln2/k_{el}. The area under plasma concentration–time curves ($AUC_{0\text{-}96}$, $AUC_{0\text{-}\infty}$) from zero to last time point (96 h) and to infinity, respectively, and the area under the first moment curve from zero to infinity ($AUMC_{0\text{-}\infty}$) were calculated using the trapezoidal method, with the exploration from the last time point of OTC plasma concentration cure to infinity (El Badawy *et al.,* 2015). Mean residence time (MRT) was calculated as the ratio of AUMC∞ to AUC∞ (El Badawy *et al.,* 2015). The maximum plasma concentration C_{max} and time of maximum concentration T_{max} were calculated from the following equation (Baggot, 1979):

$$T_{max} = \frac{2.303}{K_{ab} - K_{el}} \times \log \frac{K_{ab}}{K_{el}} \text{ (h)}$$

$$C_{max} = \log B \times (e^{-kel\ Tmax} - e^{-Kab\ Tmax})$$

Where K_{ab} = absorption rate constant (h^{-1}), K_{el} = elimination rate constant (h^{-1}). C_{max}, and AUC, were considered as primary, while, T_{max}, $T_{1/2}$, K and MRT considered as Secondary parameters.

Statistical analysis

The mean plasma concentrations and pharmacokinetic variables for oxytetracycline of the two tested formulations were statistically compared by one way analysis of variances (ANOVA) using the commercially available software package (SPSS Inc., version 17.0, Chicago, IL, USA). All data for pharmacokinetic parameters and plasma concentrations for both formulations of the study were reported as mean ±SD and means were considered significantly different at P<0.05. Confidence intervals of 90% for untransformed and log- transformed $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, C_{max} and T_{max} were calculated as the ratio of test/reference (T/R). Limits of acceptance for Westlake's 90% confidence intervals for tested formulations fall in the range from 80% to 125% according to FDA and CVMP guidance for in-vivo bioequivalence studies.

RESULTS

All calves used in the present study were clinically healthy throughout the experimental period. Oxytetracycline (spectropan 20% L.A® and terramycin®/ L.A) was well tolerated by all calves. Unexpected incidents that could have influenced the outcome of the study did not occur. The detection limit (LOD) was 0.0137 µg/mL while, the limit of quantification (LOQ) was 0.0457 µg/mL. The calibration curve between analyte areas and OTC concentrations (µg/mL) in calve plasma were found to be linear between 0.25 and 12.5 µg/mL with correlation coefficients of 0.996. The later was described by the following linear equation: y = y0 + a × c, where y is the analyte area, and c is the OTC concentration. The mean inter-assay coefficient of variation CV and mean extraction recovery at the spiked concentrations of 0.25, 1 and 12.5 µg/mL, were 1.2% and 99% respectively.

The plasma disposition kinetics of oxytetracycline in calve plasma were similar following treatment with both formulations. C_{max} values of 3.282±0.125 and 3.209± 0.231 µg/mL was achieved at 3.667±0.534 and 3.661± 0.450 h post-administration for spectropan and terramycin, respectively. The comparative means plasma concentration of OTC obtained after the intramuscular administration of both formulations to calves are shown in Table 1. The results of the study demonstrated that, values of C_{max}, T_{max}, AUC, K_{el} and MRT determined for both spectropan and terramycin (test & reference product, respectively) were similar. The mean pharmacokinetic parameters for OTC obtained after the administration of OTC test and OTC reference formulations to calves are compared in Table 2. None of the estimated kinetic parameters with exception of $T_{1/2el}$ showed statistically significant difference between both formulations. Westlake's 90% confidence intervals for untransformed and log- transformed $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, C_{max} and T_{max} reported in Table 3.

The mean OTC plasma concentration time curves for both test product (spectrospan) and reference one (terramycin) is shown in figure 1. The relationship between both formulations is shown through Scatterplot and Bland-Altman plot in figure 2. The later indicated the identical relationship between plasma OTC concentrations following IM injection of both products.

DISCUSSION

The study was designed as a one-period parallel study (Mestorino *et al.,* 2016). The results of these trial confirm the practicality of the parallel design to evaluate veterinary drugs bioequivalence particularly, when cross-over designs are not recommended, such as highly

variable veterinary drugs (HVVD) (Claxton *et al.*, 2012). Furthermore, the study of pharmacokinetics of drugs with extended half-lives and consequently long washout periods, in rapidly growing young animals using a cross-over design would be expected to exhibit physiological changes in the two different periods of study that affecting on pharmacokinetics. Thus, parallel design is preferred in such case to avoid period effects (Toutain and Koritz, 1997).

Table 1: Mean plasma concentrations of oxytetracycline following of intramuscular administration of terramycin (reference formulation) and spectropan (test formulation), 20% oxytetracycline injectable solution at dose of 20 mg oxytetracycline/kg b.wt in calves (n=12, mean ± SD, * P<0.05 significant compared to terramycin).

Time (h)	Products	
	Terramycin/ LA	Spectropan/ LA
0	ND	ND
0.5	1.5±0.21	1.5±0.29
1	2.4±0.19	2.3±0.32
2	2.9±0.19	2.8±0.24
4	3.5±0.23	3.5±0.48
6	3.1±0.18	3.0±0.25
8	2.8±0.16	2.8±0.22
12	2.7±0.10	2.6±0.14
24	2.4±0.19	2.3±0.29
36	1.6±0.14	1.3±0.13
48	1.2±0.11	1.1±0.16
60	0.86±0.087	0.77±0.089
72	0.72±0.094	0.64±0.093
84	0.45±0.059	0.38±0.046
96	0.45±0.057	0.26±0.043
120	ND	ND

ND: not detectable; No significant differences detected by using ANOVA.

Table 2: Summary of some pharmacokinetic parameters following of intramuscular administration of terramycin (reference formulation) and spectropan (test formulation), 20% oxytetracycline injectable solution at dose of 20 mg oxytetracycline/kg b.wt in calves (n=12, mean ± SD, * P<0.05 significant compared to terramycin).

Parameters	Units	Products	
		Terramycin/ LA	Spectropan/ LA
C_{max}	µg/mL	3.21±0.231	3.28±0.105
T_{max}	h	3.66±0.450	3.67±0.534
AUC_{0-96}	µg.mL/h	130.9±8.92	134.5±6.97
AUC_{0-inf}	µg.mL/h	145.9±10.48	150.1±9.69
$AUMC_{0-inf}$	µg.mL/h^2	6230±696	9±743 644
K_{el}	h^{-1}	0.024±0.002	0.001±0.023
$T_{1/2el}$	h	28.9±1.97	30.3±1.00*
MRT	h	42.6±2.75	42.8±2.30

C_{max} maximum plasma concentration, T_{max} time to peak concentration, AUC area under the curve, K_{el} Elimination rate constant, $T_{1/2el}$ elimination half-life, MRT mean residence time.

Table 3: Westlake's 90% confidence intervals, for spectropan (test formulation) versus terramycin (reference formulation), following IM administration at dose of (20 mg oxytetracycline /kg b.wt) in calves (n=12).

Parameters	Westlake's 90% confidence intervals (test vs reference) #	
	Untransformed	Log transformed
C_{max}	86.81-110.62%	88.08-108.47%
T_{max}	72.85-125.05%	78.79-121.58%
AUC_{0-96}	85.48-111.13%	96.85-102.17%
AUC_{0-inf}	83.14-112.47%	96.34-102.37%

Acceptable range by FDA and CVMP is 80-125%.

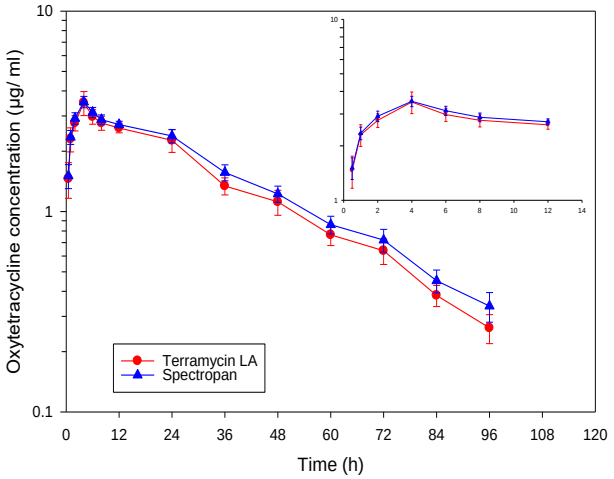

Fig. 1: Mean oxytetracycline plasma concentrations time curve obtained following intramuscular administration of terramycin (reference formulation) and spectropan (test formulation) and at a dose 20 mg oxyteracycline/ kg b.wt (n = 12, Mean, SD).

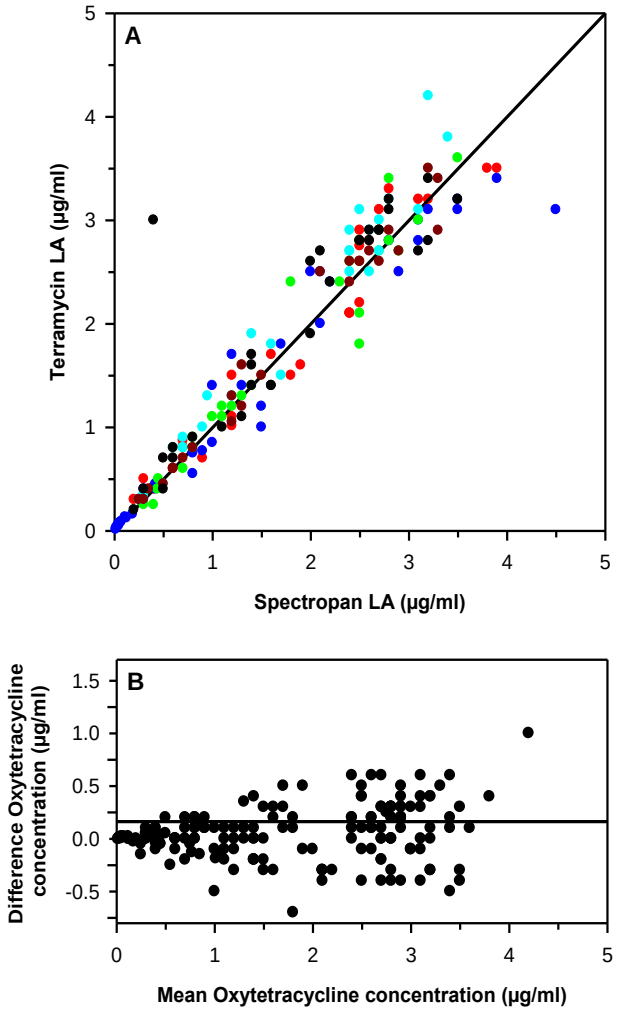

Fig. 2: A; Scatterplot indicating the relationship between plasma oxytetracycline concentrations obtained after intramuscular administration of terramycin (reference product) and spectropan (test product) and at a dose (20 mg oxyteracycline/ kg b.wt). The solid diagonal line is the line of identity. B; Bland-Altman plot for the difference between plasma oxytetracycline concentrations obtained after intramuscular administration of terramycin and spectropan. The medium horizontal line is the estimated mean difference of 0.06 µg/mL.

The maximum plasma concentration values (C_{max}) were 3.28±0.105 and 3.21±0.231 µg/mL, achieved at T_{max} of 3.67±0.534 and 3.66±0.450 h post-treatment for both test and reference products, respectively. Consistently, C_{max} mean values of 4, 3.1, 4.5 and 4.7 µg/mL were recorded, in cattle (Mestorino *et al.*, 2007), castrated male calves (Davey *et al*., 1985), young beef cattle (Toutain and Koritz, 1997) and dairy cows (Mevius *et al*., 1986). In contrast, to higher C_{max} values reported in veal calves of 5.9±0.6 µg/mL at T_{max} of 6.3±1.0 h (Meijer *et al.*, 1993) and of 5.7±0.3 µg/mL was achieved at 8±0.4 h by previous study of (Kumar and Malik, 1999). OTC in calves excreted mainly in urine and with minor extent in bile through its entero-hepatic circulation (Kumar and Malik, 1999)**.** Probably the later difference is contributed to, the effect of weaning stress and increased water consumption in those post weaning calves (Mesonero Escuredo *et al*., 2016). In addition to genetic breed variation that was suggested by (Ashwell *et al*., 2011) to be considered for drug dosing in cattle**.** $C_{max\ and}$ T_{max} did not exhibit any statistically significant differences between spectropan and terramycin.

Oxytetracycline was detected in plasma up to 96 h post-administration of the both test and reference formulations in calves. And maintained concentration above 0.5 µg/mL for more than 72 h post injection which referred as MIC_{50} of OTC for *P. multocida* susceptible strains (n = 36) (Achenbach, 2000).

Following IM administration of terramycin LA, the area under the OTC plasma concentration time curve of (AUC 0-∞), ranged from 129.3 to 161.7 µg.mL/h with a mean value of 145.9±10.48 µg.mL/h. While, after spectropan IM injection $AUC_{0-\infty}$ reported within the range of (136.9- 166.4 µg.mL/h), with a mean value of 150.1±9.69 µg.mL/h. $AUC_{0-\infty}$ showed no statistically significant difference between spectropan and terramycin. The later AUC finding is in contrast to higher other indicated values of 231.9 µg.h/mL (Achenbach, 2000) and of 311.40±93.05 µg h/mL (Mestorino *et al.*, 2016). The later make sense as a reflection for lower plasma concentration achieved and may attribute also, as explained before, to weaning stress, increased water consumption and breed variation.

The elimination half-life ($T_{1/2el}$) of oxytetracycline following the administration of terramycin LA intramuscular reference solution ranged from 24.8 to 31.9 h, with a mean value of 28.9±1.97 h. Whereas elimination half-life $T_{1/2el}$ following the administration of spectropan 20% LA intramuscular solution ranged from 28.8 to 32.0 h, with a mean value of 30.3±1.00 h. These finding agree with range of 20.5 to 30.4h that was reported in 8 calves by (Kumar and Malik, 1999). Consistently, $T_{1/2el}$ reported for terramycin LA in veal calves by (Mestorino *et al.*, 2016) of 36.88±11.50 h. Elimination half-life of spectropan was significantly longer than that of terramycin. While, MRT showed no significant differences between both formulations (42.6±2.75 h and 42.8±2.30 h), respectively. Similar MRT range of 29.6 to 43.8h was recorded by (Kumar and Malik, 1999) in calves.

The used HPLC method exhibited a good precision (CV 1.2%, recovery 99%) and sensitivity (LOD 0.0137 µg/mL, LOQ 0.0457 µg/mL), validating its use for bioequivalence assessment. The Bland-Altman plot indicated that, the mean difference between the obtained oxytetracycline concentrations, following IM administration of spectropan and terramycin, was 0.06 µg/mL (Fig. 2B). Bioequivalence assessment was performed according Guidelines of European committee for proprietary medicinal products and United States food and drug administration (FDA). The bioequivalence range, obtained as the ratio of the untransformed and log transformed AUC, Cmax and Tmax values, between the test and reference formulations, should fall between 80 and 125%. Kinetic parameters including AUC_{0-96}, $AUC_{0-\infty}$, C_{max}, Tmax showed no statistically significant differences between the two OTC formulations in calves. And, Westlake's 90% confidence interval of tested formulations, for either untransformed or log transformed primary kinetic parameters, did fall within the acceptable range (Table 2) and hence, considered bioequivalent (CVMP, 2000 and Patel *et al.*, 2010).

Conclusions

From the previous obtained results, it could be concluded that spectropan 20% L.A (OCT 20%) intramuscular solution and terramycin/L.A (OCT 20%) intramuscular solution is bioequivalent, since they deliver equivalent amount of OCT to systemic circulation at the same rate when administered at the same dose and exhibit equivalent AUC, C_{max}, T_{max}. In spite of, the statistically significant longer elimination half- life ($T_{1/2el}$) of OTC in case of spectropan than that for terramycin, no significant differences were reported regarding the mean residence time. That similar pharmacokinetic behavior and bioequivalence would suggest equivalent efficacy and safety of both formulations. The lower plasma concentrations and faster elimination recorded in those post weaning calves may be attributed for stress effect on OTC pharmacokinetics. Further investigation, for the effect of weaning stress on drug pharmacokinetics is required.

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