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Multiple-dose pharmacokinetics of long-acting oxytetracycline in *Brucella melitensis*-infected sheep

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Abstract

Multiple-dose pharmacokinetics of a long-acting oxytetracycline (LA-OTC) was studied in six *Brucella melitensis*-infected sheep. The sheep were treated with multiple intramuscular (i.m.) injection of LA-OTC (25 mg/kg) every 48 h for 3 weeks. Blood samples were withdrawn at 4, 24 and 48 h after each injection. To estimate the pharmacokinetics of LA-OTC in sheep, non-infected sheep received a single i.m. injection of LA-OTC solution at a dose of 25 mg/kg. Blood samples were collected at intervals up to 72 h after dosing. Serum samples were analyzed using a high-performance liquid chromatography (HPLC) assay with UV detection at 355 nm. The mean serum concentrations of OTC, in infected sheep, at 4 and 48 h following the first injection were 13.3 ± 3.6 and 3.3 ± 2.4 μg/ml, respectively. While after the last injection the mean serum concentrations were 15.2 ± 1.7 and 3.6 ± 0.73 μg/ml, respectively. *Brucella* infection seems to significantly alter the elimination of OTC without affecting its distribution. The accumulation index values of LA-OTC were 1.1–1.6 throughout the treatment. OTC was not detected in most serum samples (only in three out of six) collected 3 days after the last dose of treatment. A significant fluctuation of OTC between peak and trough levels was shown during treatment. Therefore, a shorter dosing interval is recommended. © 1998 Elsevier Science B.V.

Keywords: Accumulation; *Brucella melitensis*; Fluctuation; Long acting; Multiple dosing; Oxytetracycline; Sheep; Steady state

1. Introduction

Brucella is a genus of gram-negative, aerobic coccobacilli. The organisms are animal parasites

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and pathogens, causing brucellosis, transmissible to humans through contact with infected tissue or dairy products. *Brucella* organisms are known to survive within phagocytic cells of the reticuloendothelial system, the mammary glands, reproductive organs, and tissues with a weak blood supply. Therefore, *Brucella* organisms are protected from antibodies, complement, and antibiotics.

Brucellosis has been reported and confirmed in both livestock and humans in Saudi Arabia. *Brucella melitensis* biovars 1, 2 and 3 were responsible for all infections in sheep, goats, camels and dairy cattle, except cows in one dairy herd which had *Brucella abortus* (Collins and Campbell, 1982; Radwan et al., 1992).

Several treatments have been tried for curing bovine brucellosis using vitamins, trace elements, minerals, dyes, phenol, sulfonamides, penicillin and chlortetracyclines, without complete success (Dumaresq, 1940; Berman et al., 1946; Pivnyak, 1958; Schuhardt et al., 1994). However, the treatment with tetracyclines showed a significant reduction in abortions and shedding of organisms (Milward et al., 1984). The same trend was observed with the use of long-acting tetracycline combined with streptomycin in treating 70% of *Brucella*-infected cows (Nicoletti et al., 1985). Radwan et al. conducted experimental treatments of *Brucella melitensis* infections in sheep and goats with different regimens of oxytetracycline (OTC) alone or combined with streptomycin. Cessation of shedding of *Brucella* from udder secretions and absence of *Brucella* in selected tissues at autopsy were considered criteria for successful treatment. The results showed that long-term treatment with a high dose of OTC alone had succeeded in eliminating *Brucella melitensis* (Radwan et al., 1989, 1992).

The pharmacokinetics of OTC were studied after both intravenous (i.v.) and intramuscular (i.m.) administration to a group of five healthy calves. The data demonstrate a three-phase elimination of oxytetracycline. However, no explanation was given to the slowest phase of elimination ($t_{1/2} = 95$ h). In addition, the slow absorption from the injection site and/or the release of the drug incorporated into bone were not likely explanations (Meijer et al., 1993a). A correlation between tissue and plasma concentrations of OTC in veal calves was demonstrated, especially when certain criteria were fulfilled (Meijer et al., 1993b). Londoni and Errecalde (1992) showed that the observed concentrations after i.m. dose (20 mg/kg) of long-acting oxytetracycline (LA-OTC) formulation to calves were higher than the minimal inhibitory concentration (MIC) for the majority of pathogens in all the analyzed tissues for at least 72 h post-injection. Oukessou et al. (1992) studied the disposition of a new LA-OTC in camels in which OTC concentrations were measured by a microbiological assay. Archimbault et al. (1988) proved that two brands of LA-OTC are not bioequivalent in cattle. The evaluation of the pharmacokinetics of LA-OTC in *Brucella*-infected sheep, to our knowledge, has never been demonstrated. Therefore, the purpose of this investigation was to study the pharmacokinetics of oxytetracycline given as a long-term therapy in treating Najdi ewes, naturally infected with *Brucella melitensis*, to suggest an optimum dosage regimen to control this infection in sheep.

2. Materials and methods

2.1. Animals and dosing scheme

The study was conducted on pure native Najdi ewes raised on a large sheep breeding farm in the eastern province of Saudi Arabia. After serological and microbiological screening, only brucellosis-positive sheep were isolated. Six ewes from the positive group were selected at random for this study. These animals had aborted about 1 month before the beginning of the treatment. *Brucella melitensis* biovar 3 was isolated from the aborted fetuses (4–5 month). The ewes were 2–7 years old and 40–70 kg in weight. The *Brucella* antibody titer just before treatment, among the choosing ewes, ranged from 1:25 to 1:200. Two non-infected sheep were also utilized in this study; their age and weight were comparable to those of the infected sheep. A generic LA-OTC (200 mg/ml) injectable solution was purchased from United Kingdom. The LA-OTC was administered i.m. in the cervical or the thigh region.

The non-infected ewes ($n = 2$) were given a single i.m. dose of 25 mg/kg LA-OTC. Blood samples were collected by venipuncture at intervals up to 72 h after dosing. The infected sheep ($n = 6$) were given a multiple-dose regimen of 25 mg/kg LA-OTC every 48 h for 3 weeks. Blood samples were withdrawn at 4, 24 and 48 h after each injection. The blood was allowed to clot at room temperature and serum samples were separated by centrifugation at 6000 rpm for 15 min and stored at -70°C until assayed.

2.2. HPLC assay of oxytetracycline

OTC concentrations in serum were measured by a specific high-performance liquid chromatography (HPLC) assay. A serum sample (200 μl) was spiked with 50 μl doxytetracycline (500 ng) solution as the internal standard (IS). Serum proteins were precipitated with 500 μl acetonitrile and the mixture was vortexed for 1 min. Following centrifugation, the supernatant was evaporated at 37°C under a stream of nitrogen to dryness. The residue was reconstituted in 150 μl of mobile phase prior to injection into the chromatograph for analysis. OTC and its IS were eluted from a Novapak C-18 column (3.9×150 mm) packed with 5 μm spherical particles with detection at 355 nm (Archimbault et al., 1988). The HPLC system was operated at ambient temperature. Methanol (7%) in 0.005 M EDTA solution was used as the mobile phase. The pH was adjusted to 8.6 with 1 N sodium hydroxide. The flow rate was 2.5 ml/min. The injection volume was 50 μl. The assay was fully validated. The percent intraday relative standard deviation (R.S.D.%) was less than 4%, and interday R.S.D.% was less than 6% at three different serum concentrations ($p < 0.05$). The mean percent recovery was 93 ± 4%, and the detection limit was 50 ng/ml.

2.3. Pharmacokinetic analysis

The two non-infected sheep data were analyzed using model-independent methods. Computer programs RSTRIP (RSTRIP VER 5, Micromath Scientific Software, Salt Lake City, UT), a curve stripping program, and PCNONLIN (PCNONLIN VER 4, Statistical Consultant, Apex, NC, 1992), a nonlinear least squares regression program, were utilized to estimate the pharmacokinetic parameters of OTC in the two groups of sheep, non-infected and infected. The non-compartmental analysis for extravascular administration in PCNONLIN was used to measure the area under the OTC concentration–time curve ($\mathrm{AUC} = \int_0^\infty C\,\mathrm{d}t$), the area under the first moment of the curve ($\mathrm{AUMC} = \int_0^\infty tC\,\mathrm{d}t$), the mean residence time ($\mathrm{MRT} = \mathrm{AUMC}/\mathrm{AUC}$) and the elimination half-life ($t_{1/2}$). The apparent total clearance (Cl/F) and apparent volume of distribution at steady state (V_{ss}/F) were calculated using non-compartmental equations, where $\mathrm{Cl}/F = (\mathrm{Dose}/\mathrm{AUC})$ and $V_{ss}/F = (\mathrm{Cl}*\mathrm{MRT})$. After multiple dosing, the AUC was estimated during the dosing interval (τ). Drug accumulation was measured by $C_{max,ss}/C_{max,1}$, where $C_{max,ss}$ is the maximum concentration after the last dose (at steady state) and $C_{max,1}$ is the maximum concentration after the first dose. The fluctuation of OTC concentration was calculated as % fluctuation $= 100 \cdot (C_{max,ss} - C_{min,ss})/C_{min,ss}$, where $C_{min,ss}$ is the minimum concentration at steady state.

2.4. Statistical analysis

The t-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95% (Tallarida and Murray, 1987).

3. Results and discussion

The objective for developing long-acting oxytetracycline formulation is to deliver the required concentration of drug to the site of action and to achieve optimum efficacy over an extended period of time. Also, long-acting formulations provide a convenient method of treatment by reducing the frequency of administrations. The disposition of LA-OTC in non-infected and infected sheep after i.m. administration was investigated. A semilogarithmic serum concentration–time profile after

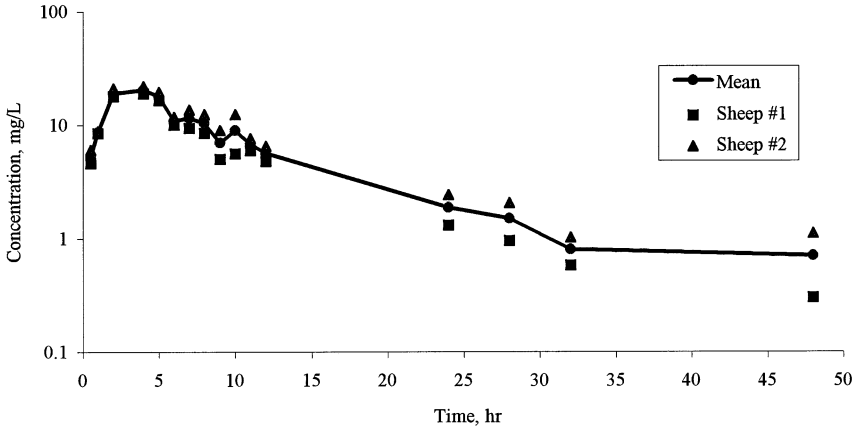

Fig. 1. Oxytetracycline serum concentration–time profile after i.m. administration (25 mg/kg) of LA-OTC in non-infected sheep ($n = 2$).

i.m. administration of LA-OTC (25 mg/kg) in non-infected sheep is depicted in Fig. 1. The mean serum concentrations of the drug showed an apparent elimination phase started after 12 h of administrations. OTC pharmacokinetic parameters calculated using non-compartmental analysis are summarized in Table 1. The OTC mean concentration 4 h after administration was at its maximum ($C_{max} = 20$ μg/ml). OTC concentrations were maintained greater than 1 μg/ml up to 28 h after drug administration. The mean elimination half-life was 11 h and the apparent systemic Cl was 111.6 ml/h per kg.

Archimbault et al. performed a comparative bioavailability study of two formulae of 20% long-acting oxytetracycline in six cattle (20 mg/kg). In their study the mean T_{max} values were 4.4 and 4.8 h for the two formulations, while the C_{max} values were 11 and 7.7 μg/ml, respectively. OTC concentrations were greater than 1 μg/ml for more than 60 h after the administration of the two formulations. This may indicate that the release of OTC from the present studied formulation was faster than from the two formulations mentioned above (Archimbault et al., 1988) or that it may be due to species differences in the pharmacokinetics.

Table 1
Pharmacokinetic parameters of oxytetracycline following i.m. administration of 25 mg/kg to non-infected sheep ($n = 2$)

Parameter	Value
C_{max} (μg/ml)	20.5
T_{max} (h)	4
AUC (μg/h per ml)	224
AUMC (μg/h^2 per ml)	2821
MRT (h)	11.7
$t_{1/2}$ (h)	11
Cl/F (ml/h per kg)	111.6
V_{ss}/F (ml/kg)	1310

Radwan et al. (1989) reported an experimental treatment of *Brucella melitensis* infections in sheep with OTC alone or combined with streptomycin. The percentage of *Brucella*-free sheep at necroscopy was compared in two groups of sheep. One group received OTC daily while the other received a brand of LA-OTC every 3 days; both treatments were given as 25 mg/kg and continued for 6 weeks. The respective groups of sheep at necroscopy were found to be 100 and 75% *Brucella*-free. Thus, dosing up to 72 h intervals seems to eradicate the disease successfully.

Fig. 2A represents the individual OTC serum concentration–time profile after multiple i.m. doses of 25 mg/kg every 48 h over a 3-week period for the six infected sheep, while Fig. 2B shows the mean of concentration (+S.D.) of OTC in the six sheep. Oxytetracycline was not detected in most serum samples (only in three out of six) collected 3 days after the last dose of

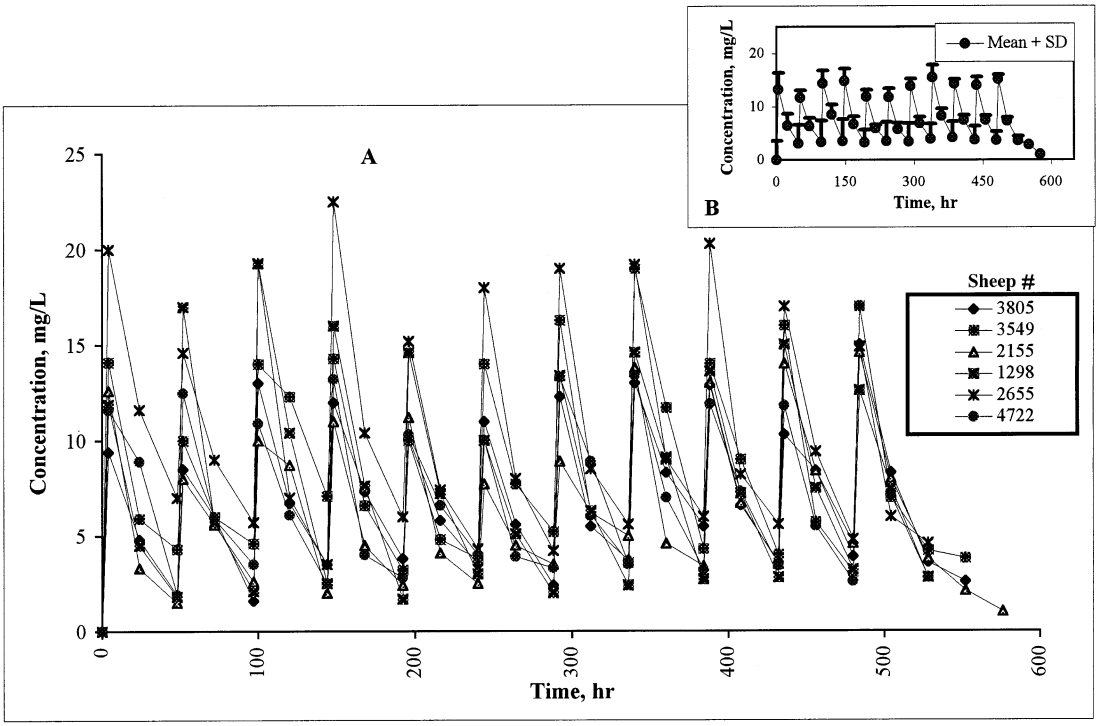

Fig. 2. Oxytetracycline serum concentration–time profile after multiple i.m. doses of 25 mg/kg every 48 h over 3-week period in infected sheep ($n = 6$). (A) Individual OTC serum concentration; (B) mean of concentration (+ S.D.) of OTC.

treatment. A measurable drug concentration was found 4 days after the last dose only in one sheep. A summary of the pharmacokinetic parameters of OTC in infected sheep is shown in Table 2. The MRT is defined as the mean time for the intact drug molecule to transit through the body and involves a composite of all kinetic processes including release from the dosage form, drug absorption into the body and drug disposition. MRT can be used in a comparative way to evaluate the in vivo performance of a sustained release dosage form (Cutler, 1978). No significant change ($p < 0.05$) in the MRT after the first dose (17 ± 1.0 h) relative to the MRT at steady state (18 ± 0.7 h) was observed in infected sheep, while a notable increase in the MRT ($\sim 54\%$) was detected between non-infected and infected sheep at steady state, 11.7 and 18 ± 0.7 h, respectively. Comparison the apparent Cl of the non-infected sheep to that of the infected sheep showed a substantial decrease in Cl, 111.6 and 64 ± 4 ml/h per kg, respectively. The apparent volume of distribution was 1310 ml/kg in case of non-infected sheep and 1150 ± 87 ml/kg in infected sheep. Therefore, *Brucella* infection seems to alter the elimination of OTC, while it does not affect its distribution.

The outcome from infected sheep indicates that there is no increase in the trough or the peak concentrations after the first and the last dose. Since the elimination $t_{1/2}$ of OTC in non-infected sheep was 11 h, a dosing interval of 48 h ($4t_{1/2}$) should result in no significant accumulation of the drug. Accordingly, the accumulation in infected sheep was 1.3 ± 0.23.

Fluctuations in drug serum concentrations are related not only to the release rate of the drug from the dosage form and the dosage interval but also due to the drug elimination (Gibaldi, 1984). This regimen produced a consequential % fluctuation, from 223.9 to 507%, between OTC peak and trough concentrations at steady state. This may

Table 2
Pharmacokinetic parameters of oxytetracycline following im administration of 25 mg/kg in *Brucella* infected sheep ($n = 6$)

Parameter	Sheep No.						
	3805	3549	2155	1298	2655	4722	Mean (S.D.)
Pharmacokinetic parameters after first dose							
AUC (μg/h per ml)	241	379	242	263	579	358	344 (130)
AUMC (μg/h^2 per ml)	4122	6820	3863	4351	10784	5894	5972 (2622)
MRT (h)	17.1	18	16	16.5	18.6	16.5	17 (1)
Cl/F (ml/h per kg)	—	—	—	—	—	—	—
V_{ss}/F (ml/kg)	—	—	—	—	—	—	—
C_{min} (mg/ml)	1.9	4.3	1.5	1.8	7	1.9	3.3 (2.4)
C_{max} (mg/ml)	9.4	14.1	12.6	11.9	20	11.6	13.3 (3.6)
Fluctuation %	—	—	—	—	—	—	—
Pharmacokinetic parameters at steady state							
AUC (μg/h per ml)	414	415	406	356	376	399	394 (24)
AUMC (μg/h^2 per ml)	7469	7509	7456	6360	7153	6797	7124 (462)
MRT (h)	18.1	18.1	18.4	17.8	19	17	18 (0.7)
Cl/F (ml/h per kg)	60	60	62	70	67	63	64 (4)
V_{ss}/F (ml/kg)	1092	1091	1131	1252	1268	1068	1150 (87)
C_{min} (μg/ml)	3.6	4.2	3.8	2.8	4.6	2.8	3.6 (0.73)
C_{max} (μg/ml)	15	17	14.6	12.6	14.9	17	15.2 (1.7)
Fluctuation %	316.7	304.8	284.2	350	223.9	507	331.1 (95.8)

enhance the emergence of resistance to the drug. Therefore, a dosage interval of 48 h of OTC alone might not be appropriate for this formulation for complete eradication of *Brucella* in infected animals.

Guerra and Nicoletti conducted a comparative study of the susceptibility of *Brucella abortus* isolates obtained in eight cows before and after treatment. The treatment regimen consisted of an i.m. injection of 20 mg/kg LA-OTC every 4 days for five doses and 25 mg/kg of streptomycin once a day only in the 1st week (Guerra and Nicoletti, 1986). They concluded that the treatment failures in the cows were not due to development of antibiotic resistance, because the MIC (minimal inhibitory concentration) and MLC (minimal lethal concentration) of all the post-treatment isolates were no greater than those of pretreatment isolates. One of their suggested reasons for the treatment failure may include improper method of administration (frequency of dosing) or insufficient doses of drug or length of treatment.

Accordingly, the currently used regimen with this particular OTC brand as a monotherapy should not relied upon for complete elimination of *Brucella* in infected sheep. Therefore, the recommended regimen is to use this LA-OTC in combination with streptomycin, known as synergistic to OTC, and/or to decrease the dosage interval to every 24 h since dosage up to 60 mg/kg showed no signs of toxicity. These recommendations should be investigated microbiologically or serologically in the treated animals in the near future.

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