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CHLORTETRACYCLINE IN SERUM AND MILK FOLLOWING SINGLE INTRAUTERINE ADMINISTRATION IN CLINICAL ENDOMETRITIS CAMELS (*Camelus dromedarius*)

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ABSTRACT

This research paper aimed to study the effect of single intrauterine administration of Chlortetracycline (CTC) in clinical endometritis camels (*Camelus dromedarius*) with regard to its levels in serum and milk. CTC pessaries were administered to 5 dromedary camels with clinical endometritis at a dosage of 2 g per animal. Blood and milk samples were collected before treatment and at 12 h intervals for 156 h. Serum and milk were analysed by ultra-performance liquid chromatography (UHPLC/MSMS). The serum and milk analyses revealed that maximum concentration of CTC was detected at 12 h post-treatment. The mean maximum CTC concentration was recorded in the serum and milk at 12 h post CTC administration. The maximum individual concentrations of CTC in milk ranged from 434.0 to 34.6 ng/ml. The mean concentration of CTC in the serum and milk decreased steadily by 24 and 36 h and thereafter post-treatment, respectively. CTC sustained in the milk during the period of 144.4 ± 13.99 h (range, 118 to 154 h) where the serum CTC retained by 111.2 ± 11.70 h (range, 106 to 130 h) after treatment. The mean milk CTC values ≥30 ng/ml was proved till 24 h after treatment (range 12 to 60 h). In conclusion, the safe residual level (≥30 ng/ml milk) established by the US Food and Drug Administration was recorded in the dromedary milk at 24 h onward after intrauterine CTC administration.

Key words: Camel, chlortetracycline, endometritis, milk, uterus

Endometritis is one of the most common uterine disorders of dromedary camels (Tibary *et al,* 2001; Tibary, 2004; Kaufmann, 2005). Numerous antimicrobial compounds have been employed for treatment and prevent of genital infection in the livestock (Pyörälä *et al,* 2014). Attribute to its broad-spectrum antibacterial effect, its efficient action in anaerobic environment of the uterus and activity in the presence of organic debris, tetracycline is recommended for intrauterine treatment (Bretzlaff *et al,* 1983; Bretzlaff, 1987; Hoedemaker, 1998). Although, the absorption and distribution of intra-uterine antibiotic treatment are well documented in cattle (Righter *et al,* 1975; Dinsmore *et al,* 1996; Jaroslav *et al,* 2003), equine (LeBlanc, 2012), and ovine (Cester *et al,* 1996), such studies are rather scarce in dromedary camels. Camels can lactate under severe drought conditions even when dehydrated (Yagil *et al,* 1994). Dromedary daily milk production average is estimated to be between 3 and 10 kg during a lactation period of 12-18 months (Farah *et al,* 2007). Besides the high nutritional quality (El-Agamy *et al,* 1998; Karue, 1998), camel milk is known for its medicinal properties (Magjeed, 2005; Shabo *et al,* 2005; Agrawal *et al,* 2003). The presence of antibiotic residues in milk may have direct toxic effects on consumers (Moats and Medina, 1996; Bencini and Pulina, 1997). The objective of the current study was to determine the concentration of CTC in the serum and milk of dairy camels suffering from clinical endometritis after a single intrauterine therapeutic dose.

Materials and Methods

The present study was conducted at the Camel Research Centre, King Faisal University. Five pluriparous lactating dromedary camels (age 7 to

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12 years and weight 400 to 500 kg) were involved. Camels were maintained under standard conditions of feeding and management. The animals were hand-milked twice a day with 12 h intervals. Their average milk yield was 4.5 litres per animal.

Diagnosis and treatment of endometritis

Camels had a history of failing to conceive after more than 2 services with fertile male camel. All camels were clinically examined by visual appraisal for any signs of abnormal vulval discharge, rectal palpation of the reproductive tract and ovaries (Tibary and Anouassi, 1997), vaginal examination (Tibary and Anouassi, 1997b; Tibary and Anouassi, 2000; Ali *et al,* 2009) as well as transrectal ultrasound (Tibary and Anouassi, 1997a; Tibary and Anouassi, 2000; Tibary *et al,* 2001; Ali *et al,* 2009) using linear-array 5 MHz transducer (UST-588U-5, SSD-500V, ALOKA, Co., Japan). Based on history, rectal, vaginal and ultrasound examination, these animals were diagnosed as suffering from clinical endometritis. Each animal was given a single intrauterine administration of 2g CTC pessaries (MetricyclinKell Belgium).

Milk and blood sampling

Milk samples (25 ml) were collected from the bulk milk of each camel before CTC administration (0 h) and at 12 h intervals up to 156 h after the treatment. Blood samples (10 ml) were taken from the jugular vein of each camel at the same intervals as the milk samples. Serum was prepared by centrifugation at 1400g for 15 min. Milk and serum samples were immediately frozen at – 80 °C and stored for subsequent assay.

CTC analysis

Chlortetracycline was purchased from Sigma–Aldrich, (95%; European Pharmacopoeia HPLC assay, lot 081M1598V, product of China; Shanghai, Trading Co., Ltd.). Mcllvaine buffer was prepared [12.0g sodium hydrogen phosphate (Na_2HPO_4), 3.72g EDTA and 11.9g anhydrous citric acid in 1L deionised water], and the pH was adjusted to 2.9 using concentrated phosphoric acid. Formic acid and methanol (HPLC grade solvent)were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Deionised water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Disposable 0.22 µm nylon membrane filter (used for extract filtration) were obtained from Millipore (MA, USA).

Extraction and clean up procedures for serum samples was done according to Ghoneim *et al* (2015).

Serum samples (500 µl each) were transferred individually to conical Eppendorf tubes (1.5 ml) and then a volume of 500-µl acetonitrile was added to each tube. The tubes were vortex-mixed for 1 min., and centrifuged at 5000 rpm for 15 min at 4°C. An aliquot of 500 µl of supernatants was transferred to 500 µl mobile phase, mixed and filtered through 0.22µm nylon membrane filter prior to injection onto the UPLC–MS/MS analysis. The injection volume was 7 µl.

Milk samples were collected according to the methods of NaVrátiloVá *et al* (2009) and Cinquina *et al* (2003).

Before the analysis, milk samples were allowed to reach room temperature and stirred for homogenisation then 2ml of each milk sample was mixed with 6ml of McIlvaine buffer. The diluted samples were centrifuged at 4000 rpm and 5°C for 10 minutes to allow complete precipitation of the denatured protein, then after centrifuging; the upper fat layer was removed. The supernatant was decanted from each tube and transferred into a solid-phase extraction (SPE) column (Oasis HLB, Waters, Milford, USA).

SPE Columns were preconditioned (before the addition of samples supernatants) with 3 ml of methanol and 2 ml of water, then an aliquot of supernatants was applied to the column, drained and washed with 1.5 ml 5% methanol in water.

Chlortetracycline was eluted with 2 ml of methanol and then evaporated to dryness on a rotary vacuum evaporator. The evaporated residues were reconstituted in 1 ml of the mobile phase and filtered through 0.2 µm nylon filters for chromatographic analysis. A 7 µl of the filtered extract was injected onto the UPLC–MS/MS system.

UPLC/ESI–MS/MS analysis was done according to Han *et al* (2015). An ultra-performance liquid chromatography (UPLCTM) system Acquity (Waters, Mildford, MA, USA) was interfaced to a triple quadrupole mass spectrometer(UPLC/MSMS) (TQDTM, Waters Micromass, Manchester, UK) using an electrospray interface. The UPLC separation was performed using an Acquity UPLC BEH C18 analytical column, 1.7 µm particle size, 2.1mm × 50 mm (Waters), at a flow rate of 300 µL/min. A gradient elution system was used with mobile phase A (0.1% formic acid in water) and mobile phase B (Acetonitrile) at a total flow rate of 0.3 ml/min. The gradient program was started at 95% mobile phase A and 5 % mobile

phase B, changed linearly to 60 % mobile phase A and 40 % mobile phase B for 3.0 min, changed linearly to 10 % mobile phase A and 90 % mobile phase B for 4.0 min, and finally, 4.1-6.5 min, 5% mobile phase A. The total run time for each sample analysis was 7 min. The injection volume was 7µL. Drying gas as well as nebulising gas was nitrogen. The gas flow was set to 900 L/h. For operation in MS/MS mode, collision gas was Argon 99.995% with a pressure of approximately 2.103 mbar. Capillary voltage of 3.5 kV in positive electro-spray ionisation mode was applied. The interface temperature was set to 450°C and the source temperature to 125°C. Temperature column was set to 40 °C. Dwell times of 30 ms/scan were chosen. Masslynx v 4.1(Waters, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and samples. Quantitation was performed in the multiple reaction monitoring (MRM) mode using peak areas.

MRM transition of chlortetracycline was applied as indicated by Quanpedia software (waters, Mildford, MA, USA) where M/Z 479.4>444.1 and 479.4>462.1 were the qualifier and quantifier ion, respectively.

Specificity:

The specifcity was confrmed based on the presence of the transition ions (quantifer and qualifer) at the correct retention times (that was defined formerly by using one milk sample and one serum sample that spiked at 100ng/ml to check the retention time of chlortetracycline.) corresponding to that of the precursor ion. The measured peak area ratios of qualifer/quantifer were within the range defined in EU Commission Decision 2002/657/EC when compared to the standards.

Matrix-matched calibration curves:

Matrix-matched calibration curves were prepared for control and quantification purposes according to Stolker *et al* (2010) and Han *et al* (2015).

Milk and serum sample extracts (after reconstitution in mobile phase)were spiked with different aliquots of chlortetracycline standard solution to give final concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml. The linearity of the employed method was performed by preparing calibration curves using the aforementioned concentration levels. The calibration curves were constructed by means of plotting the detection response of the matrix matched standard solutions (spiked samples extract) versus the corresponding concentrations by means of regression analysis. From these data, the regression coefficients (r^2) of the calibration curves were calculated where criterion for good linearity should be r^2>0.99.

Method accuracy (recovery percentages):

Method accuracy was determined according to Bousova and Mittendorf (2012), Cinquina *et al* (2003) and Han *et al* (2015) using independently spiked blank samples at three different levels (12.5, 50 and 200ng/ml milk or serum) in 6 replicates. Accuracy was evaluated by comparing found values with standard additions in spikes. Recovery values were expressed in percentages (Table 1).

Table 1. Recovery of CTC in the spiked milk and serum samples (n=6).

Analyte	Spiked level (ng\ml) (milk and serum)	Milk samples (Mean ±SD) (n=6)	Serum samples (Mean ±SD) (n=6)
CTC	12.5	89.40 ± 4.02	89.00 ± 3.09
	50.0	90.50 ± 3.90	94.00 ± 6.05
	200.0	92.30 ± 3.28	91.00 ± 3.57

n= Number of assays, SD= Standard Deviation

Limit of detection (LOD) and limit of quantification (LOQ):

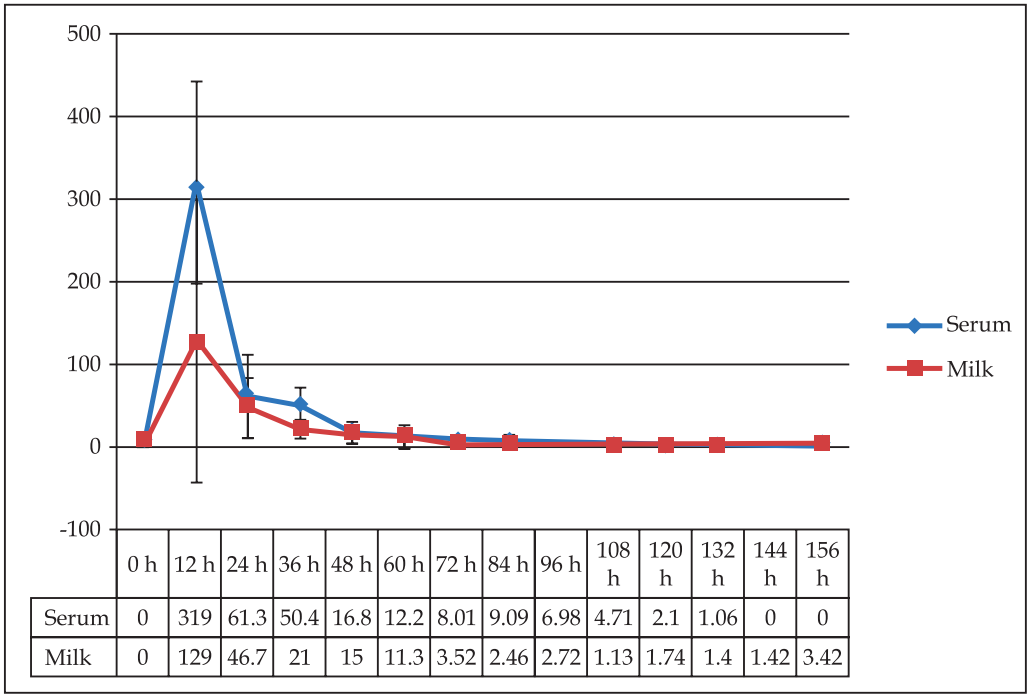
Limits of detection (LOD) and quantification (LOQ) were defined as lowest concentrations with a signal-to-noise (S/N) ratio of ≥3 for LOD or ≥10 for LOQ.

Statistical analysis

Data of CTC are presented as means ± SD. Statistics performed using SPSS program 22.0 statistical software (2013).

Results

The data demonstrated in Fig 1 represented the serum and milk CTC concentrations following intrauterine administration. CTC was firstly detected in the serum and milk 12 h after the treatment. The mean maximum CTC concentration was recorded in the serum and milk by 12 h after CTC administration. The maximum individual concentrations of CTC in milk ranged between 34.6 and 434.0 ng/ml. The mean CTC concentration decreased steadily by 12 h in serum and milk after administration and thereafter. However, after 108 h post-treatment there was no significant decrease in CTC values in milk. CTC sustained in the milk during the period of 144.4 ± 13.99 h (range, 118 to 154 h) where the serum CTC retained by 111 .2 ± 11.70 h (range, 106 to 130 h) after



	0 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h	96 h	108 h	120 h	132 h	144 h	156 h
Serum	0	319	61.3	50.4	16.8	12.2	8.01	9.09	6.98	4.71	2.1	1.06	0	0
Milk	0	129	46.7	21	15	11.3	3.52	2.46	2.72	1.13	1.74	1.4	1.42	3.42

Fig 1. Chlortetracycline concentration in the serum and milk following intrauterine administration in endometritis in dromedary she camels.

treatment. The concentrations of CTC in milk were approximately 70% of maternal serum concentrations. The mean milk CTC values ≥30 ng/ml was proved till 24 h after treatment (range 12 to 60 h).

Discussion

The main principle of practising intrauterine therapy is to achieve a high level of antibiotic in the uterine lumen (Gilbert, 1992; Jaroslav *et al,* 2003). The current experiment was carried out during the non-breeding season to avoid the effect of sex steroids on the blood flow (Dickey, 1997; Bollwein *et al,* 2002) as the rate of drug absorption greatly affected with local blood flow (Morris *et al,* 1993). Recoveries of CTC analyte spiked at 3 different concentrations ranged from 89.4% to 92.3% and 89% to 94.0% in milk and serum, respectively (Table 1). It indicated the extraction and purification method in this study was suitable and reliable for the CTC analyte in milk and serum. The protracted recovery of CTC from the serum and milk (144 and 156 h, respectively) reported in this study was also recorded by Tan *et al* (2007) in cattle. This could be due to slow absorption of the solid form of CTC from the uterus (Roncada *et al,* 2000). On the contrary, tetracycline spray cannot be recovered from the serum of cows after 24 h from intrauterine administration (Girardi *et al,* 1990). The earlier vanishing of CTC from the blood than milk may be attributed to that the concentration of CTC in the serum reflects the concurrent drug absorption from the uterus where its concentration in the milk provides the image over last 12 h. Eliminated tetracyclines in milk were approximately 70% of maternal serum concentrations as reported before (Gideon and Martin, 1997). As the blood concentration of the antibiotic is an indicator for the level in the tissue (Ryan *et al,* 1986; Nix *et al,* 1991), in the current study, the mean serum concentration of CTC recorded in the first 48 h post injection was within the minimum inhibitory concentration required for treatment against bacteria that cause uterine infections in cattle (Sheldon *et al,* 2004). The presence of antibiotics residues in milk poses a dual hazard on the health of consumers (Moats and Medina, 1996; Bencini and Pulina, 1997) as well as dairy products manufacture (Heeschen *et al,* 1991). Activity levels of certain residual antibiotics appear to be decreased by pasteurisation (Roca *et al,* 2010; 2011; Zorraquino *et al,* 2009; 2011). Yet, the effect of pasteurisation on the degradation of CTC is very limited (Loksuwan, 2002; Kellnerová *et al,* 2014). Most camel milk are traditionally consumed as raw milk (Mehaia *et al,* 1995). Although, local and regional authorities and organizations have issued quality requirements (regulations and standards) specific for camel milk (GSO 1970/2009; GSO, 2009). Yet, it does not includ antibiotics residue levels. The safe residual level of ≤ 30 ng/ml in cows' milk established by the US Food and Drug Administration (Popadoyannis

et al, 2000) was recorded in the current study at 24 h onward after CTC injection. On the other hand, the 'safe level' set by the Food and Agricultural Organisation (FAO) and European Union of 100 ng/ml milk (Naoto, 1999) was not reported in the current study. In conclusion, after intrauterine administration of CTC, residues were identified in the milk for 156 ± 13.99 h. The safe residual level of ≤ 30 ng/ml milk established by the US Food and Drug Administration was recorded in milk at 24 h onward after intrauterine CTC administration. Serum CTC retained by 111.2 ± 11.70 h after intrauterine administration.

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