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Pharmacokinetic and Metabolic Profile of Difloxacin in Camels Following Parenteral Administration

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Abstract: The pharmacokinetics of difloxacin delivered by both Intravenous (IV) and Subcutaneous (SC) routes and its metabolic profile and elimination pattern following the subcutaneous administration of 5 mg kg^{-1} were investigated in a crossover study using 10 camels (*Camelus dromedaries*). Multiple plasma, faecal and urine samples were collected for the quantitation of difloxacin and its metabolites using HPLC with fluorescence detection and mass spectrometry for the elucidation of metabolite structure. Difloxacin was eliminated from plasma with elimination half-lives of 6.65 and 7.52 h following Intravenous (IV) and Subcutaneous (SC) administration, respectively. The drug was absorbed slowly following SC administration and a maximum concentration of 2.1 μg mL^{-1} was attained at (T_{max}) 4 h with a bioavailability of 94.6%. Difloxacin was metabolised in camels by the N-demethylation pathway to produce the active metabolite sarafloxacin (M1) and by oxidation into three other metabolites, 3-oxosarafloxacin (M2), 3-oxodifloxacin (M3) and desethylenesarafloxacin (M4). The concentrations of the circulating Metabolites in plasma (M1, M2 and M3) were much lower than that of the parent drug. The administered dose of difloxacin was eliminated largely in its parent form in faeces (69.5%) and to a small extent in urine (5.9%) whereas sarafloxacin (M1) and 3-oxosarafloxacin (M2) were the main metabolites detected in faeces (7.2 and 3%) and urine (3.5 and 1.8%). The other metabolites, 3-oxodifloxacin (M3) and desethylenesarafloxacin (M4) were detected to a minimal extent in faeces only and amounted to 1.47 and 0.6% of the dose, respectively. The results of the present study revealed that the N-demethylation and oxidative pathways of biotransformation are the primary routes of difloxacin metabolism in camels with renal and hepatobiliary excretion through urine and faeces. Phase II conjugation plays a minor role in the elimination of the drug in camels.

Key words: Camels, difloxacin, faeces, metabolism, pharmacokinetics, plasma, urine

INTRODUCTION

Difloxacin is a difluoroquinolone antimicrobial agent with high *in vitro* activity against a wide range of Gram-positive bacteria, Gram-negative bacteria and Mycoplasmas (Digranes and Dibb, 1988; Mader *et al*., 1987; Fernandes *et al*., 1988, 1986; Brown, 1996; Fernandez-Varon *et al*., 2006). Difloxacin exerts its bactericidal activity by the inhibition of subunit A of DNA topoisomerase 2 (gyrase) an enzyme that is essential for DNA synthesis and repair (Hooper and Wolfson, 1993; Drica and Zhao, 1997). A p-fluorophenyl ring at position N-1 of difloxacin gives the molecule enhanced activity against Gram-positive bacteria as well as an excellent pharmacokinetic profile among other fluoroquinolones (Walker, 2000). Despite the wide knowledge of the pharmacokinetic characteristics of difloxacin in many animal species including mice (Fernandez *et al*., 1986), pigs (Inui *et al*., 1998; Zheng *et al*., 2003), chickens (Inui *et al*., 1998; Ding *et al*., 2008; Anadon *et al*., 2011), goats (Atef *et al*., 2002; Marin *et al*., 2007a), dogs (Frazier *et al*., 2000; Heinen, 2002), rabbits (El-Aty *et al*., 2005; Fernandez-Varon *et al*., 2008), mares (Adams *et al*., 2005), horses (Fernandez-Varon *et al*., 2006), calves (Ismail, 2007), sheep (Marin *et al*., 2007b) and camels (Abo-El-Soud and Goudah, 2009) there is scarce data for its metabolic profile in most animal species. Previously published studies have indicated that in humans (Granneman and Senello, 1987), pigs (Sukul *et al*., 2009), rabbits (Garcia *et al*., 2000) and birds (Anadon *et al*., 2011) difloxacin is metabolised primarily through the N-demethylation pathway or through glucuronidation (Chu *et al*., 1985). Seven metabolites have been identified for difloxacin but their existence, quantity and excretion patterns differ across species and the pharmacokinetic profile of the parent drug differs accordingly. Camels metabolise many drugs via alternative pathways distinct from the pathways common in other animal species and this phenomenon could explain the camel's

adaptation to many dietary and environmental pollutants (Ali and Elsheikh, 1992; Raza and Montague, 1993; Al-Otaiba *et al*., 2010). Little information about the biotransformation and excretion patterns of difloxacin and other members of the same class of fluoroquinolone is available for ruminants, especially dromedary camels. Antimicrobial drugs eliminated into the soil may promote the development and increase the risk of the spread of resistance genes in the environment (Heuer and Smalla, 2007; Heuer *et al*., 2008; Kotzerke *et al*., 2008). Complete knowledge of the excretion patterns of fluoroquinolones in different animal species is imperative for human health protection and environmental control. So, the aim of this research was to investigate the pharmacokinetic and metabolic profile of difloxacin in camels following intravenous and subcutaneous administration to provide knowledge about the excretion patterns of the drug and its metabolites in urine and faeces.

MATERIALS AND METHODS

Chemicals: Difloxacin (D2819, 5 g), authentic standard for difloxacin (33984, 100 mg), sarafloxacin (33497, 100 mg) and ofloxacin (33703, 100 mg) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Difloxacin metabolites were synthesised by Bootech Bioscience and Technology (Shanghai, China) with purity >99%. Nonafluoropentanoic Acid (NFPA), acetonitrile, methanol and trichloromethane were all HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Hydrochloric acid, ammonia, ammonium hydroxide and sodium hydroxide were supplied by Fluka, Switzerland.

Animals: Ten healthy 16-24 months old female camels (*Camelus dromedarius*) weighing between 320-390 kg were used in this experiment. The animals were obtained from the farm of the College of Veterinary Medicines and Animal Resources at King Faisal University. All animals were maintained for 1 month without any medication before the commencement of the experiment. Each camel was housed in an individual well-ventilated hygienic pen. Feed consisted of alfalfa hay, concentrate and green fodder, drinking water was provided *ad libitum*. The experimental protocols of this study were approved by the institutional animal care and use committee at the university.

Drug administration and sampling: This study was performed in two segments using a crossover design with 20 days washout period between each segment to ensure complete clearance of the drug. In each segment of the experiment, animals were divided into 2 groups of 5 animals, one group was given freshly prepared difloxacin (10%) intravenously and the second group was given the drug Subcutaneous (SC). The drug was given at a dose of 5 mg kg^{-1} B.W. intravenously via the jugular vein and subcutaneously into the neck region. Blood samples (3 mL each) were collected in heparinised tubes just prior to administration and at 5, 10, 15, 30 min and at 1, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h post administration. Blood samples were centrifuged at 1000 g for 10 min and the plasma was decanted and frozen at -20°C until assayed.

For urine collection following subcutaneous administration, the camels were catheterised with an indwelling balloon catheter (Foley urinary catheter, No. 8, Timedco, Atlanta, Ga.). Catheters were connected to a 2 L container. Urine containers were evacuated upon filling and daily urine samples were collected for 10 days post drug administration. The volume of urine voided daily was measured and 10 mL aliquots were used for extraction on the same day.

Faeces was collected upon voiding by the animals through a slatted floor for 10 days post Subcutaneous (SC) administration of the drug and the samples for each day were homogenised and freeze dried to calculate the dry weight. A 20-30 mg aliquot was used for extraction.

The glucuronide conjugate of difloxacin or its metabolites in plasma, urine and faeces were quantitated by comparison of the concentration of the unchanged analytes before and after enzyme hydrolysis. Aliquots of different samples (1 mL) were adjusted to pH 7 by phosphate buffer and then aliquots of the samples (0.5 mL) were incubated with 50,000 U mL^{-1} β-glucuronidase (Sigma, Munich, Germany) for 4 h at 37°C.

Sample preparation and extraction: Fifty microliters of IS solution (5 μg mL^{-1} ofloxacin dissolved in 0.05^{M} phosphate buffer) were added to the plasma sample (200 μL). The solution was vortex mixed and further diluted with 800 μL of trichloromethane. Urine samples were diluted using aqueous phosphate buffer (pH = 4) and spiked with ofloxacin as an internal standard. Faeces samples were homogenised in 2 volumes of phosphate buffered saline (PBS, pH = 2) and a 300 μL aliquot was further diluted with 700 μL of acetonitrile and vortex mixed. The 100 microlitres of sample solution were mixed with 10 μL of IS. Different matrix solutions were loaded onto preconditioned Solid Phase Extraction (SPE) cartridges (Oasis MAX and Oasis MCX, Waters Corporation, Milford, USA) for further clean up. Preconditioning of the SPE cartridge was performed using 1 mL of methanol, 1 mL of 5 N NaOH and 1 mL of water.

After sample loading, the cartridge (MAX) was washed with 1 mL of 5% ammonia in water and then washed with 1 mL of methanol and eluted with 1 mL of 0.2 N HCL in methanol into MCX cartridge. The Oasis MAX cartridge was washed with 1 mL of methanol and eluted with 500 μL of 10% N H_4OH in methanol and the elute was neutralised using formic acid and then evaporated to dryness under a gentle N_2 stream at 50°C. The residue was reconstituted with 100 μL of 70% methanol. The 10 μL of the aliquot were injected onto the HPLC System.

LC mass spectrometry: Quantitation and characterisation of difloxacin metabolites were performed by LC-MS this system consisted of an Agilent 1100 series HPLC System (G1379A degasser, G1389A, autosampler, G1315B fluorescence detector, G1357 A binary capillary pump and a G1316A column oven) and an LTQXI quadrupole ion trap mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with electrospray ion source. All components are run under the control of Xcalibur Version 1.2 Software (Thermo Fisher Scientific Inc., San Jose, CA, USA). The column used was a Luna C18 reverse phase column (3×150×2.0, 4251-B0, Phenomenex, USA).

The mobile phase consisted of solvent A, 0.2% Nona Fluoro Pentanoic Acid (NFPA) in water and solvent B, methanol. The elution was performed in a gradient mode, the gradient was 20% B-30% B in 10 min, 50% B-80% B from 10-20 min and 10% B from 20-30 min. The column was operated at 32°C with a flow rate of 0.5 mL min^{-1}. The injection volume was 30 μL and the time of analysis was 30 min. Fluorescence detection of the analytes was accomplished with an excitation/emission wavelength of 255/460 nm.

The mass spectrometer was operated in positive ion mode with a mass resolution of 4 GH (high resolution mode). Nitrogen gas was used as the sheath gas at a flow rate of 20 arbitrary units and helium was used as the collision gas at a pressure of 1.1×10^{-5} Torr. Full scan mass spectrum data were acquired from m/z 100-1100 at a rate of 1.5 scans sec^{-1}. The remaining LTQXI parameters (ion focus voltage, quadrupole lens, fragmentor voltage) were optimised by the autotuning procedure for maximum abundance of the molecular ions $[M+H]^+$. The Ms^n precursor ion (m/z) collision energy for difloxacin and its metabolites was in the range of 25-45.

Calibration curves: Primary stock solutions of difloxacin, M1-M7 and ofloxacin (1 mg mL^{-1}) were prepared by dissolving 10 mg of each compound in 10 mL of 1 M solution of sodium hydroxide in methanol. The solutions were stored in dark glass bottles at -20°C.

The calibration standards of difloxacin and its metabolites were prepared by spiking appropriate aliquots of the stock solution of each analyte and internal standard into the drug free camel plasma, urine and faeces homogenates to give a final concentration ranging from 0.005-100 μg mL^{-1}. Quality Control Samples (QCS) at concentrations of 0.005, 50 and 100 μg mL^{-1} were prepared by adding the appropriate aliquots of the stock solution to drug free matrices. The Quality Control Samples (QCS) were aliquoted (100 μL) in polypropylene tubes and stored at -20 until analysis.

The concentration was calculated from standard calibration curves constructed by linear regression of the peak height ratios of the analytes to that of internal standards as a function of the standard spiked matrices. Linear curves with correlation coefficients >0.99 were obtained for all analytes in the concentration range of 0.005-100 μg mL^{-1}. The Lower Quantitation limit (LOQ) was 0.005 μg mL^{-1} which was the lowest calibration standard on linear standard curves.

Validation of the assay method: The precision and accuracy of the protocol were evaluated by repetitive analysis of the plasma, urine and faecal homogenate QCS (n = 12) spiked with 0.005, 50 and 100 μg mL^{-1} of the different analytes. The recovery was calculated by comparison of the plasma, urine, faeces homogenate QCS and aqueous samples (n = 6).

The intra-assay precision was <4.4% for plasma and urine and <3.4% for faecal homogenate QCS. The intra-assay accuracy was >94% for all matrices. The interassay precision was <3.9% for plasma, urine and faecal homogenate QCS. The interassay accuracy was >95% for all matrices. Recovery of difloxacin from plasma, urine and faeces samples were found to be <94% for all matrices.

Pharmacokinetic analysis: Compartmental models were fitted to the plasma concentration versus time curves for each animal individually using a computer programme (WinNonlin, Pharsight Corporation, Mountain View, CA, USA). According to the value of Akaikes's Information Criterion (AIC) (Yamaoka *et al*., 1978), a two compartment open model system best fit the data from the IV drug administration group. The parameters calculated following IV administration include A and α (intercept and slope of the distribution phase) and B and β (intercept and slope of the elimination phase). The distribution and elimination half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$), the volume of distribution at steady-state (V_{dss}), the volume of the central compartment (V_c) and the total body Clearance (Cl_B) were computed according to standard equations (Baggot, 1978).

The renal Clearance (CL_R) of difloxacin and its plasma circulating metabolites following SC administration of difloxacin was determined by measuring their plasma and urine concentrations and the urine volume from samples collected over the sampling period for each analyte after SC administration of the drug. The calculation of renal clearance was performed according to the following equation (Ismail, 2005):

$$CL_R = \frac{(A \times V)_{0-t}}{AUC_{0-t} \times B.W.}$$

Where:

$(A \times V)_{0-t}$ = The cumulative amount of the drug excreted during the whole sampling period
A = The concentration of difloxacin excreted in each urine sample
V = The volume of urine sample
AUC_{0-t} = The area under the concentration time curve over the same sampling period
B.W. = The weight of each camel in kilogrammes

Fractional clearance was calculated by the ratio between renal clearance of difloxacin or its metabolites and creatinine clearance in camels. Following subcutaneous administration, plasma concentration data were analysed by compartmental analysis (Gibaldi and Perrier, 1982). The terminal elimination half-life ($t_{1/2el}$) and absorption half-life ($t_{1/2ab}$) were calculated as ln_2/k_{el} or ln_2/k_{ab}, respectively where k_{el} and k_{ab} are the elimination rate constant and absorption rate constant, respectively. The Area Under the plasma Concentration-time curve ($AUC_{0-\infty}$) and the Area Under the first Moment Curve ($AUMC_{0-\infty}$) were calculated by the trapezoidal rule for all measured data with extrapolation to infinity. The Mean Residence Time (MRT) was calculated as:

$$MRT = \frac{AUMC_{0-\infty}}{AUC_{0-\infty}}$$

The mean absorption time was calculated as:

$$MAT = MRT_{SC} - MRT_{i.v.}$$

The peak plasma Concentration (C_{max}) and time to maximum concentration (t_{max}) were taken from the plot of each camel's plasma concentration-time curve. Bioavailability (F: fraction of drug absorbed systemically) was calculated as follows:

$$F = \frac{AUC_{SC}}{AUC_{i.v.}} \times 100$$

Estimation of creatinine concentration: Plasma and urine samples were analysed for creatinine concentration according to the biochemical method described by Siest *et al.* (1985) using a commercial creatinine diagnostic kit (Bio Merieux, Paris, France).

Statistical analysis: The statistical analysis was performed using the SPSS® 6.1.3 Software package (SAS, Cary, NC, USA). The results were expressed as the mean±SE. Analysis of variance was performed by one-way Analysis of Variance (ANOVA) procedures.

RESULTS

The present study used an HPLC Method developed for the simultaneous quantitation of the concentrations of difloxacin and its metabolites, seven metabolites (M1-M7) for difloxacin were included in this study. Better separation of the analytes was accomplished by adjustment of chromatographic conditions including buffer composition, pH of the mobile phase and the gradient mode. These parameters were modified and optimised for better peak resolution, retention time and peak symmetry. The chromatographic conditions selected in this research allowed the separation of difloxacin and its metabolites without interference between the different analytes. The retention times for difloxacin, ofloxacin and M1-M7 in plasma were 19.1, 16.8, 18.66, 17.63, 14.35, 12.5, 15.3, 11.9 and 13.45 min, respectively. Mass spectrometry was used to identify difloxacin and its metabolites by comparing their product ion chromatograms with that of the reference standards, four metabolites were identified in plasma, urine and faeces. The mean concentrations of difloxacin and its circulating metabolites in plasma are presented in Fig. 1. Three circulating metabolites were

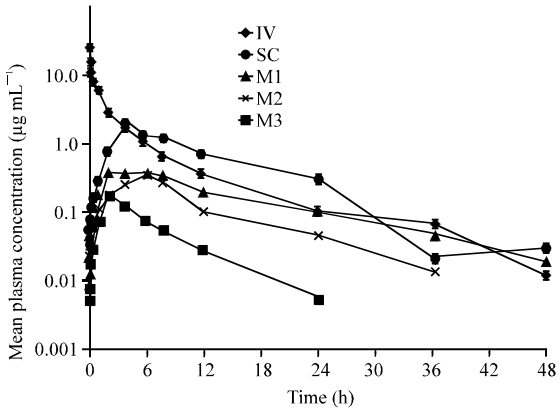

Fig. 1: Semilogarethmic graph depicting mean plasma concentrations of difloxacin in camels following IV and SC administration of 5 mg kg^{-1} B.W. and its metabolites (M1-M3 following SC route (n = 10))

detected in plasma following SC administration including sarafloxacin (M1), 3-oxosarafloxacin (M2) and 3-oxodifloxacin (M3), representing 26.5, 15 and 3.37% of the total concentration of the parent drug in plasma based on AUC ratio. None of the detected metabolites in plasma were in the conjugated form. In this research, the pharmacokinetic profile of difloxacin and its plasma circulating metabolites were determined. Table 1 summarises the mean pharmacokinetic parameters for difloxacin following the IV and SC routes of administration and Table 2 shows the pharmacokinetic data for M1, M2 and M3 following SC administration. The mean value of endogenous creatinine clearance (CL_{cr}) was 0.023±0.004 L/h/kg and the renal Clearances (CL_R) for difloxacin, sarafloxacin (M1) and 3-oxosarafloxacin (M2) were 0.031±0.002, 0.027±0.001 and 0.04±0.002 L/h/kg, respectively.

Table 1: Mean±SE kinetic parameters of difloxacin following a single i.v. and SC injection of 5 mg kg^{-1} B.W. in camels (n = 10)

Pharmacokinetic parameters	Units	i.v.	SC
α	h^{-1}	1.46±0.21	NA
β	h^{-1}	0.10±0.011	NA
K_{ab}	h^{-1}	NA	0.22±0.01
K_{el}	h^{-1}	NA	0.09±0.003
$t_{1/2\alpha}$	h	0.46±0.08	NA
$t_{1/2\beta}$ ($t_{1/2el}$)	h	6.65±0.45	7.52±0.37
$t_{1/2ab}$	h	NA	2.22±0.23
Vc	L kg^{-1}	0.29±0.03	NA
MAT	h	NA	5.4±0.22
V_{dss}	L kg^{-1}	1.14±0.3	NA
CL_B	L/h/kg	0.18±0.02	NA
CL_R	L/h/kg	NA	0.031±0.002
$AUC_{0-\infty}$	μg hmL^{-1}	31.9±3.1	30.2±2.7
$AUMC_{0-\infty}$	μg h^2 mL^{-1}	159.9±15.6	314.9±26.3
MRT	h	5.0±0.7	10.4±0.8
C_{max}	μg mL^{-1}	NA	2.1±0.3
t_{max}	h	NA	4.0±0.3
F	%	NA	94.6±8.4

NA: Not Applicable; α, β: hybrid rate constants representing the slopes of distribution and elimination phases, respectively; K_{ab}: Absorption rate constant; K_{el}: First-order elimination rate constant $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$: Elimination half-life (i.v.); $t_{1/2el}$ Elimination half-life (SC); $t_{1/2ab}$ Absorption half-life; Vc: Apparent volume of the central compartment; V_{dss}: Volume of distribution at steady state; Cl_B: Total body Clearance; CL_R: Renal Clearance; AUC_{0-8}: Area Under Curve from zero time to infinity; $AUMC_{0-\infty}$: Area Under the first Moment Curve from zero time to infinity; MRT: Mean Residence Time; MAT: Mean Absorption Time; C_{max}: Peak drug Concentration; t_{max}: Time to peak concentration; F: Systemic bioavailability following subcutaneous administration

Table 2: Mean±SE kinetic parameters of difloxacin metabolites in plasma following a single SC injection of 5 mg kg^{-1} B.W. in camels (n = 10)

Pharmacokinetic parameters	Units	M1	M2	M3
K_{el}	h^{-1}	0.072±0.003	0.09±0.003	0.14±0.013
$t_{1/2el}$	h	9.56±0.7	6.95±0.5	4.78±0.63
CL_R	L/h/kg	0.027±0.001	0.04±0.002	NA
$AUC_{0-\infty}$	μg hmL^{-1}	7.97±0.9	4.5±0.6	1.02±0.1
$AUMC_{0-\infty}$	μg h^2 mL^{-1}	82.4±9.3	51.5±4.4	8.2±0.95
MRT	h	12.4±0.9	11.4±1.4	7.94±0.8
C_{max}	μg mL^{-1}	0.39±0.02	0.35±0.03	0.18±0.014
t_{max}	h	2.0±0.1	6.0±0.4	2.0±0.15

K_{el}: First-order elimination rate constant $t_{1/2el}$: Elimination half-life; CL_R: Renal Clearance; $AUC_{0-\infty}$: Area Under Curve from zero time to infinity; $AUMC_{0-\infty}$: Area Under the first Moment Curve from zero time to infinity; MRT: Mean Residence Time; C_{max}: Peak drug concentration; t_{max}: Time to peak concentration

The mean dose percentages of difloxacin and its metabolites excreted in the urine and faeces of camels are depicted in Fig. 2 and 3, respectively. Unchanged difloxacin accounted for 5.9% of the dose fraction eliminated in urine. The major metabolite in urine was desmethyldifloxacin (sarafloxacin, M1) which accounted for 3.5% followed by glucuronide 3-oxosarafloxacin (M2) which accounted for elimination of 1.8% of the dose. Difloxacin, M1 and conjugated M2 metabolites were detected in urine for 6, 5 and 3 days, respectively and the maximal dose fraction was reached in the second day post drug administration (Fig. 2).

In faecal extract, difloxacin was by far the main analyte observed and accounted for the excretion of 69.5% of the total dose whereas the other metabolites, M1, M2, M3 and M4, accounted for the excretion of 7.2%, 3.0, 1.47 and 0.6% of the total dose, respectively. Difloxacin and its metabolites were eliminated in the unconjugated forms and the maximal dose fractions were

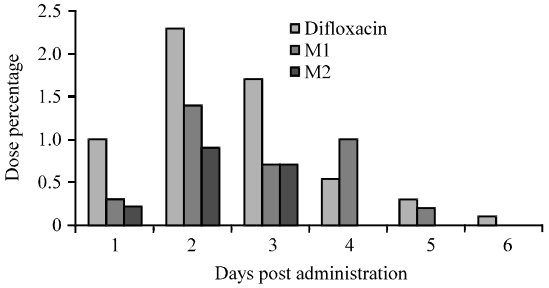

Fig. 2: Dose percentage of difloxacin and its metabolites (M1 and M2) recovered in urine on daily base following subcutaneous administration of difloxacin

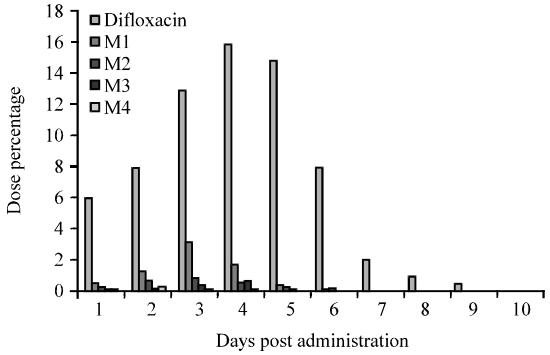

Fig. 3: Dose percentage of difloxacin and its metabolites recovered in feaces on daily base following subcutaneous administration of difloxacin

reported in the 2nd day for M4, the 3rd day for M1 and M2 and the 4th day for difloxacin and M3 post drug administration.

DISCUSSION

Difloxacin displayed a rapid distribution phase following intravenous administration with a $t_{1/2\alpha}$ of 0.46 h and a long elimination phase ($t_{1/2\beta}$) of 6.65 h. The elimination half-life reported in camels in the present study is close to that previously reported in pigs of 7.9 h (Inui *et al*., 1998) and higher than those reported in rabbits (3.25 h; El-Aty *et al*., 2005), calves (5.56 h; Ismail, 2007), camels (2.97 h; Abo-El-Sooud and Goudah, 2009) and horses (2.66 h; Fernandez-Varon *et al*., 2006). A much higher value has been reported in sheep (11.43 h; Marin *et al*., 2007b) and the $t_{1/2\beta}$ values in chickens ranged within 4.1-9.5 h (Inui *et al*., 1998; Ding *et al*., 2008; Anadon *et al*., 2011). The total body clearance of difloxacin in camels in this research (0.18 L/h/kg) was close to those reported in calves (0.13 L/h/kg, Ismail, 2007) and goats (0.13 L/h/kg, Atef *et al*., 2002). In contrast, a much higher clearance value of 58 L/h/kg was reported in rabbits (El-Aty *et al*., 2005). In contrast, the clearance value previously reported for difloxacin in camels (Abo-El-Sooud and Goudah, 2009) was double the value reported in the present study. This discrepancy could not be explained by the data, however, the higher sensitivity (0.005 μg mL^{-1}) of the analytical method used in this research might be a reason for the difference (Haddad *et al*., 1985).

Following subcutaneous administration, difloxacin was slowly absorbed with an absorption half-life ($t_{1/2ab}$) of 2.23 h and a MAT of 5.4 h. A similar finding has been reported in calves following the same route of administration (Ismail, 2007). A maximum concentration of 2.1 μg mL^{-1} was achieved after 4 h. These values are comparable to those reported in calves (2.18 μg mL^{-1} and 3.7 h; Ismail, 2007) but lower than values reported following intramuscular administration of the same dose in camels (Abo-El-Sooud and Goudah, 2009). Difloxacin has a longer elimination half-life of 7.52 h following subcutaneous administration compared to the value following intravenous route reported in the present study. The reported values in this study are close to those reported in calves (Ismail, 2007) and higher than the value reported in camels following intramuscular administration (Abo-El-Sooud and Goudah, 2009). The findings of this study indicate an extension of absorption phase within the elimination phase and suggest a flip flop phenomenon.

In most animal species, the metabolism of difloxacin is still not fully elucidated although, the piperazine ring seems to be the centre of metabolism. The ring may become oxidised, opened, demethylated or undergo sequential oxidation. Difloxacin is metabolised in camels via N-demethylation and N-oxidation. Researchers identified three metabolites in the plasma (M1, M2 and M3) they were detected in the plasma for 48, 36 and 24 h with peak plasma concentrations of 0.39, 0.35 and 0.18 μg mL^{-1} attained after 2, 6 and 2 h, respectively. Peak concentrations of the active metabolite (sarafloxacin, M1) was lower than the value reported in chickens (0.718 μg mL^{-1}) which was achieved 2.18 h after a 10 mg kg^{-1} oral dose (Anadon *et al*., 2011). Based on the AUC ratio of difloxacin and its plasma metabolites (M1, M2 and M3), sarafloxacin (demethylateddifloxacin, M1) was the major metabolite, constituting 30% of the total quantity of the parent drug in plasma. The oxidised metabolites 3-oxosarafloxacin (16%) (M2) and 3-oxodifloxacin (3.7%) (M3) comprised a small fraction of the parent drug. This finding is consistent with previous reports that showed N-demethylation as a common pathway for the metabolism of certain drugs in camels such as dexamethasone and etamiphylline (Al Katheeri *et al*., 2006; Elghazali *et al*., 2002). Similarly, difloxacin undergoes metabolism through the N-demethylation and oxidation pathways in pigs and humans but to variant degrees. In humans, difloxacin was largely metabolised to its demethylated analogues which constitute the major metabolites in agreement with the findings. In contrast to the findings, two different oxidised metabolites (4-N-oxide difloxacin, M5 and desethyleneaminosarafloxacin, M6) have been reported in humans (Granneman and Sennello, 1987) and pigs (Sukul *et al*., 2009) which indicate that difloxacin undergoes oxidation but at different sites of the piperazine ring (4-N position of piperazine ring) to form M5 or sequential oxidation of the same ring to form M6. In the present research, the main site for the oxidation of difloxacin metabolites in camels was at position 3 of the piperazine ring of the parent drug or its active metabolite, sarafloxacin (M1) with a minimal degree of sequential oxidation of the same ring to form desethylenesarafloxacin (M4). Camels have been shown to have the lowest lung and hepatic mixed function oxidase activity compared to other animals (Damanhouri and Tayeb, 1993; El Sheikh, 1997; Raza *et al*., 2004). However, it was reported that chickens do not metabolise difloxacin into its oxidised forms they instead eliminate it in the demethylated form after extensive biotransformation.

Anadon *et al*. (2011), the elimination half-life and MRT of difloxacin metabolites were 9.56 and 12.4 h for the active metabolite sarafloxacin (M1), indicating slower elimination of this metabolite relative to its parent drug. This finding could be of therapeutic relevance where it contributes to the antimicrobial activity of the parent drug. Conversely, other circulating metabolites (M2 and M3) detected in the plasma of camels in this study are not active and were eliminated faster than the active Metabolite sarafloxacin (M1) and the parent drug.

Urinary excretion of unchanged difloxacin and its metabolites accounted for 11.2% of the total administered dose. Difloxacin was eliminated in urine in the unchanged form (5.9%), demethylated form (3.5%) and as the glucuronide conjugate of 3-oxosarafloxacin (1.8%), whereas 3-oxodifloxacin and desethylene sarafloxacin metabolites were not detected in urine to any extent. Comparable to other species, the camel has been shown to under-express multiple forms of phase 2 metabolic enzymes such as glutathione transferase and UDP glucuronyl transferase (El Sheikh *et al*., 1986; Raza and Montague, 1993; Damanhouri and Tayeb, 1994). In dogs, urinary excretion of the conjugated form of demethylated derivatives accounted for the elimination of 20% of the total administered dose (Heinen, 2002). In humans, three metabolites have been identified in urine the major metabolite was sarafloxacin and urinary excretion of difloxacin along with its metabolites accounted for 33% of the dose with the remainder being excreted through a hepatobiliary process (Granneman and Sennello, 1987; Granneman *et al*., 1986).

The slower clearance of difloxacin and its metabolites in camels compared to other animal species and humans is consistent with the low values of renal clearance in such species (difloxacin, CL_R, 0.031 L/h/kg; sarafloxacin, CL_R, 0.027 L/h/kg; M2, CL_R, 0.04 L/h/kg) and could be related to the relatively low glomerular filtration rate and renal clearance in the camel (Wilson, 1984) as well as the small daily urine volume (Yagil, 1985). The reported value of creatinine clearance (CL_{cr}, 0.023 L/h/kg) is consistent with the slow rate of renal elimination for difloxacin, M1 and M2. The fractional clearances (CL_R/CL_{cr}) for difloxacin (1.34), M1 (1.17) and M2 (1.73) indicated that renal elimination occurred primarily through glomerular filtration with no renal reabsorption. In humans, difloxacin undergoes an appreciable amount of renal reabsorption. This discrepancy could be attributed to the pH-dependent renal tubular reabsorption that has been reported for fluoroquinolones in many animal species (Sorgel and Kinzig, 1993). Difloxacin and sarafloxacin are zwitterionic compounds with double pKa values and their isoelectric points are within the range of pH (5.2-8.5) therefore as urine pH value (camel urine pH, 9.5) increases, tubular reabsorption will decrease.

Difloxacin was largely eliminated unchanged in faeces and four metabolites were identified (M1, M2, M3 and M4), all were excreted with no glucuronic conjugation. A trace amount of desethylenesarafloxacin (M4) was detected in faeces the presence of this metabolite indicates that either difloxacin or sarafloxacin undergoes sequential oxidation of the piperazine moiety but to a limited extent (Fig. 3). Similarly this pattern of oxidation has been reported for difloxacin in human and pigs. Despite this similarity, the formed metabolite either undergoes conjugation before elimination in humans (Granneman *et al*., 1986) or further oxidation and excretion in the unconjugated form in pigs (Sukul *et al*., 2009).

CONCLUSION

The data in the present study quantitatively describes the difloxacin elimination pathway in camels. The results confirmed previous findings suggesting that difloxacin passes through the N-demethylation and oxidative metabolic pathways and is excreted in urine and faeces but with qualitative and quantitative differences for the metabolites detected and their final elimination patterns as the conjugated or free forms. These differences could be attributed to interspecies variation in liver microsomal enzymes and renal physiology and support many previous reports indicating clear differences in metabolism between many drugs in camels. However, the differences in the metabolic profiles of difloxacin in various species emphasise the importance of further investigation of fluoroquinolone metabolic profiles in different animal species.

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