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THE PHARMACOKINETICS AND PHARMACODYNAMICS OF BETAMETHASONE (PHOSPHATE AND DIPROPIONATE) IN CAMELS

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ABSTRACT

The pharmacokinetics and pharmacodynamics of a betamethasone (formulation dipropionate and sodium phosphate) were evaluated in 4 healthy camels after a single intramuscular dose of 35 µg/kg body weight. A sensitive, validated LC-MS/MS method for the quantification of plasma betamethasone and hydrocortisone was developed. Plasma betamethasone versus time concentration was best described by a two-compartment open model. The pharmacokinetic parameters median and range were as follows: terminal elimination half-life was 7.17 (6.93-7.58) h, Cmax was 15.9 (10.8-20.85) ng/ml, and Tmax was 0.5 (0.25-0.75) h. The estimated IC50 for hydrocortisone (mean ± SD) was 0.09 ± 0.08 ng/ml. Based on the analytical method and plasma terminal elimination half-life, a 4-day withdrawal period of the betamethasone formulation is advised prior to racing.

Key words: Betamethasone, camels, pharmacokinetics, pharmacodynamics

Betamethasone (BMT) is a synthetic corticosteroid and is a stereoisomer of dexamethasone (DEX). Synthetic corticosteroids depress formation, release and activity of endogenous mediators of inflammation, including prostaglandins, kinins, histamine, liposomal enzymes, and the complement system (Barnes, 1998). Synthetic corticosteroids are potent drugs widely used in veterinary practice. These are prohibited by most racing authorities and the Fédération Equestre Internationale. These can be administered either by a systemic or topical route (Popot *et al,* 2003). These are usually used for the treatment of joint disorders, such as osteoarthritis and muscle injuries (Sokolowski, 1982; French *et al,* 2000; Murray *et al,* 2002). Treatment during the early stages of inflammation is critical for early rehabilitation and return to racing activity following race injuries (Drezner, 2003; Toumi and Best, 2003). The use of corticosteroids administered by intra-articular injection has become commonplace recently. Direct injection into the site of action allows the use of low doses of corticosteroids, which results in relatively low urine and plasma concentrations compared with systemic administrations (Popot *et al,* 2003). There are reports describing the detection times of BMT in horses after intra-articular administration (Popot *et al,* 2003; Knych *et al,* 2017; Menendez *et al,* 2016). However, the use of corticosteroids administered by intra-articular injection in camels is not a common practice. We are not aware of any report describing intra-articular administration of a corticosteroid in camels. An injectable formulation of BMT for intramuscular (i.m.) administration is available on the UAE market and is frequently used by veterinarians on race camels. Our laboratory receives plasma samples following camel races. The authorities and camel owners both request information on the BMT withdrawal period following a therapeutic dose of BMT.

The goal of the current investigation was, therefore, to create a sensitive and validated LC-MS/MS method for the quantification of BMT in camel plasma and use it to describe the pharmacokinetics (PK) of BMT in camels following a single i.m. injection. Using plasma hydrocortisone (HCOR) suppression, we also wanted to assess the pharmacodynamics (PD) of BMT in camels. Another goal of the study was to provide advice to the camel racing authority regarding the timing of BMT's withdrawal based on the analytical method and PK values.

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Materials and Methods

BMT and triamcinolone acetonide (TRA), used as internal standard, were purchased from Sigma Aldrich with 98% purity (St. Louis, MO, USA). All solvents and chemicals were of analytical grade or HPLC grade and were obtained from Sigma Aldrich (St. Louis, MO, USA). Oasis HLB cartridges, 3 CC, 60 mg, were purchased from Gulf Scientific Corporation, Dubai, U.A.E.

Animals

Four clinically healthy male camels (*Camelus dromedarius*), 5-7 years old and ranging in body weight from 350 to 500 kg, were used in this study. The camels were out of training and were kept in open pens. They were fed good quality hay and Lucerne (alfalfa) once daily, with water allowed *ad libitum*. The Ministry of Agriculture, Veterinary Department, UAE, approved the study protocol.

Treatment

Combine BMT (Diprospan injection contains betamethasone dipropionate equivalent to 5 mg betamethasone and betamethasone sodium phosphate equivalent to 2 mg betamethasone in a sterile buffered and preserved vehicle, Schering-PlowLabo NV, Belgium) was administered as an i.m. injection at a dose of 35 µg/kg body weight. Venous blood samples were drawn from the jugular vein in heparinised blood tubes at 0, 5, 10, 15, 30, 45 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 12, 24, 48. 72, 96 and 120 h. Blood samples were immediately placed on ice; plasma was separated by centrifugation at 4500 x g at room temperature for 10 min. The harvested plasma was frozen at -20°C until analysis for BMT and HCOR.

Extraction of plasma for BMT and HCOR

Plasma samples (1 ml) were pipetted in duplicates into glass test tubes, and 3 ml of phosphate buffer pH 6.0 was added. TRA (5 ng) was added as an internal standard. Samples were vortexed and then centrifuged at 3000 rpm for 10 min, and the supernatant was decanted for solid-phase extraction. Oasis HLB cartridges were conditioned with 2 ml of methanol and 2 ml of water. Then samples were loaded onto the cartridges. Cartridges were washed with 2 ml of 5% (v/v) methanol in water and dried for 5 min at 20 mm Hg. The analytes were eluted with 2 ml of methanol. The eluent was evaporated under a nitrogen stream at 40°C, reconstituted in 100 µl of mobile phase, and analysed by a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method.

LC-MS/MS conditions

LC–MS/MS analysis for BMT and TRA was performed using a 5500QTrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent 1200 series HPLC system consisting of a binary gradient pump (Agilent Technologies, Palo Alto, CA, USA) and an autosampler. A PhenomenexKintex C18 column (2.6 µm × 2.1 mm × 50 mm) linked to a Phenomenex pre-column filter (KrudKatcher ULTRA, 2.0µm Depth Filter x 0.004 in ID) operating in gradient mode at 35°C was used. The mobile phase was 0.1% formic acid in water (solvent A) and methanol with 0.1% formic acid (solvent B). A linear gradient was used at 0.3 ml/min with 40% solvent B at the start (t = 0 min), increasing to 90% solvent B at t = 3 min. The gradient was then returned to 40% solvent B at t = 4.0 min and stabilised until t = 7.5 min before starting the next injection. The source was operated in positive ESI mode at 500°C with the nebuliser gas and heater gas set to 45 and 55 psi, respectively. Ion spray voltage was set to 5500V, curtain gas was set to 22 psi, and collision gas was set to medium. The resolution for the selection of the precursor ions in Q1 and the product ions in Q3 was set to unit mass. Detection of the analyte and internal standard was performed in the MRM mode with a single time segment and the scan time was 50 ms per transition. To selectively monitor precursor ions and corresponding product ions, the mass transitions m/z 393→355 and m/z 393→147 for BMT; m/z 435.3→415.2 and m/z 435.3→397 for TRA were used. The SRM transitions, declustering potentials, collision energies, and the collision cell exit potentials for BMT and TRA were optimised by infusion of reference material. Data processing was performed using Analyst software (Version 1.5.1). Plasma HCOR was analysed as previously reported (Wasfi *et al,* 2018).

LC-MS/MS method validation

The method was validated for selectivity, linearity, accuracy, precision, recovery, matrix effect, limit of detection (LOD), limit of quantification (LOQ), and stability. Pooled drug-free camel plasma from 15 camels was used for BMT validation. Validation runs were conducted on four consecutive days. Each validation run consisted of one set of calibration standards and two replicates of QC samples (n = 24 total values in 4 days). For intra-assay coefficients of variation, 10 replicas were used on the same day.

The selectivity of the method was evaluated by analysing 15 blank camel plasma samples. Calibration curves were constructed by analysing

spiked calibration samples on four separate days. Peak area ratios of BMT and TRA were plotted against BMT concentrations. Standard curves were fitted to the equations by linear regression. The calibration levels for BMT were: 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 15.0, 30.0 ng/ml, and the QC levels were 0.1, 1.0, and 10 ng/ml. The lower limit of quantification (LOQ) was defined as the lowest concentration on the calibration curve that can be quantified reliably with acceptable accuracy (80–120%) and precision (± 20%). The LOD was determined as the lowest concentration detectable with a signal-to-noise ratio (S/N) > 3.

Accuracy and precision were assessed by the determination of QC samples in four validation days. The precision was expressed by the coefficient of variation (CV). The accuracy was expressed as a percentage of the nominal concentration (RE%).The recovery of BMT (A/B × 100%) was evaluated by comparing peak area of QC samples (A) with those of reference QC solutions reconstituted in blank plasma after extraction (B, n = 6). Recovery of the internal standard was determined in the same way.

To evaluate the matrix effect (B/C × 100%), the extract of blank camel plasma samples were spiked after extraction with QC levels (B). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations dissolved in the mobile phase (C), and this peak area ratio was defined as the matrix effect. The matrix effect of the internal standard was evaluated at the working concentration (5 ng/mL) in the same manner. Fifteen pooled camel plasma samples were used for matrix effects experiments. Carry-over was assessed following injection of a blank plasma sample immediately after three repeats of the upper limit of quantification (ULOQ) and the response was checked. Carryover giving no greater than 5% of the response of the ULOQ in the blank samples was accepted. The stability of BMT and TRA in plasma was examined for various storage or handling conditions. This included short-term tests and assessments were performed with the three QC levels. Short-term stability testing included one freeze–thaw cycle following storage at −20°C for 14 days and concentrations were quantified before and after this process. Stability was also assessed by reinjection of processed QC samples after 24 and 48h kept in the auto-sampler (10°C).

Pharmacokinetic and pharmacodynamics analysis

PK analysis of plasma BMT concentrations for each animal was performed using the least-square nonlinear regression analysis programme (WinNonLin Standard edition, version 4.0.1, Pharsight, Sunnyvale, CA, USA). One and two-compartment models were tested for the best fit to the i.m. administration data. The best fit was based on Akaike (1976) and Schwarz (1978) criteria, analysis of residual plots, and the correlation matrix. The PK-PD surrogate, the IC50 for the reduction of plasma HCOR concentration, was calculated. Calculations were performed on individual data using least-square nonlinear regression analysis (WinNonLin Standard edition, version 1.5, USA). An indirect response model describing the PD effect of the drug with the mechanism producing the effect was used (Dayneka *et al,* 1993; Al Katheeri *et al,* 2004). Good modelling could be obtained if corticosteroids were assumed to modify the influx of HCOR by decreasing Kin, causing a reduction of HCOR concentration in blood (Al Katheeri *et al,* 2004; Wasfi *et al,* 2018). As corticosteroids exert their effects via receptors, the Emax model was reported as appropriate (Möllmann *et al,* 1998) which is described by the equation:

$$\frac{dC_{BMT}}{dt} = K_{in} \quad \frac{(1- C_{BMT})}{IC_{50} + C_{BMT}} \quad - K_{out} \cdot C_{BMT}$$

where C_{BMT} is the plasma free BMT concentration, and IC_{50} is the concentration of free BMT that will result in 50% of maximum inhibition. IC_{50} is unique to each corticosteroid and reflects its potency.

Statistical calculations

PK parameters are reported as medians and ranges and PD parameters are reported as mean ± SD.

Results

LC-MS/MS method validation

The LOQ and LOD for BMT were 0.05 ng/ml and 0.025 ng/mL, respectively. The intra-assay coefficient of variation (n = 10) for BMT was less than 9% for all QC samples. The inter-assay coefficient of variation (n = 10) for BMT was less than 15% for all QC samples. The accuracy (RE) was less than 12%. Extraction recovery for BMT and TRA ranged from 75-88%. Specificity was confirmed by the absence of significant chromatographic peaks interfering with BMT and TRA in fifteen different camel plasmas. The BMT calibration curves were linear (r^2 = > 0.995) and the slopes of the standard curves varied by 13% over four different runs. The matrix effect was insignificant for both BMT and TRA. Stability for BMT was acceptable (± 11% change from initial concentration) at 10°C for 24 and 72 h (in the autosampler) and after

one freeze–thaw cycle following storage at -20°C for 14 days.

PK-PD Analysis

BMT was well tolerated in camels, and no side effects were observed during the experimental period. Pharmacokinetic parameter estimates of BMT (median and range) following i.m. administration are shown in Table 1. Fig 1 depicts the BMT plasma concentrations–time profile. The plasma drug profiles were characterised by a fast distribution phase and a terminal elimination half-life of 7.17 (6.93-7.58) h. The plasma HCOR concentration was significantly lower than basal levels at time 2.0 h and remained significantly depressed until day 4 (Fig 2). The estimated IC50 of BMT for COR (mean ± SD) was 0.09 ± 0.08 ng/ml.

Table 1. Pharmacokinetic parameters of betamethasone following intramuscular administration to 4 healthy camels at a dose of 35 µg/kg body weight. Data are expressed as median and ranges.

Variable	value
$T_{½\alpha}$ (hour)	1.00 (0.47-1.99)
$T_{½\beta}$ (hour)	7.17 (6.93-7.58)
$AUC_{0-\infty}$ (ng $hour^{-1}$ per mL)	67.91 (50.23-78.42)
Tmax (h)	0.5 (0.25-0.75)
Cmax (ng/ml)	15.92 (10.8-20.85)
T ½ absorption (hour)	0.05 (0.02-0.20)

$T_{½\alpha}$ = half–life of distribution phase; $T_{½\beta}$ = half–life of elimination phase; AUC= area under the curve to infinity; Tmax= time of maximum concentration; Cmax= maximum concentration; $T_{½}$ absorption = absorption half-life.

Discussion

The validated method developed in this report proved to be sensitive and robust with a LOQ and LOD of 0.05 ng/mL and 0.025 ng/mL, respectively. The method will allow the control of low doses of BMT in camel races, which results in relatively low plasma concentrations. In contrast, BMT phosphate ester was not detected in camel plasma in the current study. However, BMT phosphate ester is shown in humans to have an exceedingly short half-life of 4.7 min (Petersen *et al,* 1983) and that is rapidly and completely converted to BMT *in vivo* so that BMT phosphate ester were rarely determined for the quantification in human plasma *in vitro* and *in vivo* (Salem *et al,* 2012; Ahmed and Atia, 2013). Additionally, neither the BMT dipropionate ester nor its monopropionate esters were found in camel plasma, which may also indicate that they have a very short half-life or that they were degraded by esterases at the injection site or in the plasma. However, Chen *et al* (2016) used a similar formulation in humans with an i.m. dose that was 2.6 times larger than the dose we used in camels. These authors measured BMT, betamethasone dipropionate, and betamethasone 17 propionate in plasma, the latter at low concentration (Cmax, 2.2 ng/ml). Betamethasone 21 propionate, however, could not be quantified by the authors.

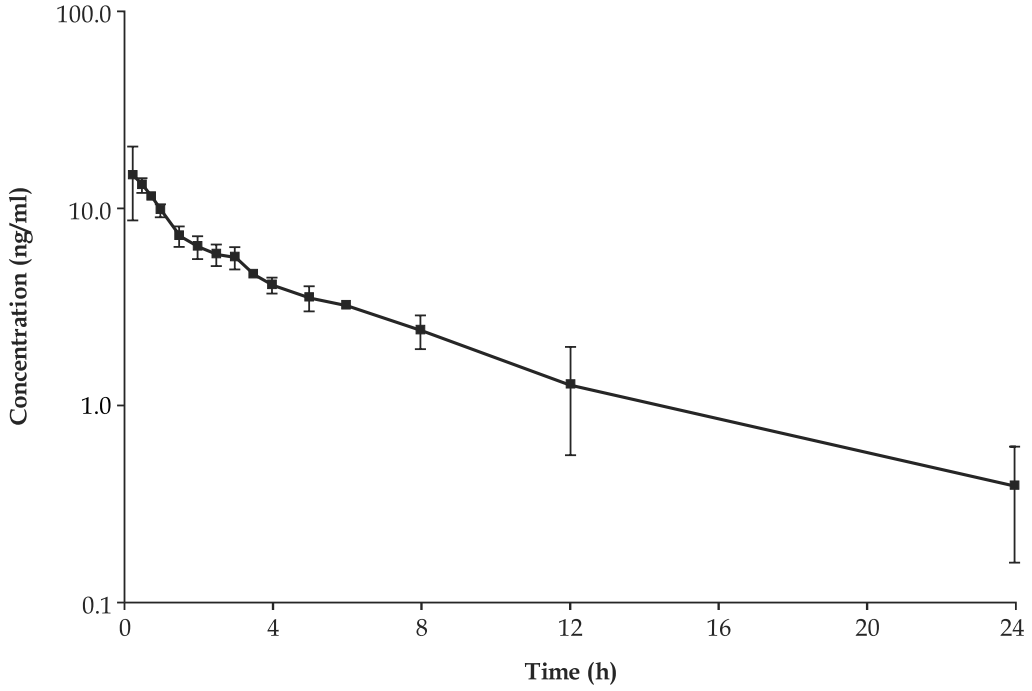

Fig 1. Betamethasone plasma concentrations -time profile of four camels after an i.m. dose of 35 µg/kg BW. Values are presented as means ± standard deviations of the mean.

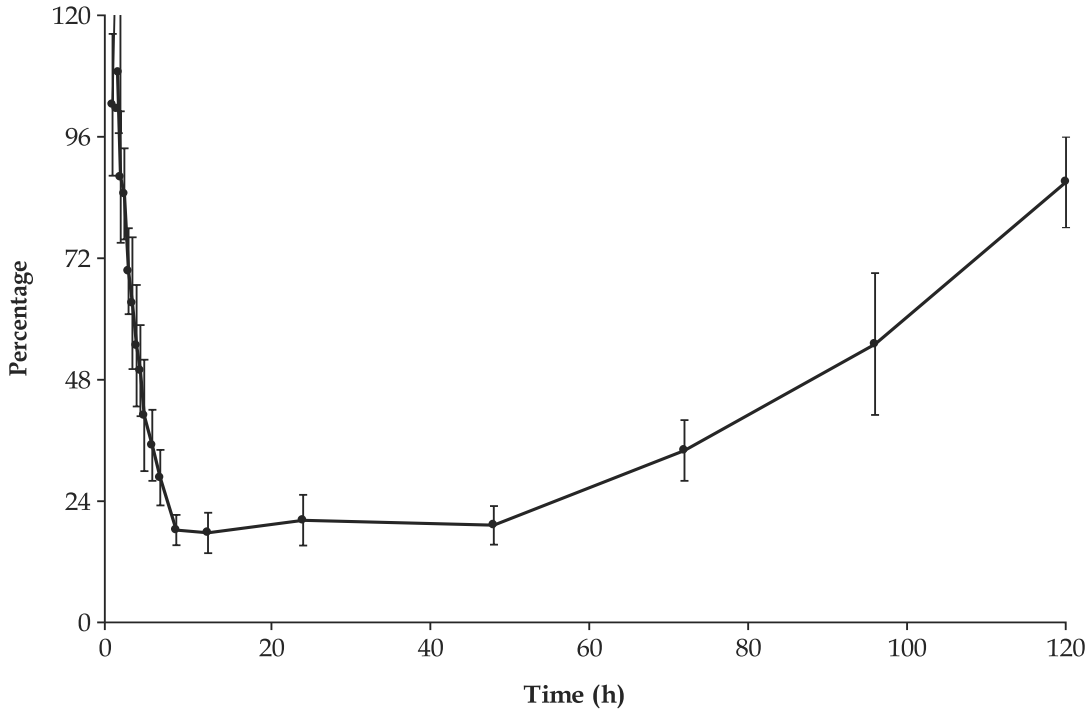

Fig 2. Hydrocortisone plasma concentrations -time profile of four camels after an i.m. dose of betamethasone of 35 µg / kg BW. Values are presented as means ± standard deviations of the mean. The plasma hydrocortisone concentration was significantly lower than basal levels at time 2.0 h and remained significantly depressed until 96 h.

The research on PK/PD of BMT in camels is not traceable in available literature hence our results could not be compared with other studies. However, DXM, a stereoisomer of BMT, was reported to have a half-life of 7-8 h in camels after an i.v. of 50 µg/kg body weight (Al Katheeri *et al,* 2004), which is similar to the half-life of BMT in camels reported in the current study. There are two reports which describe the PK of BMT in horses after intra-articular administration (Knych *et al,* 2017; Menendez *et al,* 2016). In one study (Knych *et al,* 2017), the authors administered a single intra-articular administration of 9 mg of betamethasone sodium phosphate and betamethasone acetate injectable suspension in the right antebrachiocarpal joint of exercised thoroughbred horses. The reported terminal elimination half-life in plasma was 7.48 ± 0.39 h (mean ± SE), which was close to the plasma terminal elimination half-life in camels reported in this study (7.17, 6.93-7.58 h, median and range). According to those authors, BMT could be found in plasma at a maximum concentration of 3.97 ng/ml, compared to the value of 15.92 (10.8-20.85) ng/ml reported in this study (median and range). In the other study (Menendez *et al,* 2016), the authors administered a similar preparation of the BMT formulation used by the authors in the first study. However, the dose used was 30 mg divided into two different joints, which resulted in a maximum plasma concentration of BMT of 26.64 ± 4.79 ng/ml (mean ± SE) and a slightly longer half-life of 9.22 ± 0.86 h. Samtani *et al* (2005) studied the PK of two formulations of BMT in sheep. Following i.m. administration of BMT phosphate (0.25 mg/kg) and betamethasone phosphate/acetate (0.5 mg/kg), they reported a plasma terminal half-life of 4h and 14 h, respectively. The formulation used in the current study produced a peak concentration of about 10.8–20.85 ng/ml at 0.25-0.75 h, which reflects the fast input from the prodrug (Ka half-life of 0.05 h).

It has been reported that administering BMT preparations results in a significant decrease in endogenous HCOR concentrations (Petersen *et al,*1983; Popot *et al,* 2003). Exogenous synthetic corticosteroids have been shown to suppress HCOR in horses (Bayer *et al,* 2001) and camels (Al Katheeri *et al,* 2004; Wasfi *et al,* 2018). In the current study, the IC_{50} of BMT for the suppression of endogenous HCOR was 0.09 ng/ml, indicating that it is a very potent corticosteroid when compared to values reported by us previously for dexamethasone (3.74 ng/ml) (Al Katheeri *et al,* 2004) and flumethasone (4.52 ng/ml) in camels (Wasfi *et al,* 2018).

The maximum suppression effect occurred at around 6 h and persisted up to day 4 after BMT

administration. A similar finding was reported in camels following i.v. DXM administration (Al Katheeri *et al,* 2004). Corticosteroids have both rapid and slower PD effects. It is possible that plasma HCOR suppression would persist when the plasma BMT concentration is below detectable levels and below its IC_{50}. In fact, we frequently observe extremely low plasma HCOR levels in samples from races where there are no measurable BMT levels in the blood or any other corticosteroid.

The Asian Racing Federation and the International Federation of Horse racing Authorities describe a screening limit for BMT in horse urine of 0.2 ng /ml but no plasma values are described (Wong and Wan, 2014). The Camel Racing Association in Saudi Arabia adopts a zero medication rule in plasma samples taken post-racing. The LOQ reported in this study was 50 pg/ml and the average plasma BMT concentration at time 24 h was 390 pg/ml. Accordingly, based on the analytical results and plasma terminal elimination half-life, it will take 2-3 additional half-lives, or 2 days after BMT treatment, for the plasma BMT concentration to fall below the LOQ. In fact, BMT could be quantified in one camel at day 2 but not in all camels at days 3, 4, or 5 after injection. However, a 4-day withdrawal period is advised before racing due to animal variability and as a safety measure. It should be noted, however, that this withdrawal period is based on a limited number of animals and further work is required on various doses and routes of BMT administration in camels.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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