Synthesis and detection of toltrazuril sulfone and its pharmacokinetics in horses following administration in dimethylsulfoxide

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Triazine-based antiprotozoal agents are known for their lipophylic characteristics and may therefore be expected to be well absorbed following oral administration. However, although an increase in lipid solubility generally increases the absorption of chemicals, extremely lipid-soluble chemicals may dissolve poorly in gastrointestinal (GI) fluids, and their corresponding absorption and bioavailability would be low. Also, if the compound is administered in solid form and is relatively insoluble in GI fluids, it is likely to have limited contact with the GI mucosa, and therefore, its rate of absorption will be low. Based on the above considerations, we sought a solvent with low or no toxicity that would maintain triazine agents in solution. As the oral route is most preferred for daily drug therapy, such a solvent would allow an increased rate of absorption following oral administration. In present study, it was demonstrated that dimethylsulfoxide (DMSO) increased the oral bioavailability of toltrazuril sulfone (Ponazuril) threefold, relative to oral administrations of toltrazuril sulfone suspended in water. The cross-over study of toltrazuril sulfone formulated in DMSO indicated that the absolute oral bioavailability of toltrazuril sulfone in DMSO is 71%. The high bioavailability of the DMSO-preparation suggests that its daily oral administration will routinely yield effective plasma and cerebral spinal fluid (CSF) concentrations in all horses treated. Also, this improved formulation would allow clinicians to administer loading doses of toltrazuril sulfone in acute cases of Equine Protozoal Myeloencephalitis. Another option would involve administration of toltrazuril sulfone in DMSO mixed with feed (1.23 kg daily dose) meeting the US Food and Drug Administration (FDA) recommendations for the levels of DMSO permissible in pharmaceutical preparations.

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INTRODUCTION

Toltrazuril, (1-(3-methyl-4′-trifluoromethyl-thiophenoxy)-phenyl)-3-methyl-1,3,5-triazine-2,4,6 (1H, 3H, 5H) trione, toltrazuril sulfone also known as ponazuril, (1,3,5-Triazine-2,4,6(1H, 3H, 5H)-trione,1-methyl-3-(3-methyl-4-((4-trifluoromethyl)sulfonyl) phenoxy) phenyl), and diclazuril, ((+)-4-chlorophenyl-[2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile, are triazine-based antiprotozoal agents with highly specific actions against apicomplexan group of organisms which are undergoing intensive investigation. Diclazuril con-

tains a 1,2,4-triazine ring, whereas the toltrazuril compounds contain 1,3,5-triazine rings. Besides the common triazine rings, members of this group of drugs, including, e.g., clazuril and letrazuril, generally have in common the benzeneacetonitrile group (benzene ring with an acetonitrile group attaching it to the central ring structure) which is absent in toltrazuril and toltrazuril sulfone (Fleeger, 1997).

Toltrazuril sulfone (Ponazuril) and toltrazuril sulfoxide are the major metabolites of toltrazuril following oral administration as Baycox® at 10 mg/kg in horses (Furr & Kennedy, 2000). Both of these metabolites also have the ability to pass the blood-brain

barrier. Mean steady-state concentrations of toltrazuril sulfone in cerebrospinal fluid (CSF) after 10 days of treatment were 0.09, 0.157, and 0.223 µg/mL following daily oral doses of toltrazuril at 2.5, 5, and 7.5 mg/kg, respectively in horses. Mean steady-state concentrations of toltrazuril sulfoxide in CSF after 10 days of treatment were 0.073, 0.110, and 0.147 µg/mL following daily oral administrations of toltrazuril at 2.5, 5 and 7.5 mg/kg, respectively in horses. There were no signs of toxicity of toltrazuril in this study following 2 months or longer oral administration of toltrazuril (Furr & Kennedy, 2000). The efficacy of toltrazuril sulfone in inhibiting merozoite production of *Sarcocystis neurona* in cell cultures was recently reported (Lindsay *et al.*, 2000). This study showed complete inhibition of merozoite production in cell cultures of *S. neurona* treated with 0.1–1 µg/mL toltrazuril sulfone.

Toltrazuril sulfone (Ponazuril) recently has been evaluated for the treatment of equine protozoal myeloencephalitis (EPM) in a study sponsored by Bayer Animal Health (Pittsburgh, PA, USA) (Furr *et al.*, 2001). Approximately 100 horses that had not been previously treated for EPM were treated for 28 days with a paste formulation of ponazuril either at 5 or 10 mg/kg. Seventy-one percent of horses improved with no signs of toxicity. CSF concentration of toltrazuril sulfone was found to be 150–180 ng/mL from days 7–28, falling to 20 ng/mL 7 days after treatment following oral administration of ponazuril at 5 mg/kg/day (Furr *et al.*, 2001). Recently, toltrazuril sulfone (Marquis®) was approved by Food and Drug Administration (FDA) to be used for the treatment of EPM. Toltrazuril sulfone is manufactured by Bayer Corporation, Agriculture Division, Shawnee Mission, KS, USA.

Triazine-based antiprotozoal agents are known for their lipophylic characteristics and they may be expected to be well absorbed following oral administration. Additionally, the absorption of chemicals from the gastrointestinal (GI) tract depends on physiochemical properties of compounds, such as lipid solubility, and dissociation rate (Houston *et al.*, 1974). Although it is often generalized that an increase in lipid solubility increases the absorption of chemicals, extremely lipid-soluble chemicals may have poor oral bioavailability, both because highly lipophilic molecules (e.g., if Log $P > 7$) can get stuck in the lipid portion of the plasma membrane (Martinez & Amidon, 2002), and because highly lipophilic compounds are more difficult to dissolve in GI fluids (Houston *et al.*, 1974). If the compound administered is a solid and is relatively insoluble in GI fluids, it will have limited contact with the GI mucosa, and therefore, its rate of absorption will be low (Gorringe & Sproston, 1964; Bates & Gibaldi, 1970).

Based on the above considerations we sought a solvent that would maintain triazine agents in solution, thus allowing an increased rate of absorption following oral administration, as the oral route is most preferred for chronic drug therapy. Bioavailability is an important parameter in clinical trials because the majority of a drug's therapeutic and toxic effects are proportional to both dose and bioavailability. Additionally, poor oral bioavailability results in more variable and poorly controlled plasma drug concentrations and therefore therapeutic effects. When bioavailability is low, inter- and intra-subject variability in bioavailability are magnified and incomplete bioavailability becomes a major concern. Another problem associated with the poor and variable bioavailability is that it is generally difficult to predict and control plasma drug concentration of any given dose. It was therefore, important for us to try to maximize oral bioavailability of triazine-based agents with the goal of maximizing our ability to control plasma drug concentrations and therefore the clinical efficacy of these agents.

As dimethylsulfoxide (DMSO) is relatively safe to use and the parenteral administration of DMSO enhances the ability of high molecular-weight substances to be absorbed, we chose DMSO as the solvent to keep our triazine-based agents in solution. DMSO, a by-product of the paper-making industry, is a polar solvent with a high affinity for water molecules. It readily penetrates skin, mucous membranes, cell and organelle membranes without causing irreversible membrane damage (David, 1972). There are many reports in the literature indicating an increase in the absorption characteristics of various compounds, mainly skin penetration, in the presence of DMSO (Banthorpe & Lamont, 1967; Braude & Monroe, 1967; Potts *et al.*, 1969; Rubin, 1975; Yellowlees *et al.*, 1980; Jimenez & Wilkens, 1982; Brayton, 1986; Elzinga *et al.*, 1989; Gyrd-Hansen *et al.*, 1993; Ehninger *et al.*, 1995; Winker *et al.*, 1995; Schuler *et al.*, 1998; Watanabe *et al.*, 2000). Therefore, it is widely accepted that DMSO enhances the penetration of nonpolar compounds through the biological membrane, but the extent of this penetration depends on both the chemical's properties and route of administration. Additionally, it has been shown that DMSO does not potentiate absorption and tissue distributions of various compounds (Rubinstein & Lev-El, 1980; Egorin *et al.*, 1982). In one of these studies (Egorin *et al.*, 1982), it was shown that the pharmacokinetic parameters of cyclophosphamide are not altered when administered either orally or i.v. with and without DMSO. DMSO did not alter peak plasma and CSF concentrations or plasma and CSF half-lives of cyclophosphamide in 10 human subjects.

Therefore, the enhancement of absorption and distribution for some compounds could be significant ranging from 1- to 100-fold, while for others the effects might be negligible. The primary objectives of the present study were to determine the pharmacokinetic parameters of toltrazuril sulfone in DMSO and also to determine whether or not DMSO enhances the oral bioavailability of a triazine-based agent toltrazuril sulfone (Ponazuril) in a clinically significant manner.

MATERIALS AND METHODS

Horses and sample collection

Horses were provided by Saxony farm (Versailles, KY, USA) and were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. Horses were not feed for at least 1 h after oral administration of compound. Horses were kept in a 20-acre field until they were placed in box stalls where they were provided water and hay *ad libitum*. Animals used in these

experiments were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care Use Committee, which also approved the experimental protocol.

In this study, seven groups of horses each received one of the following formulations of toltrazuril sulfone: (i) 2.2 mg/kg toltrazuril sulfone orally and 1 mg/kg toltrazuril sulfone intravenously (cross-over study) formulated in DMSO ($n = 4$), (ii) 2.2 mg/kg of toltrazuril sulfone formulated in DMSO administered daily orally for 28 days ($n = 2$), (iii) 2.2 mg/kg of toltrazuril sulfone formulated in DMSO administered daily intravenously for 28 days ($n = 2$), (iv) 15 mg/kg of toltrazuril sulfone as a loading dose, 2.2 mg/kg of toltrazuril sulfone as a maintenance dose formulated in DMSO administered orally daily for 28 days ($n = 2$), (v) 3.65 mg/kg of toltrazuril sulfone as a loading dose and 0.55 mg/kg of toltrazuril sulfone as a maintenance dose formulated in DMSO administered orally daily for 28 days ($n = 3$), (vi) 2.2 mg/kg of toltrazuril sulfone suspended in water administered by nasogastric intubation ($n = 2$), and (vii) 2.2 mg/kg of toltrazuril sulfone formulated in DMSO as a feed additive formulations administered orally ($n = 4$).

We used a randomized cross-over study with a 2×2 latin square design in order to determine absolute bioavailability and pharmacokinetic characteristics of toltrazuril sulfone formulated in DMSO in the horse. Four mature Thoroughbred mares weighing 453–526 kg were used for the toltrazuril sulfone in DMSO study. Toltrazuril sulfone (150 mg/mL in DMSO) was administered either orally or intravenously to horses at a single dose of 2.2 or 1 mg/kg, respectively. Horses were allowed a 3-weeks interval between subsequent dosing regimens after the last sample collection. Blood samples were obtained for analyses at 0, 0.16, 0.33, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 h into heparinized tubes that were centrifuged at 4 °C 224 g for 15 min, and the plasma stored at −20 °C until assayed.

In a second and a third experiment, toltrazuril sulfone (2.2 mg/kg body weight in DMSO) was administered daily for 28 days to two horses either intravenously or orally. Blood samples were collected daily as described above. A licensed veterinarian collected CSF samples at the lumbosacral space at days 0, 7, 14, 21, and 28. The cerebrospinal fluid was retained for analysis if there was no visible evidence of blood contamination and stored in test tubes at −20 °C until analyzed.

In a fourth experiment, toltrazuril sulfone was administered to two horses orally for 28 days with the following dosing schedule; first day 15 mg/kg in DMSO (loading dose), and 2.2 mg/kg in DMSO (maintenance dose) for the remaining 27 days. Blood samples were collected every day as described above. CSF samples were collected at days 0, 1, 7, 14, 21, and 28 days as described above. In a fifth series of experiments, toltrazuril sulfone was administered to three horses weighing 453–589 kg orally for 28 days with the following dosing schedule; first day 3.65 mg/kg in DMSO (loading dose), and 0.55 mg/kg in DMSO (maintenance dose) for the remaining 27 days. Blood samples were collected every day as described above and CSF samples were collected on various days. In a sixth series of experiments, toltrazuril sulfone was administered to two mature horses weighing 500–545 kg at a single oral dose of 2.2 mg/kg of toltrazuril sulfone suspended in 0.5 L water, by nasogastric intubation. Blood samples were collected at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 h as described above. In a seventh series of experiments, toltrazuril sulfone was administered to four horses weighing 559–591 kg at a single oral dose of 2.2 mg/kg in DMSO combined with 0.5 oz. beet pulp added to 1 lb. sweet feed. Blood samples were collected at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 h as described above.

Toltrazuril sulfone analysis

Preparation of toltrazuril sulfone from Baycox®
Recovering of toltrazuril from Baycox®. The Baycox® suspension was placed in centrifuge tubes and spun for 0.5 h at 894 g. The upper solution was decanted, to the obtained precipitate, water was added and the resulting suspension spun for 5 min at 894 g. This rinsing was repeated twice. The white precipitate of toltrazuril was dried for 12 h at 70 °C and 8 h at 105 °C. From 24 bottles of Baycox® (250 mL, 50 mg/mL) 290 g of crude toltrazuril was obtained. This material was placed into 1.5 L of acetone, stirred at reflux for 3 h and filtered through celite to remove the gray insoluble admixture. The acetone was then evaporated, and the obtained pure toltrazuril dried for 8 h at 70 °C to yield 270 g of toltrazuril.

Oxidation of toltrazuril to toltrazuril sulfone. Toltrazuril (255 g) was dissolved in hot acetic acid (1.2 L) and hydrogen peroxide (100 mL, 30%) was added. The reaction mixture was stirred for 4 days at 100 °C and every 24 h 50 mL (150 mL altogether) of hydrogen peroxide (30%) was added. After 96 h the reaction mixture was poured into 8 L ice-water and filtered. The obtained precipitate was washed with water, dried at 105 °C for 8 h and ground to obtain 252 g of crude toltrazuril sulfone, which was purified from minor admixtures upon adding of ethyl alcohol (1 L), stirring at reflux for 2 h and filtration. The white precipitate was washed with ethyl alcohol (200 mL) and dried for 5 h at 80 °C to obtain 249 g of 99.7% pure toltrazuril sulfone (Fig. 1) determined by thin layer chromatography; m.p. (melting point) 242–244 °C and Gas Chromatography/Mass Spectrometry.

Toltrazuril sulfone measurements by high performance liquid chromatography
Sample preparation. A standard solution of 1 mg toltrazuril sulfone was prepared in 1 mL high performance liquid chromatography (HPLC) grade methanol. Standards were prepared by the addition of a specified amount of toltrazuril sulfone in 60% solvent B/40% solvent A (see instrumentation) to blank plasma samples, 1 mL each, over a range from 25 to 10 000 ng/mL. Janssen compound R 62646, a structural analog of diclazuril, was used as the internal standard. The internal standard was prepared in 1 mL methanol (1 mg/mL) and diluted 1–10 in 60% solvent B 40% solvent A to yield 100 ng/μL standard solution. To each sample, 20 μL of 100 ng/μL internal standard was added. Then, 2 mL of 0.1 M
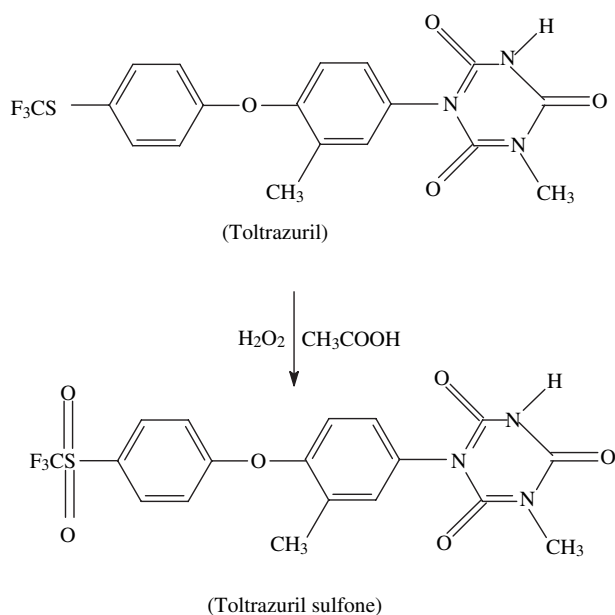
Fig. 1. Oxidation of toltrazuril to the major metabolite toltrazuril sulfone.

potassium phosphate buffer (pH 6.0) was added to each sample and the pH was adjusted to 6.0 as necessary.

Extraction method. Varian 'Bond Elut' columns were placed into an SPS24 VacElut vacuum chamber and treated sequentially with 2 mL of HPLC grade methanol and 2 mL of 0.1 M potassium phosphate buffer (pH 6.0). The vacuum was turned off as soon as the buffer reached the top of the sorbent bed to prevent column drying. The specimen was drawn slowly through the column taking at least 2 min to pass the specimen through the Bond Elut column. The column was then rinsed sequentially with 1 mL of 0.1 M potassium phosphate buffer (pH 6.0): methanol, 80:20, 1 mL of 1.0 M acetic acid, and 1 mL of hexane. The column was allowed to dry for 5–10 min after each rinse. A labeled silanized glass tube was placed below the column and an eluate was collected by slowly rinsing the column with 4 mL of dichloromethane. The solvent was evaporated under a stream of nitrogen gas at 40 °C using silanized taper bottom tubes. The residue was resuspended in 150 μL of 60% solvent B/40% solvent A mixture with moderately vigorous vortexing and sonication. This solution was placed into a 300 μL vial for HPLC analysis.

Instrumentation. The HPLC procedure was adapted from that described for diclazuril. The instrument employed was a Beckman System Gold HPLC system with two 110B solvent delivery pumps, a 168 photodiode array detector and a 502 autosampler. The column was a Beckman Ultrasphere ODS, 5 μm particle size, 4.6 mm × 15 cm column size, protected with an Altech C-18 guard column. The mobile phase consisted of 40% solvent A and 60% solvent B run with a flow rate of 1 ml/min. Solvent A was 80% (0.5% ammonium acetate in water): 20% acetonitrile. Solvent B was 80% methanol, 20% acetonitrile. Acetonitrile (AX0145-1; EMS Chemicals, Gibbstown, NJ, USA) and methanol (MX0488-1, EMS Chemicals) were

HPLC grade. After preparation, solvents A and B were filtered and degassed with 0.45 μm type HV Millipore filters. The diode array detector was set up for single wavelength acquisition at 255 nm with a 12 nm span. Injections were prepared with a 20 μL sample loop.

Pharmacokinetic analysis
Pharmacokinetic analyses were performed, using a nonlinear regression program (Winnonlin, version 5.1) (Pharsight Corporation, Cary, NC, USA). Area under the curve (*AUC*) following intravenous administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (β) of the log plasma drug concentrations vs. time curve was determined by the method of least-squares regression (Gibaldi & Perrier, 1982).

The compartmental model used is represented by general equation a where *C*p is plasma concentration of compound at any time (*t*), A and B are the Y intercepts associated with distribution and elimination phase, respectively, and α and β represent the rate constant of distribution and terminal elimination phase, respectively. The rate constant of distribution (α), and distribution half-life ($t_{1/2}\ \alpha$) were determined using the method of residuals (Gibaldi & Perrier, 1975). The terminal half-life ($t_{1/2}\ \beta$) (Martinez, 1998a,b) was calculated according to equation 1.

$$C_p = A \times e^{-\alpha.t} + B \times e^{-\beta.t} \qquad (a)$$

$$t_{1/2}\beta = \ln2/\beta \qquad (1)$$

Total body clearance (Cl_s) was calculated by use of equation 2.

$$Cl_s = \text{i.v. Dose}/AUC_{0-\inf}(\text{i.v.}). \qquad (2)$$

The absolute bioavailability (F) was calculated from the $AUC_{0-\inf}$ ratio obtained following oral and i.v. administration according to equation 3.

$$F = AUC_{0-\inf}(\text{Oral})/AUC_{0-\inf}(\text{i.v.}) \times \text{i.v. Dose}/\text{Oral Dose} \qquad (3)$$

The volume of distribution in central compartment (Vd_c), volume of distribution in terminal elimination phase (Vd_β) and volume of distribution at steady-state (Vd_{ss}) were calculated according to equations 4, 5, and 6, respectively (Martinez, 1998a,b).

$$Vd_c = \text{Dose(i.v.)}/A + B \qquad (4)$$

$$Vd_\beta = \text{i.v. Dose}/AUC_{0-\inf} \times \beta \qquad (5)$$

$$Vd_{ss} = \text{i.v. Dose} \times AUMC_{0-\inf}/(AUC_{0-\inf})^2 \qquad (6)$$

Area under the moment curve (AUMC) is area under the first moment curve and calculated by the trapezoidal method and extrapolated to infinity (Gibaldi & Perrier, 1982).

K_{10} is first order elimination rate constant which describes elimination of drug from the central compartment. K_{12} and K_{21} are distribution rate constant from central to peripheral and from peripheral to central compartment, respectively. K_{10}, K_{12}, and K_{21} (Martinez, 1998a,b) were calculated according to equations 7, 8, 9, respectively.

$$K_{10} = \alpha \times \beta / K_{21} \qquad (7)$$

$$K_{12} = \alpha + \beta - K_{21} - K_{10} \qquad (8)$$

$$K_{21} = B \times \alpha + A \times \beta / (A + B) \qquad (9)$$

Average plasma concentration at steady-state (Css_{avg}) was calculated according to equation 10.

$$Css_{avg} = \text{Dose} \times F / Cl_s \times \tau(\text{Dosing interval}) \qquad (10)$$

Loading dose and maintenance dose was calculated according to equation 11 and 12, respectively.

$$\text{Loading dose} = Vd_{ss} \times Css_{avg}/F \qquad (11)$$

$$\text{Maintenance dose} = Cl_s \times Css_{avg}/F \qquad (12)$$

The compartmental model used following oral administration is represented by general equation b where A is Y intercept associated with terminal elimination phase, K_{01} is the apparent rate constant of absorption, and K_{10} is the apparent rate constant of elimination (Shargel & Yu, 1993). The rate constant of absorption (K_{01}) and the absorption half-life ($t_{1/2} K_{01}$) were determined, using the method of residuals (Gibaldi & Perrier, 1975). The linear terminal slope (K_{10}) was calculated from the log plasma drug concentrations vs. time curve by using the method of least-squares regression (Gibaldi & Perrier, 1982). The terminal elimination half-life ($t_{1/2} K_{10}$) was calculated according to equation 13.

$$Cp = A \times e^{-K10.t} - A \times e^{-K01.t} \qquad (b)$$

$$t_{1/2}K_{10} = \ln 2/K_{10} \qquad (13)$$

Total oral clearance (Cl_o) was calculated by use of equation 14

$$Cl_o = \text{Dose}(\text{Oral})/AUC_{0-\inf} \qquad (14)$$

The maximum drug concentration after oral administration (C_{max}) and the time at which C_{max} was achieved (T_{max}) (Martinez, 1998a,b) were determined by use of equations 15 and 16, respectively.

$$C_{max} = A.e^{-K10.Tmax} - A.e^{-K01.Tmax} \qquad (15)$$

$$T_{max} = 1/K_{01} - K_{10} \times (\text{Ln}(K_{01}/K_{10})) \qquad (16)$$

The relative bioavailabilities (F) of toltrazuril sulfone suspended in water and as a feed additive formulation were calculated from the $AUC_{0-\inf}$ ratio comparison with the toltrazuril sulfone in DMSO by equation 17.

$$\begin{aligned} F = {} & AUC_{0-\inf}(\text{other formulations}) \\ & /AUC_{0-\inf}(\text{toltrazuril sulfone in DMSO}) \\ & \times \text{Dose}(\text{toltrazuril sulfone in DSMO}) \\ & /\text{Dose}(\text{other formulations}) \end{aligned} \qquad (17)$$

RESULTS

The HPLC diode array detection method reported here readily detects toltrazuril sulfone in plasma, with a limit of detection of about 10 ng/mL. Satisfactory recovery (86%) was obtained for solid phase extraction of toltrazuril sulfone from plasma samples of horses (at 1500 ng/mL, $n = 6$). The standard curve was linear from 100 to 10 000 ng/mL with an r value of 0.999 (data not shown).

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) in DMSO to four horses, analysis of plasma samples showed good oral absorption (Fig. 2), with an observed mean peak plasma concentration of 2795 ± 102 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration (Fig. 2). Observed peak plasma concentrations from four horses were closely distributed ranging from the lowest 2560 ng/mL to highest 3051 ng/mL. Thereafter, the plasma concentration declined to 803 ± 83 (SEM) ng/mL at 168 h after administration with an apparent average half-life of ∼82 h (Fig. 2).

Analysis of plasma samples indicated rapid absorption characteristics of toltrazuril sulfone administered in DMSO, the mean plasma concentration being 137 ng/mL ± 35 (SEM) at 10 min following oral administration. Pharmacokinetic parameters following oral administration of toltrazuril sulfone in DMSO are summarized in Table 1. Figure 3 shows the mean plasma concentrations (±SEM) of toltrazuril sulfone following single i.v. injections of 1 mg/kg of toltrazuril sulfone in DMSO. Pharmacokinetic parameters following i.v. administration of toltrazuril sulfone in DMSO are summarized in Table 2. The cross-over design study results were consistent with an improved oral bioavailability of toltrazuril sulfone in horses following oral administration in DMSO. Mean bioavailability of toltrazuril
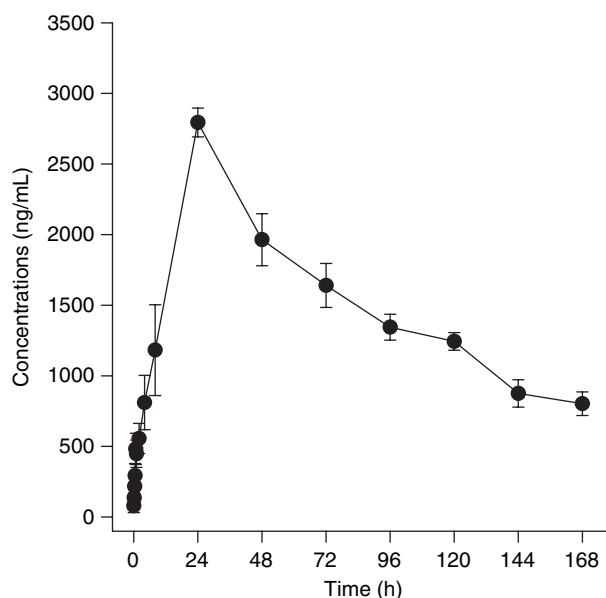
Fig. 2. Mean plasma concentrations (±SEM) of toltrazuril sulfone from four horses following single oral administration (dose: 2.2 mg/kg in dimethylsulfoxide).

Table 1. Pharmacokinetic parameters of toltrazuril sulfone following single oral administration (2.2 mg/kg in DMSO)

Horse	1	2	3	4	Mean ± SEM
Weight (kg)	518	526	517	453	503.5 ± 16.95
Absolute F (%)	69.64	61.25	73.09	78.21	70.6 ± 3.56
$t_{1/2}$ K_{01} (h)	8.944	7.511	5.089	10.08	7.91 ± 1.08
$t_{1/2}$ K_{10} (h)	106.8	71.54	80.14	66.47	81.24 ± 8.98
K_{01} (h^{-1})	0.0775	0.0923	0.1362	0.06875	0.094 ± 0.015
K_{10} (h^{-1})	0.00649	0.00968	0.00865	0.01043	0.0088 ± 0.00086
$AUC_{0-inf.}$ (ng/mL/h)	376373	325729	359649	368735	357620 ± 11167
Mean residence time (h) (MRT)	167	114.1	123	110.4	128.6 ± 13.061
Oral clearance (mL/h)	3027.9	3552.6	3162.5	2702.8	3111.4 ± 175.9
T_{max} (h)	34.9	27.3	21.6	32.3	29.04 ± 2.94
C_{max} (ng/mL)	1806	2520	2619	2656	2400.3 ± 200.18
R^2	0.915	0.961	0.981	0.969	0.96 ± 0.015

AUC, area under the curve.

sulfone in DMSO was 71% ± 3.6 (SEM). The bioavailability of toltrazuril sulfone in DMSO in horses included in our study was relatively consistent, and it ranged from 61.3 to 78.2% (Table 1). Predicted C_{max} from these horses ranged from 1806 to 2656 ng/ml with the mean C_{max} of 2400 ± 200 (SEM) ng/mL. The mean plasma half-life of toltrazuril sulfone in these horses was 81 ± 9 (SEM) h.

Based on these pharmacokinetic parameters following oral and i.v. administrations of toltrazuril sulfone in DMSO, the average steady-state concentrations (Css_{avg}) of toltrazuril sulfone for each horse included in cross-over study was calculated. The Css_{avg} values from four horses were estimated to be between 13 572 and 15 690 ng/mL following daily oral administration of 2.2 mg/kg of toltrazuril sulfone in DMSO. To confirm these calculations, two horses were dosed daily with 2.2 mg/kg toltrazuril sulfone in DMSO orally. Analysis of plasma samples from these two horses

Fig. 3. Mean plasma concentrations of toltrazuril sulfone (±SEM) from four horses following single i.v. administration (dose: 1 mg/kg in dimethylsulfoxide).

indicated steady-state plasma concentrations of toltrazuril sulfone of approximately 12 000–14 000 ng/mL and 16 000–18 000 ng/mL after approximately 10–12 days (data not shown). Steady-state concentrations of toltrazuril sulfone in the cerebrospinal fluid samples from these two horses ranged between 125–130 and 180–220 ng/mL (Fig. 4).

The Css_{avg} values from four horses included in cross-over study were estimated to be between 19 641 and 22 525 ng/mL following daily i.v. administration of 2.2 mg/kg toltrazuril sulfone in DMSO. To confirm these results, two horses were dosed daily with 2.2 mg/kg toltrazuril sulfone i.v. in DMSO. Analysis of plasma samples indicated steady-state plasma concentrations of toltrazuril sulfone of approximately 19 000–22 000 ng/mL after approximately 2–3 weeks (data not shown). Steady-state concentrations of toltrazuril sulfone in the cerebrospinal fluid samples from these two horses ranged between 150–170 and 190–220 ng/mL (Fig. 5). Therefore, steady-state plasma concentration studies for both oral and i.v. studies confirmed our calculated kinetic parameters. Additionally, analysis of CSF samples confirmed the lipophilic characteristic of toltrazuril sulfone and its ability to pass across the blood-brain barrier.

Based on clinical data information provided by Saxony Farm (Mr. B. Hundley, personal communication, Lexington, KY, USA), it was suggested that daily oral administration of 0.55 mg/kg of toltrazuril sulfone in DMSO resulted in clinical improvement of EPM horses. We therefore wanted to determine steady-state concentrations of toltrazuril sulfone in both plasma and CSF following daily oral administration of 0.55 mg/kg in DMSO using three horses. Additionally, based on our pharmacokinetic data, we calculated the loading dose considering 0.55 mg/kg of toltrazuril sulfone in DMSO as a maintenance dose. The steady-state plasma concentrations of toltrazuril sulfone from these horses ranged between 3750 and 4250 ng/mL with mean steady-state concentration of approximately 3900 ng/mL (data not shown), suggesting dose proportional kinetics. This is further confirmed by comparing the Css values observed with a 2.2 mg/kg oral dose in DMSO to the 0.55 mg/kg oral dose in DMSO, where the ratio of amount administered (fourfold difference) is consistent with the nearly fourfold difference in
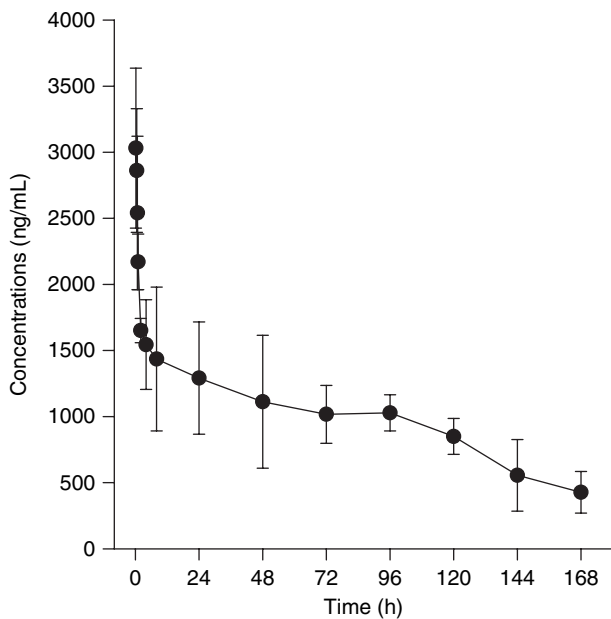
Table 2. Pharmacokinetic parameters of toltrazuril sulfone following single i.v. administration (1 mg/kg in DMSO)

| Horse	1	2	3	4	Mean ± SEM
Weight (kg)	518	526	517	453	503.5 ± 16.95
K_{10} (h^{-1})	0.010295	0.00971	0.01754	0.0148	0.0131 ± 0.001869
α (hr^{-1})	0.905	0.9003	0.95762	0.7637	0.8816 ± 0.0414
β (h^{-1})	0.006128	0.005843	0.003851	0.00665	0.00562 ± 0.000612
K_{12} (h^{-1})	0.362133	0.354702	0.73363	0.4124	0.4657 ± 0.0902
K_{21} (h^{-1})	0.5387	0.541683	0.21031	0.34283	0.4084 ± 0.081
$t_{1/2}$ K_{10} (h)	67.323	71.385	39.53	46.84	56.27 ± 7.75
$t_{1/2}$ α (h)	0.7659	0.7699	0.9576	0.9076	0.8503 ± 0.0486
$t_{1/2}$ β (h)	113.101	118.64	179.97	104.233	128.99 ± 17.25
A + B (ng/mL)	2779	2573	4097	3834	3320.75 ± 378.44
$AUC_{0-inf.}$ (ng/mL/h)	245674	241729	223679	214316	231350 ± 7427
Cl_s (mL/h)	2108.5	2176	2311.4	2113.7	2177.4 ± 47.214
Vd_c (L/kg)	0.3599	0.3887	0.2441	0.2608	0.3134 ± 0.036
Vd_β (L/kg)	0.664	0.708	1.161	0.702	0.809 ± 0.118
Vd_{ss} (L/kg)	0.661	0.705	1.144	0.694	0.801 ± 0.115
R^2	0.9942	0.993	0.992	0.984	0.991 ± 0.0023

AUC, area under the curve.
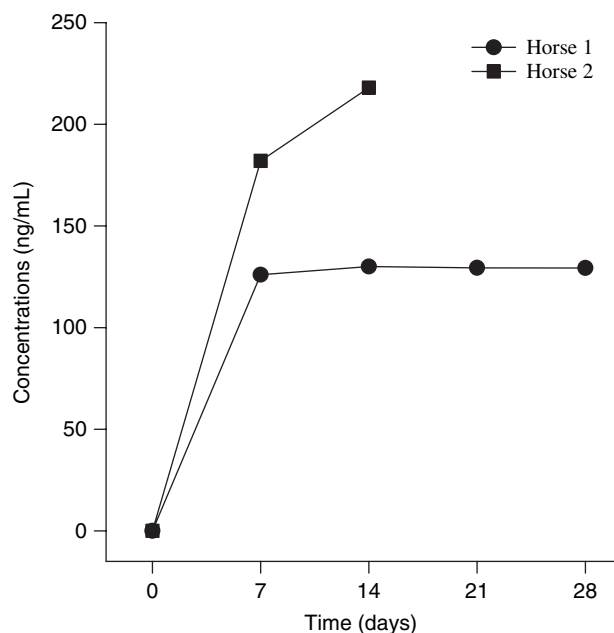
Fig. 4. Steady-state CSF concentrations of toltrazuril sulfone from two horses following daily oral administration (dose: 2.2 mg/kg in dimethylsulfoxide).

Fig. 5. Steady-state CSF concentrations of toltrazuril sulfone from two horses following daily i.v. administration with apparent CSF half-life of approximately 82 h (dose: 2.2 mg/kg in dimethylsulfoxide).

Css (approximately 12 000–18 000 ng/mL after the 2.2 mg/kg daily oral dosing regimen vs. an average Css value of 3900 ng/mL following a 0.55 mg/kg daily oral dosing regimen). Steady-state concentrations of toltrazuril sulfone in the cerebrospinal fluid samples from one of these horses were attained at 24 h following loading dose administration at a concentration of 70 ng/mL and in later time points CSF concentrations ranged between 24 and 60 ng/mL (data not shown).

Since half-life determines the time to reach a steady-state plasma concentration, and toltrazuril sulfone has very long plasma half-life, it will be more clinically effective to administer a loadi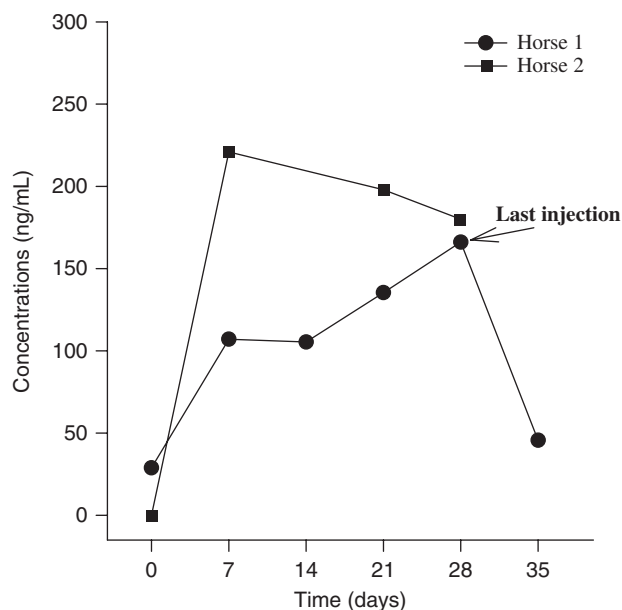ng dose with the goal of rapid attainment of the effective therapeutic concentrations of this agent in plasma and cerebrospinal fluid, especially in acute cases of EPM. Mean bioavailability, mean Vd_{ss}, and estimated Css_{avg} range values obtained from four horses included in cross-over study were used to determine loading dose to achieve steady-state plasma concentration following oral administration. Based on these calculations, the loading dose for oral administration was estimated to be between 13.5 and 19 mg/kg.

Based on these calculations, two horses were dosed orally with 15 mg/kg loading dose and 2.2 mg/kg maintenance dose for 28 days to establish the effect of loading dose in reducing the time to achieve steady-state concentrations in both plasma and CSF

(data not shown). Steady-state plasma concentrations of toltrazuril sulfone from these two horses were approximately 15 000–17 000 ng/mL and were attained 24 h postadministration of oral loading dose. We were only able to obtain useful CSF samples on day 14 from one horse and on days 14 and 28 from another horse. Steady-state CSF concentrations were 155 ng/mL from the day 14 sample (horse 1), and 178 and 202 ng/mL from days 14 and 28 (horse 2), respectively (data not shown).

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) suspended in water to two horses, analysis of plasma samples showed detectable plasma concentrations following oral administration of this aqueous suspension (Fig. 6), with the observed mean peak plasma concentration of 772 ± 14 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration. Observed plasma concentrations from two horses at 24 h postadministration were similar to each other, with values of 758–786 ng/mL (Fig. 6). Thereafter, the plasma concentration declined to 286 ± 61 (SEM) ng/mL at 168 h after administration with an apparent average half-life of ~77 ± 3.5 (SEM) h. The pharmacokinetic parameters of toltrazuril sulfone following oral administration of this compound suspended in water are shown in Table 3. The observed mean peak plasma concentration of toltrazuril sulfone at 24 h following oral administration in DMSO was approximately four times higher than following oral administration in water. The mean relative bioavailability of toltrazuril sulfone in water compared to toltrazuril sulfone in DMSO was 32%, indicating approximately threefold less bioavailability of toltrazuril sulfone following oral administration in water vs. in DMSO.

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg in DMSO) on 0.5 oz. beet pulp added to 1 lb. sweet feed to four horses, analysis of plasma samples showed relatively
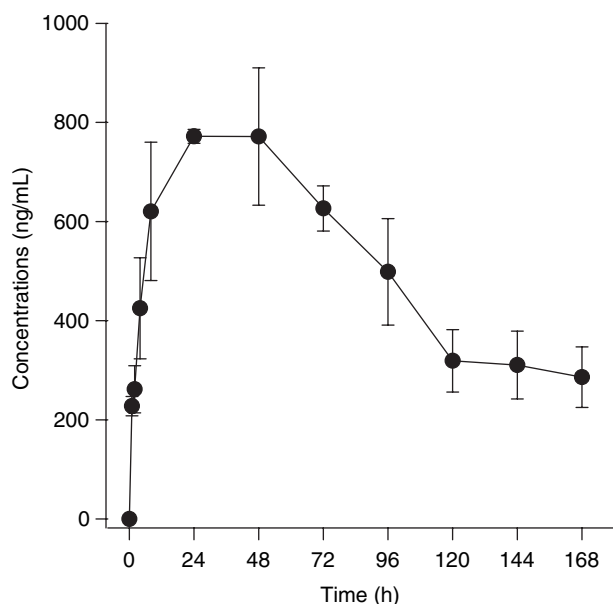
Table 3. Pharmacokinetic parameters of toltrazuril sulfone following single oral administration (2.2 mg/kg in water)

Horse	1	2	Mean ± SEM
Weight (kg)	500	555	527.5 ± 27.5
Relative F (%)	29	35	32 ± 3
$t_{1/2}$ K_{01} (h)	3.15	9.14	6.15 ± 2.99
$t_{1/2}$ K_{10} (h)	73.4	80.3	76.84 ± 3.45
K_{01} (h^{-1})	0.2202	0.076	0.0904 ± 0.00406
K_{10} (h^{-1})	0.0094	0.0086	0.009 ± 0.0004
$AUC_{0-inf.}$ (ng/mL/h)	102047	137164	119605 ± 17559
Mean residence time (h)	110	129	119.7 ± 9.3
Oral clearance (L/h)	10.78	8.75	9.77 ± 1.02
T_{max} (h)	14.94	32.34	23.64 ± 8.7
C_{max} (ng/mL)	824	877	850.5 ± 26.75
R^2	0.994	0.987	0.99

AUC, area under the curve.

rapid absorption of toltrazuril sulfone following oral administration on feed (Fig. 7), with the observed mean peak plasma concentration of 3013 ± 157 (SEM) ng/mL of toltrazuril sulfone at 8 h after administration (Fig. 7). Observed plasma concentrations from four horses at 8 h postadministration were in close agreement with values of lowest 2622 ng/mL to highest 3276 ng/mL. The plasma concentrations of toltrazuril sulfone from these horses ranged from lowest 2628 ng/mL to highest 2886 ng/mL at 24 h postadministration with the mean plasma concentration of 2746 ng/mL ± 55 (SEM) (Fig. 7). Thereafter, the plasma concentration declined to 503 ± 142 (SEM) ng/mL at 168 h after administration with an apparent average half-life of ~65.4 ± 8 (SEM) h (Table 4). The mean relative bioavailability of toltrazuril sulfone in DMSO as a feed additive compared to toltrazuril sulfone in DMSO without feed was 69%, indicating approximately 31% less bioavailability of toltrazuril sulfone in DMSO following oral administration with feed vs. without feed.

DISCUSSIONS AND CONCLUSIONS

Our earlier preliminary experiments suggested that the oral bioavailability of diclazuril as Clinacox® may vary between individual horses in a clinically significant manner (Dirikolu *et al.*, 2006). For example, in our small sample of four horses there was a twofold difference between the peak plasma concentrations of diclazuril as Clinacox® observed in the high (1.6 μg/mL) and the low (0.75 μg/mL) horses. These differences presumably translate into equivalent differences in steady-state concentrations of diclazuril attained in plasma and ultimately in the CSF of treated animals (Dirikolu *et al.*, 2006). For example in our clinical efficacy study, when the dosage was adjusted from 5 to 5.5 mg/kg and the treatment interval was extended from 21 to 28 days to allow for longer duration of detectable CSF level (six horses in our clinical efficacy trial), the rate of post-treatment relapse rate was reduced indicating the variability of
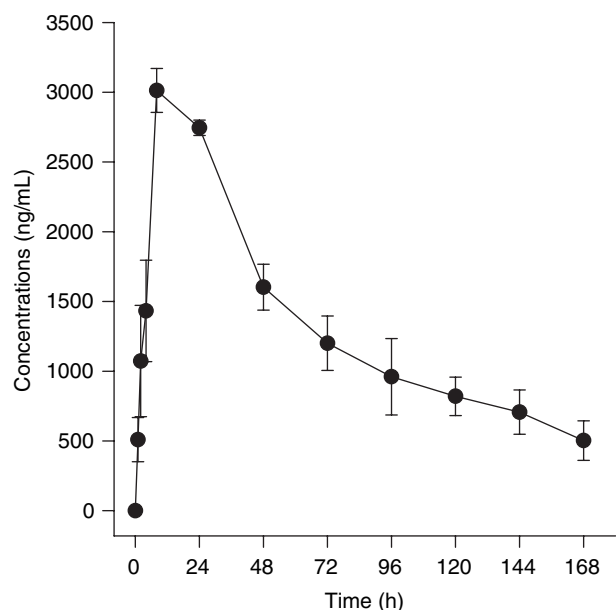

Fig. 6. Mean plasma concentrations of toltrazuril sulfone (±SEM) following single 2.2 mg/kg oral administration in water from two horses.

Fig. 7. Mean plasma concentrations of toltrazuril sulfone from four horses following single oral 2.2 mg/kg administration in dimethylsulfoxide mixed with 14 g beet pulp added to 455 g sweet feed.

oral absorption of diclazuril in horses. As evidenced by our current dataset, the slow partitioning of drug into the CNS and the high intersubject variability in CNS drug concentrations underscores the importance of minimizing the intersubject variability in drug absorption.

Three possible solutions to this problem presented themselves. The most practical is the development of a highly bioavailable oral preparation such as sodium salt formulations of diclazuril (Dirikolu *et al.*, 2006) or related triazine-based agent which will routinely yield effective plasma and CSF concentrations of this agent in all horses treated. A secondary approach is to monitor plasma concentrations of triazine-based agents during therapy and adjust the oral dose to compensate for any deficits in bioavailability in individual animals. A third approach is to develop highly bioavailable oral preparation of a diclazuril

related compound in a suitable solvent, which results in a higher bioavailability and therefore consistently high plasma and CSF concentrations of this agent in all horses treated. With literature review, DMSO was suggested as a candidate solvent which increases the absorption characteristics of these compounds in the horse.

To further investigate the effect of DMSO on the absorption characteristics of triazine agents, we compared the results of oral administration of toltrazuril sulfone suspended in water and DMSO. It was found that the mean peak plasma concentration of toltrazuril sulfone at 24 h following oral administration in DMSO was approximately four times higher than following oral administration suspended in water. The relative bioavailability of toltrazuril sulfone suspended in water compared to in DMSO was 32% indicating an approximately threefold reduction in the bioavailability of toltrazuril sulfone following oral administration in water vs. in DMSO. Our comparison of toltrazuril sulfone oral bioavailability in water vs. DMSO was linked to a comparison of F values estimated for both formulations on the basis of the drug exposure observed when toltrazuril sulfone was administered as an i.v. solution in DMSO. In so doing, there was a risk that any change in clearance attributable to the presence of DMSO would have biased our estimate of F for the aqueous suspension. To confirm the lack of bias in our estimate, we compared $t_{1/2}$ values in the aqueous suspension vs. the DMSO oral solution (note that we could not compare clearance estimates as these are confounded by differences in F). The finding of nearly identical $t_{1/2}$ values (76.84 vs. 81.24 h for the aqueous suspension and DMSO oral solution, respectively) provided assurance that observed profile differences primarily reflected the impact of formulation on oral absorption kinetics. The mean absolute bioavailability of toltrazuril sulfone in DMSO was 71% indicating low hepatic extraction ratio characteristic of this drug in DMSO following oral administration. Additionally, toltrazuril sulfone in DMSO can be administered as a feed additive formulation without significant effect on its rate and extent of absorption. The mean relative oral bioavailability of toltrazuril sulfone formulated in DMSO as a feed additive was 69% compared to administration of toltrazuril sulfone in DMSO without feed.

Table 4. Pharmacokinetic parameters of toltrazuril sulfone following single oral administration of 2.2 mg/kg in DMSO mixed with 0.5 oz. beet pulp added to 1 lb. sweet feed

Horse	1	2	3	4	Mean ± SEM
Weight (kg)	559	591	583	569	575.5 ± 7.14
Relative F (%)	98	66	52	58	68.5 ± 10.24
$t_{1/2}$ K_{01} (h)	4.66	1.286	2.85	6.53	3.83 ± 1.13
$t_{1/2}$ K_{10} (h)	82.74	72.82	46.66	59.32	65.4 ± 7.9
K_{01} (h^{-1})	0.1487	0.5389	0.2432	0.106	0.259 ± 0.098
K_{10} (h^{-1})	0.00838	0.00952	0.01485	0.01168	0.0111 ± 0.00142
$AUC_{0-inf.}$ (ng/mL/h)	390813	276498	215966	234379	279413.7 ± 39234
Oral clearance (mL/h)	3146.8	4702.4	5938.9	5340.9	5327 ± 357
T_{max} (h)	20.5	7.62	12.24	23.4	15.94 ± 3.64
C_{max} (ng/mL)	2757	2447.6	2674.6	2084.4	2490.9 ± 150.46
R^2	0.953	0.922	0.977	0.8	0.913 ± 0.039

AUC, area under the curve.

In the two horses examined in our study, the administration of toltrazuril sulfone in DMSO daily either orally or intravenously produced steady-state concentrations of this compound that are within the range of concentrations shown to be effective *in vitro*. For example, it has been shown that toltrazuril sulfone completely inhibits merozoite production of *S. neurona* in cell cultures treated with 100–1000 ng/mL of toltrazuril sulfone (Lindsay *et al.*, 2000). In comparison, the CSF concentrations of toltrazuril sulfone observed in our study following a daily oral and i.v. dose of 2.2 mg/kg ranged from 125–220 and 150–220 ng/mL, respectively, when toltrazuril sulfone was formulated in DMSO. Analysis of CSF samples from horses included in present study confirmed the lipophilic characteristic of toltrazuril sulfone and its ability to traverse blood-brain barrier at sufficient amount for the treatment of EPM. Additionally, during this repeated administration of toltrazuril sulfone formulated in DMSO for 28 days, we didn't observe any clinically significant side effects (including change in appetite, body weight, temperature, general physical condition and behavior, and observation of colic).

Dimethylsulfoxide according to FDA residual solvents VICH GL18 guidance for industry is classified as solvents with low toxic potential (class 3 solvent). According to this guidance, it is considered that amounts of these residual solvents of 0.5% (corresponding to 5000 ppm) in pharmaceuticals would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice. The solubility of toltrazuril sulfone in DMSO is 250 mg/mL, thereby minimizing the amount of DMSO needed per dose and per horse. Additionally, administration of toltrazuril sulfone in DMSO mixed with feed (1.23 kg) follows the FDA regulations for the levels of DMSO normally accepted in pharmaceuticals.

Dimethylsulfoxide is a relatively safe agent for systemic use. DMSO is available for veterinary use in two formulations; a gel and a liquid. Both are formulated as 90% solutions and are approved in the horse and the dog for external use (Brayton, 1986). The manufacturer recommends a maximum total daily dose of 100 g and a maximum dosing duration of 100 days. Adverse effects have seen during too rapid an intravenous administration of dosages equal or greater than 2.0 g/kg with concentrations of DMSO at 20% or more. The LD_{50} of DMSO varies across animal species, being 4.0 g/kg in cats and 2.5 g/kg in dogs, and ranges from 2.5 to 8.9 g/kg body weight in various other animals (David, 1972).

In conclusion, the toxicity of DMSO is minimal when used in clinically normal doses and concentrations. The toxicity effects are most frequently seen in abnormally high experimental doses and concentrations. In our triazine bioavailability study, the amount of DMSO used in preparation of solution is relatively small ranging from 15 mg/kg (in the case of daily oral administration with feed) to 100 mg/kg (in the case of loading dose administration with feed). Additionally, in our clinical efficacy study, we did not observe any toxic effects related to administration of DMSO for more than 28 days. Therefore, DMSO is very suitable solvent to keep our triazine-based agents in

solution and increase the rate and extent of absorption of these agents in clinically significant level. Toltrazuril sulfone formulated in DMSO also has potential to be used as a feed additive. Additionally, administration of triazine agents such as toltrazuril sulfone formulated in DMSO will provide less variable and better-controlled plasma drug concentrations and therefore, more predictable drug effects due to improved bioavailability. On the other hand, research on the triazine-based agents clinical efficacy, toxicity and the establishing of an appropriate therapeutic window for these compounds clearly require further study.

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