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Toltrazuril sulfone sodium salt: synthesis, analytical detection, and pharmacokinetics in the Horse

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Toltrazuril sulfone (ponazuril) is a triazine-based antiprotozoal agent with clinical application in the treatment of equine protozoal myeloencephalomyelitis (EPM). In this study, we synthesized and determined the bioavailability of a sodium salt formulation of toltrazuril sulfone that can be used for the treatment and prophylaxis of EPM in horses. Toltrazuril sulfone sodium salt was rapidly absorbed, with a mean peak plasma concentration of 2400 ± 169 (SEM) ng/mL occurring at 8 h after oral-mucosal dosing and was about 56% bioavailable compared with the i.v. administration of toltrazuril sulfone in dimethylsulfoxide (DMSO). The relative bioavailability of toltrazuril sulfone suspended in water compared with toltrazuril sulfone sodium salt was 46%, indicating approximately 54% less oral bioavailability of this compound suspended in water. In this study, we also investigated whether this salt formulation of toltrazuril sulfone can be used as a feed additive formulation without significant reduction in oral bioavailability. Our results indicated that toltrazuril sulfone sodium salt is relatively well absorbed when administered with feed with a mean oral bioavailability of 52%. Based on these data, repeated oral administration of toltrazuril sulfone sodium salt with or without feed will yield effective plasma and cerebrospinal fluid (CSF) concentrations of toltrazuril sulfone for the treatment and prophylaxis of EPM and other protozoal diseases of horses and other species. As such, toltrazuril sulfone sodium salt has the potential to be used as feed additive formulations for both the treatment and prophylaxis of EPM and various other apicomplexan diseases.

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INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is the most important infectious neurologic disease of Western Hemisphere horses; it is caused by *Sarcocystis neurona* and, less commonly, *Neospora hughesi*, two apicomplexan protozoal parasites. EPM interferes with a horse's ability to perform, race, and work, and untreated EPM can be lethal. As the location of the causative organism in the CNS is random, the clinical signs of EPM are highly variable. As such, any combination of neurologic signs is possible, although spinal cord involvement is the more usual presentation. Onset may be acute or gradual, the common usual pattern being mild signs appearing acutely and progressing with time. Although there are a number of commercially available tests to detect antibodies to *S. neurona*, all have similar shortcomings; therefore, diagnosis of EPM is very challenging, requiring careful evaluation of the animal's history, clinical signs, and laboratory data, along with rigorous exclusion of other causes. Definitive diagnosis of EPM depends on the necropsy demonstration of typical CNS lesions of the disease and/or presence of the causative organism. In the case of relapse and lack of response to treatment, the diagnosis of EPM should be reevaluated.

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), is a triazine-based antiprotozoal agent with highly specific actions against the apicomplexan group of organisms. Toltrazuril sulfone and toltrazuril sulfoxide are the major metabolites of toltrazuril following its oral dose as Baycox® at 10 mg/kg (Furr & Kennedy, 2000). Both of these metabolites have the ability to pass across the blood–brain barrier, and 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. No signs of toxicity of toltrazuril were observed 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 *S. neurona* in cell cultures was recently reported (Lindsay *et al.*, 1999). This study showed the complete inhibition of merozoite production in cell cultures of *S. neurona* treated with 0.1–1 μg/mL toltrazuril sulfone.

Toltrazuril sulfone (ponazuril, Marquis™) has recently been evaluated for the treatment of EPM in a study sponsored by Bayer Animal Health (Pittsburgh, PA) (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 showed clinical improvement 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 the oral dose of ponazuril at 5 mg/kg/day (Furr *et al.*, 2001). Recently, toltrazuril sulfone (Marquis®) was approved by Food and Drug Administration to be used for the treatment of EPM. Toltrazuril sulfone (Marquis®) is marketed by Bayer Corporation, Agriculture Division, Shawnee Mission, Kansas.

Previous studies have identified triazine-based antiprotozoal agents for the treatment and prophylaxis of EPM in the horse (Granstrom *et al.*, 1997; Bentz *et al.*, 1998; Dirikolu *et al.*, 1999), and in vitro studies confirm that *S. neurona* is sensitive to triazine-based antiprotozoal agents. Triazine-based antiprotozoal agents are known for their lipophylic characteristics, and they may be expected to be well absorbed following oral administration. The absorption of chemicals from the gastrointestinal (GI) tract depends on the physiochemical properties of compounds, such as lipid solubility, and dissociation rate (Houston *et al.*, 1974). Although it is often considered that an increase in lipid solubility increases the rate of absorption of chemicals, extremely lipid-soluble chemicals may have poor oral bioavailability, both because highly lipophilic molecules (e.g. if Log $P > 7$) can be retained 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). On this basis, we elected to develop a highly bioavailable oral formulation of toltrazuril sulfone, namely toltrazuril sulfone sodium salt.

Toltrazuril sulfone sodium salt was synthesized with the goal of developing a highly bioavailable oral formulation of ~~toltrazuril~~ that can be used for the treatment and prophylaxis of EPM and other apicomlexan-mediated diseases in the horse and other animals. The primary objectives of the present study were therefore to establish the bioavailability and pharmacokinetic parameters of toltrazuril sulfone sodium salt and to establish whether or not this sodium salt formulation of toltrazuril sulfone can be used as a feed additive formulation in the horse.

MATERIALS AND METHODS

Horses and sample collection

Horses were provided by Saxony Farms (Versailles, KY) 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 daily. Horses were kept in a 20-acre field until they were placed in box stalls, where they were provided water and hay *ad libitum*. Horses were not fed for at least 2 h before and 1 h after oral administrations of each drug formulation included in this study. The horses were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care and Use Committee, which approved the experimental protocol.

In this study, five 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 (crossover study) formulated in medicinal-grade dimethylsulfoxide (DMSO) ~~(n:4)~~, (ii) 2.2 mg/kg toltrazuril sulfone suspended in water administered with nasogastric intubation ~~(n:2)~~, (iii) 2.2 mg/kg toltrazuril sulfone sodium salt administered by direct application on the oral mucosa (between gum and cheek), (iv) toltrazuril sulfone sodium salt as a feed additive in 0.5 oz beet pulp added to 1 lb sweet feed.

We used a randomized crossover study with a 2×2 latin square design to determine absolute bioavailability and pharmacokinetic characteristics of toltrazuril sulfone formulated in DMSO following oral administration in the horse (Dirikolu *et al.*, 2009). Four mature Thoroughbred mares weighing 453–526 kg were used for the toltrazuril sulfone study. Toltrazuril sulfone (150 mg/mL in DMSO) was administered either orally or intravenously to horses at a single dose of 2.2 mg/kg or 1 mg/kg, respectively. Horses were allowed a 3-week interval between subsequent dosage regimens after the last sample collection. Blood samples were collected at 0, 0.16, 0.33, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and 168 h into heparinized tubes that were centrifuged at 4 °C 2000 *g* for 15 min, and the plasma stored at −20 °C until assayed.

In a second experiment, four mature Thoroughbred mares weighing 482–564 kg were used for the toltrazuril sulfone sodium salt study. Toltrazuril sulfone sodium salt was administered by the direct application of 2.2 mg/kg of salt to the oral mucosa (between gum and cheek). Blood samples were obtained for analyses at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and

168 h, and plasma samples were prepared and stored as described previously.

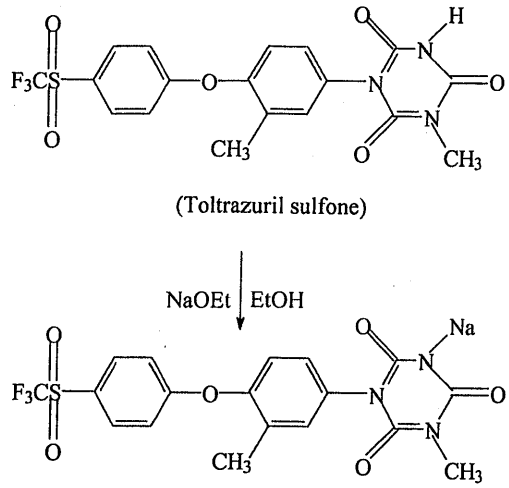
In a third experiment, four mature Thoroughbred mares weighing 536–573 kg were used to evaluate toltrazuril sulfone sodium salt as feed additive formulation. Toltrazuril sulfone sodium salt was administered as a feed additive at 2.2 mg/kg in 0.5 oz. beet pulp added to 1 lb. sweet feed. Blood samples were collected for analyses at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and 168 h into heparinized tubes. Plasma samples were prepared and stored as described previously.

In a fourth 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 (Dirikolu *et al.*, 2009). Blood samples were collected at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and 168 h as described earlier.

Toltrazuril sulfone synthesis

Preparation of toltrazuril sulfone from Baycox®. Recovery of toltrazuril from Baycox®: Toltrazuril was recovered from Baycox® suspension and converted to toltrazuril sulfone as described by Dirikolu *et al.*, 2009. Briefly, the Baycox® suspension was centrifuged for 0.5 h at ~~4000 rpm~~ × g, the upper solution was decanted, the precipitate was resuspended in water, and the resulting suspension was centrifuged for 5 min at ~~4000 rpm~~ × g. This wash step was repeated twice, following which the white toltrazuril precipitate was dried for 12 h at 70 °C and 8 h at 105 °C. This material was placed in 1.5 L of acetone, stirred at reflux for 3 h and filtered through celite to remove a gray insoluble admixture. The acetone was then evaporated, and the resulting pure toltrazuril dried for 8 h at 70 °C.

To obtain 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 determined by thin layer chromatography [melting point (m.p.) 242–244 °C] and gas chromatography/mass spectrometry (GC/MS). Mass spectrometry was performed with an Agilent (Santa Clara, CA, USA) 6890 gas chromatrograph interfaced with a 5972 mass selective detector. Separations were performed on a DB-5MS (5% phenyl-95% methylpolysiloxane) column (J&W Scientific, now part of Agilent). Column dimensions were 30 m × 0.25 mm × 0.25 μ film thickness. Electron impact mass spectrometry was performed at 70 electron volts, and spectra were recorded in the m/z 50–550 mass range and analyzed with Agilent Chemstation software. As shown in Fig. 1, a selected ion chromatogram for toltrazuril sulfone [457 m/z,



~~Fig. 1.~~ Conversion of toltrazuril sulfone to toltrazuril sulfone sodium salt.

M-ion (molecular ion)] and toltrazuril demonstrated lack of toltrazuril starting compound (425 m/z, M-ion), indicating relative purity of approximately 99.7%. Approximately 10 μg of toltrazuril sulfone was injected on column; GC conditions: 150 °C (2 min), followed by 20 dpm to 280 °C (held 17.5 min). The injector was 250 °C, the detector was 280 °C, the solvent was dichloromethane, and the volume injected was 1 μl. Toltrazuril sulfone was identified by electron impact spectrometry, and the full-scan mass spectrum is shown in Fig. 2.

Synthesis of toltrazuril sulfone sodium salt from toltrazuril sulfone: To a hot suspension of 60 g toltrazuril sulfone in 400 mL absolute ethanol (EtOH), a freshly prepared solution of sodium ethanolate (NaOEt; obtained from 3.17 g, 1.05 mol. eq. sodium) in 100 mL absolute ethanol was added. The reaction mixture was stirred for 1.5 h at 70 °C, yielding a homogenous product solution. Solvent was then evaporated under reduced pressure, and the residue was dried under high vacuum to obtain 63.2 g (100% yield determined by thin layer chromatography) of toltrazuril sulfone sodium salt as an amorphous powder. The resultant salt is not hygroscopic and very soluble in water, yielding an alkaline (pH. 9.5) solution. The reaction sequence for conversion of toltrazuril sulfone to toltrazuril sulfone sodium salt is shown in Fig. 3.

Toltrazuril sulfone analysis. Sample preparation: Toltrazuril sulfone was analyzed using high-pressure liquid chromatography (HPLC) as described by Dirikolu *et al.*, 2009. Briefly, a standard solution of 1 mg toltrazuril sulfone was prepared in 1 mL HPLC-grade methanol. Standards were prepared by the addition of a specified amount of toltrazuril sulfone in 60% solvent B/40% solvent A mixture (see instrumentation) to blank plasma samples, 1 mL each, over a range from 100 to 10 000 ng/mL. Janssen compound R 62646, a structural analog of diclazuril,

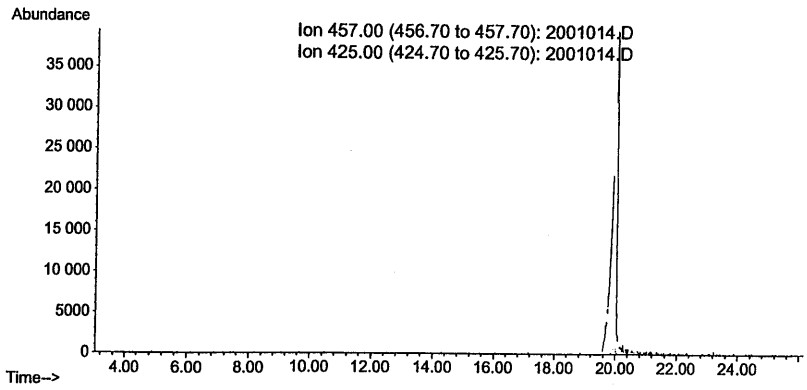


Fig. ~~2~~ 1 Ion chromatogram for toltrazuril sulfone (457 m/z, M-ion), demonstrating the lack of toltrazuril starting compound (425 m/z, M-ion), indicating relative purity of 100%.

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

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 collected 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 the 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 in a 300-μL vial for HPLC analysis.

Analytical instrumentation: The HPLC procedure was adapted from that described for diclazuril (Dirikolu *et al.*, 2009). 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] and 20% acetonitrile. Solvent B was 80% methanol and 20% acetonitrile. Acetonitrile ~~(A998-4; Fisher Scientific, NJ, USA)~~ and methanol (MX0488-1; ~~EM Science, NJ, USA)~~ 4 were of HPLC grade. After preparation, solvents A and B 5 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 3.1) (Pharsight Corporation, Cary, NC, USA). The compartmental model used following oral administration is represented by general equation *a*, 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 6 of absorption (K_{01}) and the absorptive half-life ($t_{1/2}$ K_{01}) was

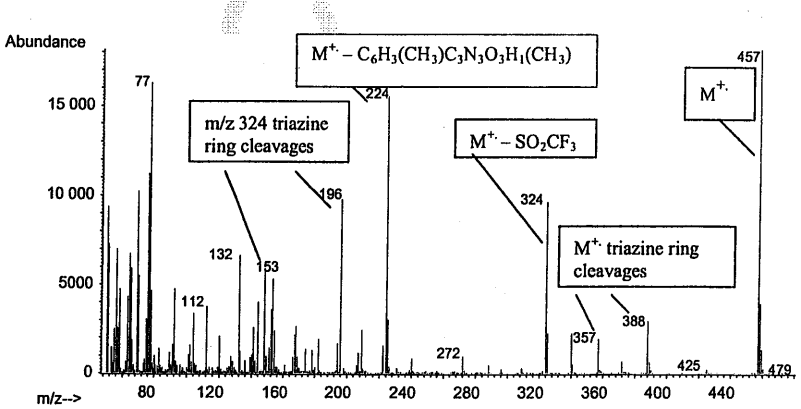

Fig. ~~3~~ 2 Full-scan mass spectrum of toltrazuril sulfone, using electron impact mass spectrometry.

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determined, using the method of residuals (Gibaldi & Perrier, 1975). The linear terminal slope (K_{10}) was calculated from the log plasma drug concentrations versus 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 1.

$$Cp = A \times e^{-K10 \times t} - A \times e^{-K01 \times t} \quad (a)$$

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

Total oral clearance (Cl_o) was calculated by use of Equation 2.

$$Cl_o = \text{dose(oral)}/AUC_{0-inf} \quad (2)$$

The maximum drug concentration after oral administration (C_{max}) and the time at which C_{max} was achieved (T_{max}) (Martinez, 1998) was determined by use of equations 3 and 4, respectively.

$$C_{max} = A \times e^{-K10T_{max}} - A \times e^{-K01T_{max}} \quad (3)$$

$$T_{max} = 1/K_{01} - K_{10} \times (\text{Ln}(K_{01}/K_{10})) \quad (4)$$

The absolute bioavailability (F) was calculated from the AUC_{0-inf} ratio obtained following oral and i.v. administration according to Equation 5 (Benet & Zia-Amirhosseini, 1995).

$$F = AUC_{0-inf}(\text{oral})/AUC_{0-inf}(\text{i.v.}) \times \text{i.v.dose/oraldose} \quad (5)$$

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 and toltrazuril sulfone sodium salt by Equation 6.

$$F = AUC_{0-inf}\ (\text{other formulations})/AUC_{0-inf}\ (\text{toltrazuril sulfone in DMSO or as a sodium salt}) \times \text{dose (toltrazuril sulfone in DMSO or as a sodium salt)}/\text{oral dose (other formulations)} \quad (6)$$

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 (Dirikolu *et al.*, 2009). The toltrazuril sulfone HPLC peak eluted at around 4.3 min (±0.5 min), and the internal standard peak eluted at 5.50 min (±0.5 min) (Fig. 4). The peaks were symmetric, and the standard curve was linear from 100 to 10 000 ng/mL, with an *r* value of 0.999. The areas of the peaks corresponding to toltrazuril sulfone and internal standard were recorded, and the internal standard (methylated diclazuril) values were used to normalize the toltrazuril sulfone areas. Integrated peak values were entered into QuattroPro for Windows for the statistical analysis of standards and for the interpolation of unknown amounts of toltrazuril sulfone. Standard curves were generated with Sigma Plot for Windows.

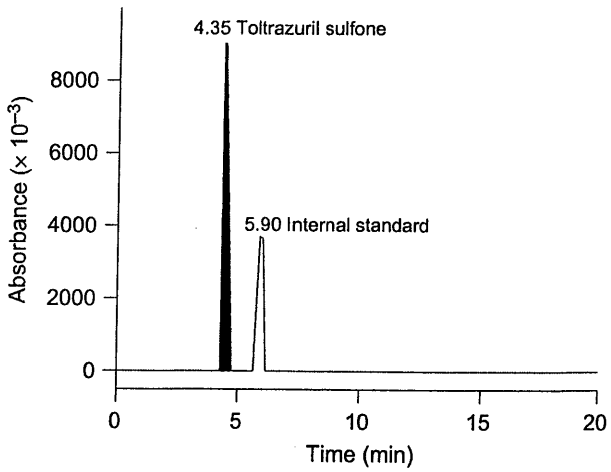

Fig. 4. A typical high-pressure liquid chromatography (HPLC) chromatogram of toltrazuril sulfone with internal standard (methylated diclazuril) in mobile phase, 60% solvent B/40% solvent A, as extracted from dosed horse plasma sample. Absorbance at 255 nm is plotted vs. retention time. The instrument is a Beckman System Gold HPLC system with Beckman ODS column, 5 μm particle size, 4.6 mm × 15 cm column size. The mobile phase consisted of 40% solvent A [80% (0.5% ammonium acetate in water) and 20% acetonitrile] and 60% solvent B (80% methanol and 20% acetonitrile) at a flow rate of 1 mL/min. The photodiode array detector wavelength was set at 255 nm.

After administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) in DMSO to four horses, analysis of plasma samples showed excellent oral absorption (Fig. 5), with an observed mean peak plasma concentration of 2795 ± 102 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration (Dirikolu *et al.*, 2009). Observed peak plasma concentrations from four horses were closely distributed, ranging from a lowest value of

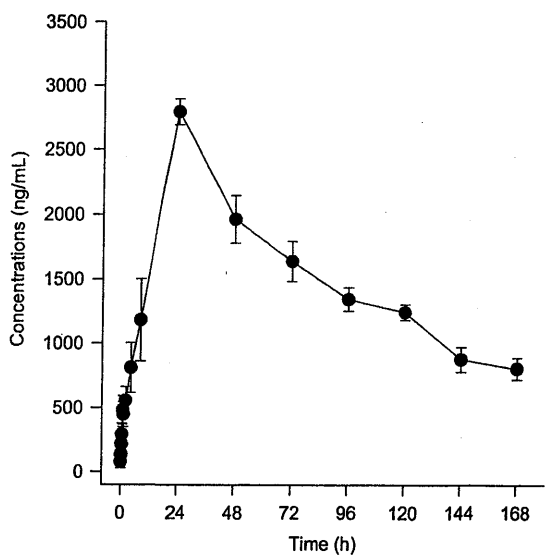

Fig. 5. Mean plasma concentrations (±SEM) of toltrazuril sulfone from four horses following a single oral dose (dose: 2.2 mg/kg in DMSO).

Horse	1	2	3	4	Mean ± SEM
Weight (kg)	518	526	517	453	503.5 ± 16.95
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)	0.0775	0.0923	0.1362	0.06875	0.094 ± 0.015
K_{10} (/h)	0.00649	0.00968	0.00865	0.01043	0.0088 ± 0.00086
AUC $_{o\text{-}inf.}$ (ng/mL/h)	376373.25	325729.38	359648.52	368735.10	357620 ± 11167
Oral clearance (mL/h)	3027.85	3552.64	3162.53	2702.75	3111.44 ± 175.9
T_{max} (h)	34.93	27.29	21.61	32.33	29.04 ± 2.94
C_{max} (ng/mL)	1805.98	2520	2618.745	2656.41	2400.28 ± 200.18
R^2	0.915	0.961	0.981	0.969	0.96 ± 0.015

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

2560 ng/mL to a highest value of 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. The predicted mean time required to achieve peak plasma concentration (T_{max}) following oral administration was 29 ± 3 (SEM) hours, with a mean K_{01} $t_{1/2}$ of 8 ± 1 (SEM) hours (Dirikolu *et al.*, 2009).

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. Analysis of plasma samples following both i.v. and oral administration indicated high bioavailability of toltrazuril sulfone in horses following oral administration in DMSO (Table 1). Mean bioavailability of toltrazuril sulfone in DMSO was 71% ± 3.6 (SEM). Predicted C_{max} from these horses ranged from 1806 ng/ml to 2656 ng/mL, with a mean C_{max} of 2400 ± 200 (SEM) ng/mL (Table 1). The mean plasma half-life of toltrazuril sulfone in these horses was 81 ± 9 (SEM) hours (SEM) (Table 1) (Dirikolu *et al.*, 2009).

Analysis of the plasma samples showed rapid absorption of toltrazuril sulfone sodium salt following oral-mucosal applications (Fig. 6). Observed plasma concentrations from four horses at 8 h postadministration were in close agreement, ranging from a lowest value of 2158 ng/mL to a highest value of 2900 ng/mL with a mean plasma concentration of 2400 ± 169 (SEM) ng/mL (Fig. 6). Thereafter, plasma concentration declined to 535 ± 153 (SEM) ng/mL at 168 h after administration, with an apparent average plasma elimination half-life of approximately 72 ± 12 (SEM) hours.

Following oral-mucosal administration of toltrazuril sulfone sodium salt, the predicted time to reach maximum plasma concentration (T_{max}) was 7.7 ± 1.8 (SEM) hours, with a mean $t_{1/2}$ K_{01} of 1.4 ± 0.5 (SEM) hours (Table 2). Predicted C_{max} of four horses was in close agreement, ranging from a lowest value of 2067 ng/mL to a highest value of 2875 ng/mL, with the mean C_{max} being 2342 ± 185 (SEM) ng/mL (Table 2).

Figure 7 compares the mean plasma concentrations of toltrazuril sulfone following oral administration as toltrazuril sulfone in DMSO and oral-mucosal administration as toltrazuril sulfone sodium salt. As different horses were dosed with

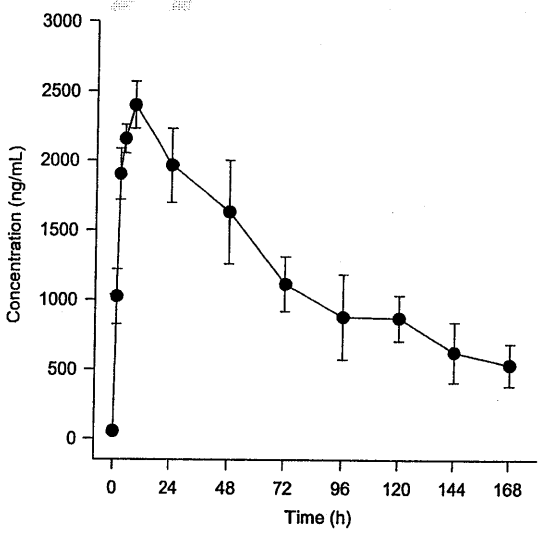

Fig. 6. Mean plasma concentrations of toltrazuril sulfone (±SEM) following a single 2.2 mg/kg oral-mucosal dose of toltrazuril sulfone sodium salt (n = 4).

toltrazuril sulfone sodium salt, each horse's pharmacokinetic parameters were compared with the mean pharmacokinetic parameters of toltrazuril sulfone when toltrazuril sulfone was administered as a single intravenous or oral administration in DMSO. The absolute bioavailability of toltrazuril sulfone sodium salt ranged from 35.5% to 82% with the mean bioavailability of about 56% ± 10.2% (SEM) (Table 2). The mean oral bioavailability of 56% for toltrazuril sulfone sodium salt indicates approximately 15% reduction in the bioavailability of toltrazuril sulfone sodium salt in comparison with toltrazuril sulfone in DMSO. Additionally, the peak plasma concentration of toltrazuril sulfone following administration as sodium salt was approximately 15% less than following toltrazuril sulfone administration in DMSO.

Analysis of the plasma samples showed rapid absorption of toltrazuril sulfone following administration of toltrazuril sulfone sodium salt with feed (Fig. 8). Peak plasma concentrations of toltrazuril sulfone were obtained at about 8 h postadministration and ranged from a lowest value of 1940 ng/mL to a highest

Table 2. Pharmacokinetic parameters of toltrazuril sulfone sodium salt following a single oral-mucosal dose (2.2 mg/kg powder)

Horse	1	2	3	4	Mean ± SEM
Weight (kg)	564	482	500	545	522.8 ± 19.09
F (%)	35.5	45.13	60	82.23	56 ± 10.2
$t_{1/2}$ K_{01} (h)	1.017	2.866	0.634	1.23	1.44 ± 0.49
$t_{1/2}$ K_{10} (h)	54	49.40	87	97	71.9 ± 11.85
K_{01} (/h)	0.6814	0.242	1.093	0.563	0.645 ± 0.176
K_{10} (/h)	0.0128	0.014	0.00797	0.00714	0.0105 ± 0.0017
AUC $_{0\text{-inf.}}$ (ng/mL/h)	173561.31	195538.56	276245.37	425472	267704 ± 57034
Oral clearance (mL/h)	7149	5423	3892	2818	4820.5 ± 942
T_{max} (h)	5.93	12.5	4.53	7.86	7.71 ± 1.74
C_{max} (ng/mL)	2067	2302.3	2123	2874.63	2341.7 ± 184.6
R^2	0.984	0.95	0.976	0.979	0.972

value of 3500 ng/mL, with a mean peak plasma concentration of 2740 ± 413 (SEM) ng/mL (Fig. 8). Thereafter, plasma concentrations declined to 677 ± 105 (SEM) ng/mL at 168 h after administration, with an apparent average elimination half-life of ~ 64 ± 7 (SEM) hour (Table 3).

Following administration of toltrazuril sulfone sodium salt with feed, the calculated time to reach maximum plasma concentration ranged between 2 and 17 h with the mean T_{max} being 7 ± 3.4 (SEM) h (Table 3). Predicted C_{max} (concentration maximum) of four horses was in relatively close agreement, ranging from a lowest value of 2017 ng/mL to a highest value of 3185 ng/mL, with the mean C_{max} being 2600 ± 303 (SEM) ng/mL (Table 3).

Figure 9 shows a comparison of the mean plasma concentrations of toltrazuril sulfone following the oral-mucosal administration of toltrazuril sulfone sodium salt and the oral administration of toltrazuril sulfone sodium salt with feed. Relative bioavailability of toltrazuril sulfone sodium salt with feed compared with the oral-mucosal administration of toltrazuril sulfone sodium salt was 93%, indicating approximately 4% reduction in absolute bioavailability of toltrazuril sulfone sodium salt when given as feed additive formulation. Additionally, the peak plasma concentrations following toltrazuril sulfone sodium salt administrations were similar when drug was given with and without feed. The absolute bioavailability of toltrazuril sulfone sodium salt when given as a feed additive formulation ranged from 33% to 72% with a mean bioavailability of 52% ± 8% (SEM), indicating approximately 19% reduction in absolute bioavailability of toltrazuril sulfone sodium salt as feed additive formulation compared with toltrazuril sulfone in DMSO.

After the administration of a single oral dose of toltrazuril sulfone (2.2 mg/kg) in aqueous solution to two horses, analysis of plasma samples showed detectable plasma concentrations following the oral administration of this aqueous solution (Fig. 10), with an observed mean peak plasma concentration of 772 ± 14 (SEM) ng/mL of toltrazuril sulfone at 24 h after administration (Dirikolu *et al.*, 2009). Observed plasma concentrations from two horses at 24 h postadministration were in

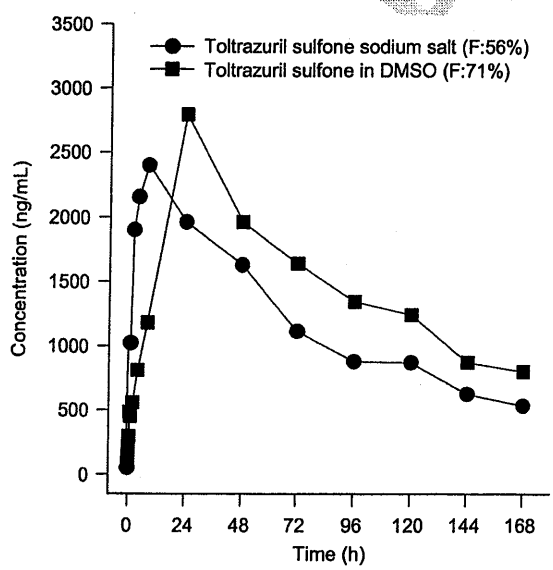

Fig. 7. Mean plasma concentration comparison of toltrazuril sulfone following a single 2.2 mg/kg oral dose in DMSO (n = 4) and oral-mucosal administration of toltrazuril sulfone sodium salt (n = 4).

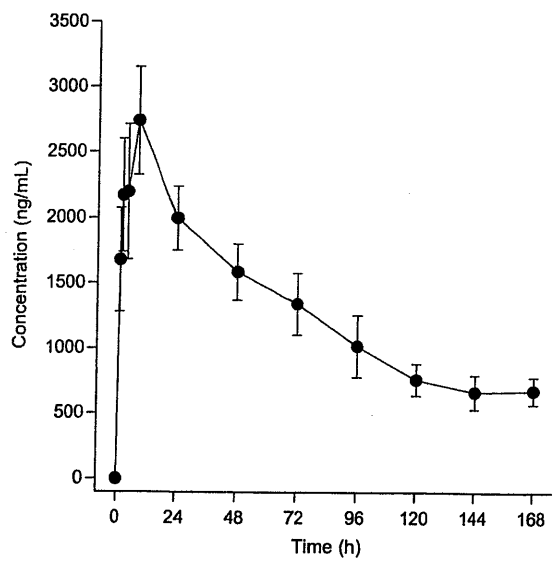

Fig. 8. Plasma concentrations of toltrazuril sulfone following 2.2 mg/kg oral dose of toltrazuril sulfone sodium salt mixed with 0.5 oz. beet pulp added to 1 lb. sweet feed (n = 4).

Horse	1	2	3	4	Mean ± SEM
Weight (kg)	573	546	554	536	552 ± 8
F (%)	72	52	33	50	52 ± 8
$t_{1/2}$ K_{01} (h)	0.932	0.514	0.28	3.72	1.36 ± 0.8
$t_{1/2}$ K_{10} (h)	81	56	48	71	64 ± 7
K_{01} (/h)	0.743	1.35	2.47	0.19	1.19 ± 0.49
K_{10} (/h)	0.0086	0.0123	0.014	0.0098	0.011 ± 0.0013
AUC $_{o-inf.}$ (ng/mL/h)	389901	258950	153350	242250	261110 ± 48785
Oral clearance (mL/h)	3233	4639	7948	4868	5172 ± 994
T_{max} (h)	6	3.5	2	17	7.2 ± 3.4
C_{max} (ng/mL)	3185	3057	2144	2017	2600 ± 303
R^2	0.99	0.98	0.99	0.96	0.98

Table 3. Pharmacokinetic parameters of toltrazuril sulfone sodium salt following a single oral dose with feed (2.2 mg/kg powder)

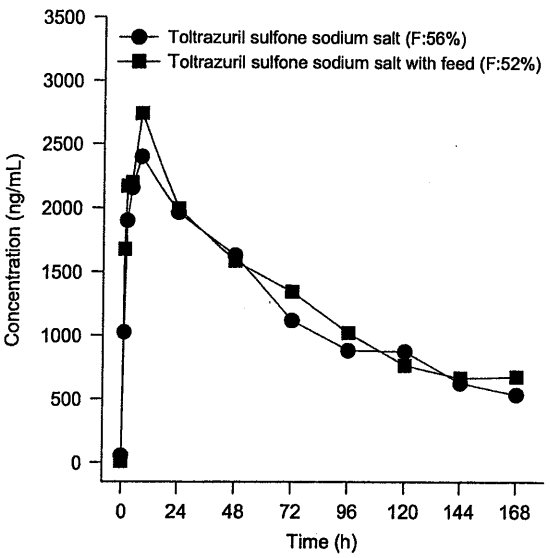

Fig. 9. Mean plasma concentration comparison of toltrazuril sulfone following a single 2.2 mg/kg oral-mucosal dose of toltrazuril sulfone sodium salt (n = 4) and oral administration of toltrazuril sulfone sodium salt with feed (n = 4).

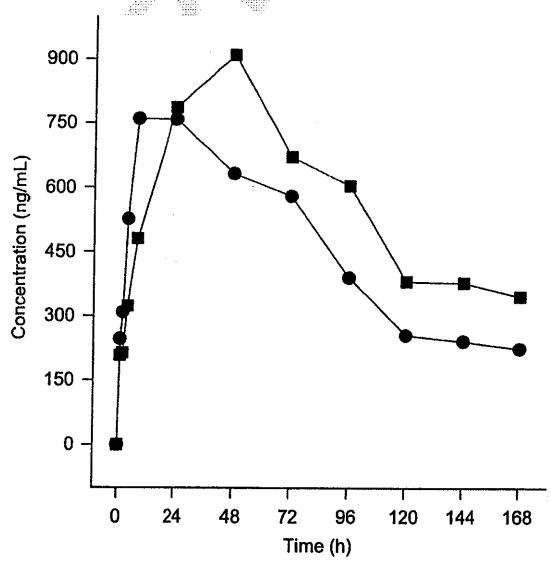

Fig. 10. Plasma concentrations of toltrazuril sulfone following a single 2.2 mg/kg oral dose in water from two horses.

close agreement with values from 758 to 786 ng/mL. The plasma concentrations of toltrazuril sulfone from these two horses were 633 and 910 ng/mL at 48 h postadministration with a mean plasma concentration of 771 ng/mL ± 138.5 (SEM) (Fig. 10). Thereafter, the plasma concentration declined to 286 ± 61 (SEM) ng/mL at 168 h after administration, with an apparent average half-life of approximately 77 ± 3.5 (SEM) hours. The predicted mean time required to achieve peak plasma concentration (T_{max}) following the oral administration was 24 ± 9 (SEM) hours, with a mean $t_{1/2}$ K_{01} of 6.2 ± 3 (SEM) hours (Table 4) (Dirikolu *et al.*, 2009).

Fig. 11 shows a comparison of the mean plasma concentrations of toltrazuril sulfone following the oral administration of toltrazuril sulfone suspended in water (2.2 mg/kg) and oral-mucosal administration of toltrazuril sulfone sodium salt (2.2 mg/kg). Relative bioavailability of toltrazuril sulfone suspended in water compared with toltrazuril sulfone sodium salt

Table 4. Pharmacokinetic parameters of toltrazuril sulfone following a single oral dose (2.2 mg/kg in water)

Horse	1	2	Mean ± SEM
$t_{1/2}$ 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)	0.2202	0.076	0.0904 ± 0.00406
K_{10} (/h)	0.0094	0.0086	0.009 ± 0.0004
AUC $_{o-inf.}$ (ng/mL/h)	102047	137164	119605 ± 17559
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

was 46%, indicating approximately 54% less bioavailability of toltrazuril sulfone in water following oral administration. On the other hand, the peak plasma concentration following toltrazuril

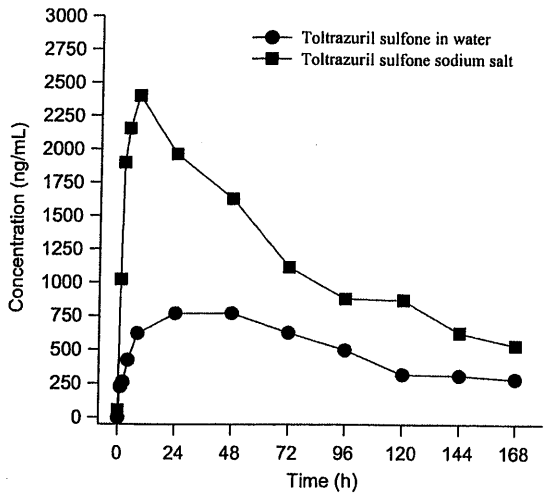

Fig. 11. Mean plasma concentration comparison of toltrazuril sulfone following a single 2.2 mg/kg oral dose in water (n = 2) and oral-mucosal administration of toltrazuril sulfone sodium salt (n = 4).

sulfone sodium salt administration was approximately three- to fourfold higher than following toltrazuril sulfone administration suspended in water.

DISCUSSION AND CONCLUSIONS

In previous studies, we identified triazine-based antiprotozoal agents as a potentially important therapeutic agent for use in the prevention and treatment of EPM (Granstrom *et al.*, 1997; Bentz *et al.*, 1998; Dirikolu *et al.*, 1999, 2006). It was also suggested that the oral bioavailability of triazine-based antiprotozoal agents may vary among individual horses in a clinically significant manner (Dirikolu *et al.*, 1999, 2006, 2009). Three possible approaches to improving the oral bioavailability of triazine-based antiprotozoal agents were proposed. The most practical one was the development of a formulation that would routinely yield effective plasma and CSF concentrations of these agents in all treated horses (Dirikolu *et al.*, 1999, 2006, 2009). The results of the present study show that the sodium salt formulation of toltrazuril sulfone is well absorbed following oral administration and has the potential to be used as a feed additive.

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 (Aungst, 1993). Additionally, poor oral bioavailability results in more variable and poorly controlled plasma drug concentrations and therefore highly variable therapeutic responses (Aungst, 1993). When bioavailability is low, inter- and intrasubject variability in bioavailability is 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 (Aungst, 1993). It was, therefore, important for us to maximize the oral bioavailability of triazine-based agents, with the goal of maximizing our ability to control plasma drug concentrations and thereby the clinical efficacy of these agents (Dirikolu *et al.*, 2006, 2009).

In the present study, we publish the results of our continuous effort to improve oral bioavailabilities and therefore clinical efficacy of triazine-based antiprotozoal agents in the horse. The results of this study showed that the sodium salt formulation of toltrazuril sulfone is well absorbed following oral administration and has the potential to be used as a feed additive formulation.

As described previously (Dirikolu *et al.*, 2006), the best way to determine the comparative bioavailability of different drug formulations is to use a Latin square design (crossover) by comparing each animal with itself as a control following appropriate washout periods. On the other hand, it should be remembered that the main goal of this study was not to determine the exact magnitude of the bioavailability of toltrazuril sulfone sodium salt but simply to show that this new formulation of toltrazuril sulfone is absorbed well in a clinically significant manner and has the potential to be used as a feed additive. Additionally, as indicated previously, our comparison of toltrazuril sulfone oral bioavailabilities in the aqueous suspension as a sodium salt and feed additive formulations vs. DMSO was linked to a comparison of F values estimated for these formulations on the basis of the drug exposure observed when toltrazuril sulfone was administered as an intravenous solution in DMSO. In doing so, there was a risk that any change in clearance or volume of distribution attributable to the presence of DMSO would have biased our estimate of F for the toltrazuril sulfone sodium salt, toltrazuril sulfone sodium salt as a feed additive and toltrazuril sulfone in aqueous suspension. To confirm the lack of bias in our estimate, we compared $t_{1/2}$ values in the aqueous suspension, toltrazuril sulfone sodium salt, and toltrazuril sulfone sodium salt as a feed additive 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 similar $t_{1/2}$ values (77, 72, and 64 vs. 81 h for the aqueous suspension, toltrazuril sulfone sodium salt, toltrazuril sulfone sodium salt as a feed additive and DMSO oral solution, respectively) provided assurance that observed profile differences primarily reflected the impact of the sodium salt formulation on oral absorption kinetics. Unfortunately, as toltrazuril sulfone sodium salt was not suitable for i.v. administration, we administered toltrazuril sulfone prepared in DMSO i.v. and evaluated the bioavailability of toltrazuril sulfone as a sodium salt in comparison with this i.v. formulation. On the other hand, as toltrazuril sulfone sodium salt is converted to toltrazuril sulfone following absorption (indicating identical physiochemical characteristics for distribution) and also as the amount of DMSO (approximately 3 mL in each adult horse given slowly) used for the i.v. administration of toltrazuril sulfone was relatively small, it is very unlikely that we had significant changes in the distribution of toltrazuril sulfone following i.v. administration either in DMSO or as a sodium salt, providing another assurance that observed profile differences primarily reflected the impact of the sodium salt formulation on oral absorption kinetics.

In conclusion, these data show that the sodium salt formulation of toltrazuril sulfone is very well absorbed following oral administration, similar to the results obtained with our diclazuril sodium salt, as previously described (Dirikolu *et al.*, 2006). As such, toltrazuril sulfone sodium salt is a very effective formulation to increase the rate and extent of absorption of toltrazuril sulfone to a clinically significant level. Toltrazuril sulfone sodium salt also has the potential to be used as feed additive formulations, especially for prophylaxis purposes, without significant effect on its rate and extent of absorption. The mean relative oral bioavailability of toltrazuril sulfone sodium salt as a feed additive was 93% compared with the administration of toltrazuril sulfone sodium salt without feed. This relatively highly bioavailable formulation of triazine-based agents also allows us to predict and control plasma drug concentration of any given dose with the goal of maximizing our ability to control the clinical efficacy of these agents. The findings presented here indicate that additional studies on the use of these compounds for the prevention and treatment of EPM and related apicomplexan diseases are warranted.

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