Cerebrospinal Fluid and Blood Concentrations of Toltrazuril 5% Suspension in the Horse After Oral Dosing*

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■ ABSTRACT

Toltrazuril 5% suspension (Baycox®, Bayer Canada, Ontario, Canada) was administered to six adult horses followed by blood collection and assay to determine the concentration of toltrazuril and its principal metabolites, toltrazuril sulfone and toltrazuril sulfoxide. From this data, the maximum concentration (C_{max}), elimination half-life ($T_{1/2}$), and mean residence times of the plasma were determined from standard pharmacokinetic formulas. After a single oral dose of 10 mg/kg body weight a rapid absorption was found, with a mean peak serum concentration of 11.17 mg/L at 18 hours. Elimination was prolonged, with a mean $T_{1/2}$ for elimination of 61.4 hours.

In addition, toltrazuril was administered to nine horses, and blood serum and cerebrospinal fluid (CSF) concentrations of toltrazuril and its principal metabolites were determined. Horses were randomly assigned to one of three treatment groups and received either 2.5 mg/kg body weight (Group A), 5.0 mg/kg body weight (Group B), or 7.5 mg/kg body weight (Group C) orally, once daily, for 10 days. Jugular venous blood was collected routinely on treatment days 2, 6, and 10, and CSF was collected on treatment day 10.

Assay of toltrazuril and its metabolites revealed a dose-dependent effect within both the blood and CSF compartments. Mean concentrations within the CSF after 10 days of treatment were 0.146 mg/L in Group A, 0.190 mg/L in Group B, and 0.386 mg/L in Group C. Toltrazuril sulfone was the primary metabolite after 10 days of treatment, with concentrations that ranged from 39% to 116% of the parent drug in individual animals. Toltrazuril sulfone was also the predominant metabolite in the serum at treatment day 10; however, it did not always exceed the concentration of toltrazuril sulfoxide in the serum on treatment day 2. In the serum, drug concentrations at treatment day 2 were variable in the low-dose group (Group A), ranging from 4.0 to 11.61 mg/L; less variable in the high-dose group (Group C), ranging from 9.9 to 10.46 mg/L; and intermediate in Group B.

This study confirms that toltrazuril is absorbed in the horse after oral administration and reaches effective in vitro concentrations within the CSF of the horse after once-daily dosing of 5 or 7.5 mg/kg. Although these data suggest that toltrazuril may have clinical value in the treatment of equine protozoal myeloencephalitis, clinical efficacy remains to be confirmed using appropriate methods. Effective in vitro concentrations are known; however, therapeutic concentrations in vivo have not been established. Further research in this area is needed to determine various drug values in the CSF (e.g., half-life, C_{max}, time to reach steady state).

■ INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is one of the most commonly diagnosed neurologic diseases of the horse. It is caused by infection of nervous tissue with the protozoan parasite *Sarcocystis neurona* and leads to chronic neurologic dysfunction. Clinical signs of ataxia, proprioceptive disorders, and neurogenic muscle wasting are most commonly seen; however, other clinical signs (e.g., cranial nerve deficits, blindness, behavioral disorders, and seizures) have also been reported.[1]

No medication is approved for the treatment of EPM; however, a combination of sulfadiazine and pyrimethamine has been used for many years.[1] Variable success has been accounted with this drug combination, but it is commonly reported that only 70% of treated animals will respond.[1] Horses are often left with residual neurologic deficits, and recrudescence has been reported as well. In an effort to identify better medications for treatment, other antiprotozoal compounds have been used in horses, including toltrazuril.

Toltrazuril is a triazinone compound used to treat neonatal swine coccidiosis[2] in Canada and several other countries. Toltrazuril has been shown to be effective against *Toxoplasma gondii*[3] and has documented efficacy in the treatment of coccidiosis in calves[4] and lambs.[5] Although toltrazuril has not been evaluated for its effectiveness against *S. neurona*, toltrazuril sulfone (i.e., ponazuril) has been shown to be effective in vitro against *S. neurona*. A concentration of 1 µg/mL toltrazuril sulfone inhibited the growth of *S. neurona* by 98.6%; a 94% reduction was found at 0.1 µg/mL.[6] Because of the broad antiparasitic spectrum of this compound and support from in vitro findings with *S. neurona*, toltrazuril has become an attractive treatment alternative for horses with EPM. Toltrazuril can be obtained for use in the United States for treatment of horses with EPM from Bayer Canada by filing a Personal Use Importation Request with the FDA-CVM.

Clinical treatment is commonly attempted at a dose of 5 to 10 mg/kg once daily, based upon a preliminary evaluation of pharmacokinetics by Tobin et al, in which it was found that the drug was well-absorbed after oral administration[7]; however, there has been no further evaluation of this drug since that initial study. At the onset of this study it was not known if the drug penetrates the CSF of horses and, if it does, what the appropriate dose for achieving adequate concentration in the CSF should be. The objectives of this study were to determine the oral absorption and pharmacokinetic parameters of toltrazuril after oral administration and to determine the concentration of toltrazuril and its primary metabolites in the CSF and serum of horses after a defined dose and treatment interval.

■ MATERIALS AND METHODS
Study 1: Oral Absorption and Pharmacokinetic Determination
Horses

Six adult horses (five Thoroughbred geldings and one mixed breed mare) were used to evaluate the oral absorption and pharmacokinetic

parameters of toltrazuril. The average weight was 542 kg (range 447 to 599 kg), and the horses' ages ranged from 3 to 21 years. Four horses had cervical compressive myelopathy and had lived at the research facility for many years (more than 5 years). Two horses were recent donations to the Equine Medical Center and had a tentative diagnosis of EPM of unknown duration. All horses were considered to be systemically healthy as determined by physical examination, baseline serum chemistry, and complete blood cell count. All horses were housed in indoor stalls during the experimentation period and were fed free-choice grass hay and water ad libitum and 1 lb of commercial sweet feed two times per day. Horses were routinely dewormed (Eqvalan®, Merck and Co Inc, Rahway, NJ) and vaccinated (Encevac TC-4, Bayer Animal Health, Shawnee Mission, KS) 1 week before commencement of the study. The protocol for this and the subsequent study (study 2, to follow) were evaluated and approved by the Virginia Tech Animal Care and Use Committee.

Drug Administration and Sampling

All horses were given one dose (10 mg/kg body weight) of toltrazuril by nasogastric tube followed by an equal volume of water to rinse the tube. Test article was supplied by the manufacturer in commercially packaged screw-top vials. Blood was collected immediately before dosing (time 0), then at 0.25, 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 hours after drug administration. Serum was collected by withdrawal after clotting and was frozen at –70˚C until analysis.

Pharmacokinetic Analysis

The best fit compartmental model and initial estimates of the model dependent on pharmacokinetic parameters of A1, A2, apparent K1, and apparent K2[8] were made using RSTRIP.[9] The procedure used to determine the best fit compartmental model involved computing the sum of the squares of the deviations:

$$F = \Sigma(Cobs - C\ est)^2.$$

The set of parameters obtained that minimized F was chosen as the best estimate. The compartmental model used is represented by the general equation

$$Cp = A1e^{-K1t} + A2e^{-K2t},$$

where A1 and A2 are the time 0 plasma drug concentrations, K1 is the apparent rate constant of absorption, and K2 is the apparent rate constant of elimination. Respective half-lives were computed from the relationship $T_{1/2} = 0.693/\text{rate constant}$.

The plasma concentration-time data were also analyzed using statistical moments.[10] The system moment mean residence time (MRT) was determined from the equation MRT = AUMC/AUC, where AUMC is the area under the curve of a plot of the product of time and plasma drug concentration time, from zero to infinity, and AUC is the area under the concentration-time curve from zero to infinity. The AUC and AUMC were calculated using the method described by Dunne and King.[11] Maximum plasma concentrations (C_{max}), plasma concentration at 24 hours (C_{24}), and time to maximum concentration (T_{max}) were determined from the mean values of concentration-time curves using 3 to 6 independent observations per dose time. All routine statistical methods were as described by Snedecor and Cochran.[12]

Study 2: Serum and Cerebrospinal Fluid Concentration after Multiple Doses
Horses

Nine adult mixed-breed horses, weighing between 419 and 614 kg, were used in the study. The study group comprised eight geldings and one mare, and the ages ranged from 3 to 24 years. Six of the nine horses used in this

phase of the study had been used in study 1 (previously described). There was a 1-month delay between the completion of study 1 and the commencement of this study (study 2). Four horses suffered from cervical compressive myelopathy; three horses had a putative diagnosis of EPM. The duration of illness in the horses with EPM was unknown. Those animals with cervical compressive myelopathy had been in residence at the equine medical center for several years, with the exception of one horse that had been recently diagnosed and in which the disease had been observed for only a short period. Cervical compressive myelopathy had been confirmed in all horses by cervical radiography and myelography. The remaining two horses had been donated to the medical center for chronic orthopedic disease. All animals were considered to be systemically healthy as determined by a physical examination and baseline serum chemistry analysis and complete blood cell count. Routine CSF analysis was normal in all horses. All horses were housed in indoor stalls and fed free-choice grass hay, water ad libitum, and 1 lb commercial sweet feed two times per day. Horses that had not been used in study 1 were routinely dewormed (Eqvalan®) and vaccinated (Encevac TC-4) 1 week before the commencement of the study. After completion of study 2, all horses were returned to pasture.

Administration and Sampling

At the beginning of the study, horses were assigned to one of three treatment groups by random draw. Horses were dosed with toltrazuril at 2.5 mg/kg (Group A), 5.0 mg/kg (Group B), or 7.5 mg/kg (Group C). Test article was supplied by the manufacturer in commercially packaged screw-top vials. Doses were given once per day at 24-hour intervals (± 0.5 hour) for a total of ten doses. The entire calculated dose was administered by placing a 2-oz dose syringe in the interdental space and depositing the dose into the rear of the horses' mouths at the base of the tongue.

Jugular venous blood was collected routinely, using sterile evacuated tubes, at 48 hours, 6 days, and 10 days after the first dose. Serum was collected by withdrawal after clotting and was frozen at –70°C until analysis. CSF was collected 24 hours after the final (tenth) dose using routine clinical methods. Horses were anesthetized by premedication with xylazine (1.0 mg/kg IV), followed by IV injection of ketamine (2.2 mg/kg). The atlanto-occipital site was surgically prepared, and approximately 10 mL of CSF was collected using a 3-in stiletted needle (Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, an aliquot of CSF was used to perform routine CSF analyses, including RBC count and calculation of albumin quotient to ensure that there was no peripheral blood contamination of the CSF sample. The remaining CSF and serum were frozen at –70°C until analysis.

Assay of Toltrazuril and Metabolites (Toltrazuril Sulfone and Toltrazuril Sulfoxide)

Serum and CSF were assayed for toltrazuril and its primary metabolites (toltrazuril sulfone and toltrazuril sulfoxide) by the analytical laboratory of Bayer AG (Monheim, Germany) using a proprietary technique. The sample was evaporated to dryness, then dissolved in potassium carbonate solution. The sample was then passed through an Extrelut disposable column with dichloromethane and ethyl acetate. Further cleanup was performed with a combination of an SCX-ion-exchange disposable column and a silica gel cartridge. The residue was then dissolved in the HPLC mobile phase. The concentration of the sample was then determined by HPLC analysis with fluorescence detection. The lower limit of detection was 0.01 mg/L. Internal

TABLE 1. Pharmacokinetic Parameters of a Single Oral Dose of 10 mg/kg Toltrazuril Suspension in Horses (N=6)

Parameter	Units	Mean (+ 1 SD)
Maximum observed concentration (C_{max})	mg/L	11.17 (2.16)
Time to maximum concentration (T_{max})	hours	18 (16)
Area under the curve (AUC)	mg/L∗h	503.6 (163.2)
Mean residence time (MRT)	hours	29.4 (5.7)
Absorption half-life ($T_{1/2}$abs)	hours	3.2 (1.0)
Elimination half-life ($T_{1/2}$elim)	hours	61.4 (29.4)
Time zero drug concentration (A1)	mg/L	13.42 (5.35)
Time zero drug concentration (A2)	mg/L	−13.42 (5.35)
Apparent rate constant for absorption (K1)	h-1	0.23 (0.08)
Apparent rate constant for elimination (K2)	h-1	0.012 (0.007)

standards were prepared in acetonitrile and water for calibration and standardization.

Data Analysis

Descriptive statistics were reported for each dosage group, and one-way analysis of variance was used to determine if CSF and/or serum concentrations varied with dosage level. Level of significance was preset at $P < .05$. Posttest pairwise comparisons were made using Tukeys test, with a family error rate of $P < .05$ and a individual error rate of $P < .022$. Pharmacokinetic analysis was determined as described previously. Data analysis was performed on a desktop PC using commercial statistical analysis software (Minitab, Minitab Inc, State College, PA).

■ RESULTS

Results indicate that the oral absorption of toltrazuril suspension was rapid, with an absorption half-life for serum of 3.2 ± 1.0 hours. At the first sampling point of 0.25 hours, the serum concentration was 0.055 mg/L. At 6 hours, the serum concentration was 9.49 mg/L. The time to the observed peak serum concentration (T_{max}) was 18 ± 16 hours and the maximum observed serum concentration (C_{max}) was 11.17 ± 2.16 mg/L. The C_{max} was consistent among five horses (range: 10.9 to 14.2 mg/L), with one horse demonstrating a low peak (7.6 mg/L) that was delayed (48 hours) relative to the other five horses (mean = 12 hours). This led to a fairly broad standard deviation for this mean C_{max}. The elimination of toltrazuril was slow, with a mean terminal half-life for serum of 61.4 hours. There was notable variability in the elimination of toltrazuril, ranging from 33.8 to 94.8 hours. Notably, no toxicity was seen in any horse. Results of the pharmacokinetic analysis are summarized in Table 1.

CSF analysis conducted on samples taken after 10 days of treatment found each sample to have a total RBC count of <5 RBC/μL, and the albumin quotient was within normal limits in all horses. These findings confirmed that there was no peripheral blood contamination of the CSF sample. A summary of the serum concentration of toltrazuril and its primary metabolites, toltrazuril sulfone and toltrazuril sulfoxide, after dosing is presented in Table 2. The concentration of toltrazuril, toltrazuril sulfone, and toltrazuril sulfoxide in CSF after 10 days of dosing are presented in Table 3.

Analysis revealed a dose-dependent effect within the serum for toltrazuril and both

TABLE 2. Serum Concentration of Toltrazuril and its Metabolites After Administration of Various Doses for 10 Days*

Dose (mg/kg)	Day 2 (mg/L)	Day 6 (mg/L)	Day 10 (mg/L)
Toltrazuril			
2.5	6.7 (4.3)	10.7 (2.1)	13.4 (3.3)
5.0	8.6 (1.1)	15.5 (2.4)	19.7 (4.6)
7.5	10.2 (0.4)	21.9 (5.0)	26.5 (4.6)
Toltrazuril Sulfoxide			
2.5	0.5 (0.4)	0.8 (0.3)	1.1 (0.3)
5.0	0.6 (0.05)	1.2 (0.1)	1.7 (0.2)
7.5	0.75 (0.18)	1.8 (0.4)	2.5 (0.7)
Toltrazuril Sulfone			
2.5	1.4 (2.0)	1.8 (1.2)	3.0 (0.9)
5.0	0.5 (0.1)	2.3 (0.3)	5.0 (0.9)
7.5	0.6 (0.2)	2.9 (0.2)	6.6 (0.4)

*Data are presented as mean (N = 3) plus standard deviation (in parentheses)

TABLE 3. Cerebrospinal Fluid Concentration of Toltrazuril and its Metabolites After Administration of Various Doses for 10 Days*

Dose	Number	Toltrazuril (mg/L)	Toltrazuril Sulfoxide (mg/L)	Toltrazuril Sulfone (mg/L)
2.5	3	0.146 (0.085)	0.073 (0.021)	0.090 (0.020)
5.0	3	0.190 (0.070)	0.110 (0.010)	0.157 (0.042)
7.5	3	0.386 (0.155)	0.147 (0.040)	0.223 (0.050)

*Data are presented as mean ± standard deviation (in parentheses)

metabolites (P = .02). A pairwise posterior test found only the low and high doses to differ. Drug concentration in the CSF did not differ for toltrazuril but did differ for both metabolites (P = .02). Similar to the blood results, posttest pairwise comparison results found the drug concentration of the CSF to differ between the 2.5 and 7.5 mg/kg body weight doses only. The serum concentration of toltrazuril was 70 to 100 times greater than in CSF. This differed from the serum:CSF ratio of the metabolites in that serum toltrazuril sulfoxide concentration was 16 times greater than CSF across all doses and

toltrazuril sulfone was 33 times greater in serum than CSF across all doses. Toltrazuril sulfone was the predominant metabolite in CSF after 10 days of treatment, with concentrations of toltrazuril sulfone that ranged from 39% to 116% of the concentration of the parent drug in individual animals. Toltrazuril sulfone was also the predominant metabolite in the serum at treatment day 10; however, it did not always exceed the concentration of toltrazuril sulfoxide in the serum on previous days. In the serum, drug concentration at treatment day 2 was variable in the low-dose group, ranging from 4.0 to 11.6

mg/L, and less variable in the high-dose group, ranging from 9.9 to 10.5 mg/L.

■ DISCUSSION

The results of this study indicate that toltrazuril is well-absorbed in the horse, with a long elimination half-life. A preliminary study of the absorption of toltrazuril in the horse, reported by Tobin et al, found an elimination half-life of 55 hours, with a lower C_{max} (4.5 µg/mL) than that found in this report. Given the very limited nature of the report of Tobin et al, it is difficult to make direct comparisons, but one difference could be the method of analysis.

In addition, the study reported here finds that toltrazuril and its metabolites do achieve detectable concentration in CSF of horses after oral dosing. The concentration achieved is adequate to inhibit the growth of *S. neurona* in vitro.[6] The effects of cervical compression on drug distribution and subsequent penetration into the CSF is unknown; however, in the horses of this study, albumin quotient and CSF index were not altered in horses with cervical compression and it appears that cervical compression had no effect upon drug distribution. Although all doses achieved adequate concentration, the mean concentration found in horses given the lowest dose (2.5 mg/kg) was marginal and was inadequate in one of three horses given that dose.

In EPM, the etiologic agent is found within the neurons, neuropil, and endothelial cells of the central nervous system (CNS). Hence, achieving effective concentrations of drug within the CNS is required for clearance of infection. The blood–brain barrier (BBB) excludes many compounds from the CNS; however, lipophyllic, nonpolar compounds and small molecular-weight drugs readily penetrate the BBB and accumulate within the CNS. In addition, the pH of the plasma and the CSF affect compounds that are organic acids or bases and alter their ability to penetrate the BBB.[13] Toltrazuril is a weak acid (pK_a 6.8) with high lipid solubility. As such, it is expected to penetrate CSF reasonably well. This is supported by the findings of this study. The variable serum:CSF ratios noted with toltrazuril, toltrazuril sulfone, and toltrazuril sulfoxide suggest different physicochemical characteristics that alter their ability to penetrate the BBB. It was not possible to determine from this study if there is any transport mechanism for these compounds, and it is presumed that they enter the CSF by passive diffusion.

Another factor that contributes to drug penetration into the CNS is damage to the BBB. Infection or inflammation can cause a breakdown in the integrity of the BBB and allow penetration of compounds that might normally be excluded. For example, penicillin is normally excluded from the CSF, but in purulent meningitis there is an increased permeability of the BBB and penicillin achieves an adequate concentration.[13] It has been reported that BBB competence can be determined from calculation of the albumin quotient of CSF. This has been determined in normal horses and has been found to be < 2.0.[14] A more recent report, however, has questioned the sensitivity of this method and has found that RBC of the CSF is a more sensitive means of determining the presence of peripheral blood contamination of the CSF than the albumin quotient.[15] For this reason, both methods were used to evaluate the CSF and confirm the absence of peripheral blood contamination. All CSF samples had <5 RBC/µL, and the albumin quotient was normal, confirming that there was no peripheral blood contamination of CSF, which might lead to altered drug concentrations. In most cases of EPM, the albumin quotient is normal and CSF analysis does not usually reflect inflammatory changes. Presence of *S. neurona* in the CNS does elicit localized inflammation, however, and this may lead to increased penetration of drug in a localized microenvironment surrounding the parasite.

According to the manufacturer, the primary metabolites of toltrazuril are toltrazuril sulfone and toltrazuril sulfoxide. The primary metabolite found in the serum and CSF of treated horses was toltrazuril sulfone (i.e., ponazuril). Although the concentration of toltrazuril necessary to inhibit *S. neurona* is not specifically known, ponazuril has significant activity in vitro.[6] Ponazuril apparently penetrates the CSF well, achieving effective in vitro concentrations and enhancing the total antiprotozoal drug within the CNS.

■ CONCLUSION

This study describes the absorption and pharmacokinetic parameters of toltrazuril in the horse. Further, it confirms the accumulation of toltrazuril and its metabolites within the CSF of horses after oral dosing with toltrazuril. This study demonstrates that a dosage of 5.0 or 7.5 mg/kg body weight once daily will achieve adequate concentrations of drug within the CSF. Further studies are needed to determine the concentrations that are achieved after the longer duration of dosing that is commonly used in the field as well as the duration of persistence of drug after withdrawal of treatment. In addition, the toxicity of toltrazuril in the horse is unknown at this time. Although toxic signs were not seen in this study, formal studies to evaluate toltrazuril for toxicity in horses are warranted and another manuscript has been prepared describing a formal toxicity study using this compound. The lack of toxic signs noted in this study is consistent with the primary author's experience in which horses given drug for 2 months or more have not shown any untoward signs. We know the drug concentration in plasma; however, further steps are needed to establish a half-life, C_{max}, time to reach steady state in CSF, and determine if CNS damage can affect the flow of CSF. There appears to be minimal effects upon CSF drug concentration from cervical compression.

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