NEW THERAPEUTIC APPROACHES FOR EQUINE PROTOZOAL MYELOENCEPHALITIS: SYNTHESIS AND PHARMACOKINETICS OF DICLAZURIL SODIUM SALT IN THE HORSE

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ABSTRACT

Diclazuril, ((+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrohydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile is a triazine- based anti-protozoal agents. It may have clinical application in the treatment of equine protozoal myeloencephalomyelitis (EPM). In this study, we describe the use of the sodium salt of diclazuril to increase the oral bioavailability of the drug for the treatment and prophylaxis of EPM and various other Apicomplexan mediated diseases. We had earlier identified triazine-based antiprotozoal agents for the treatment and prophylaxis of EPM in the horse, and *in vitro* studies confirm that *S. neurona* is sensitive to triazine-based antiprotozoal agents. On this basis, we elected to develop a highly bioavailable oral formulations of diclazuril. Diclazuril sodium salt was synthesised for the evaluation of these formulations for the treatment and prophylaxis of EPM. This paper also describes absorption, distribution and elimination characteristics of diclazuril sodium salt in the horse.

The experimental data showed that diclazuril was absorbed rapidly, with peak plasma concentrations occurring at 8–24 h following an oral mucosal administration of diclazuril sodium salt. The mean oral bioavailability of diclazuril as Clinacox® was 9.5% relative to oral mucosal administration of diclazuril sodium salt. Additionally, diclazuril in DMSO administered orally was 50% less bioavailable than diclazuril sodium salt following an oral mucosal administration.

It was also shown that diclazuril sodium salt has the potential to be used as a feed additive for the treatment and prophylaxis of EPM and various other Apicomplexan mediated diseases. Administrations of diclazuril sodium salt with feed reduced the mean oral bioavailability of diclazuril by 50%, relative to that obtained from the oral mucosal administrations of diclazuril sodium salt. Based on these data, we concluded that repeated oral mucosal administration of diclazuril sodium salt with or without feed will yield effective steady state plasma and CSF concentrations of diclazuril for the treatment and prophylaxis of EPM and other protozoal diseases of animals and man.

INTRODUCTION

The neurological disease now known as equine protozoal myeloencephalomyelitis (EPM) was first discussed at the American Association of Equine Practitioners annual convention in 1968 in Philadelphia (Rooney *et al.* 1970). Forty-four cases of what came to be known as EPM were accumulated by research at that time (Prickett 1968). The causative parasite, *Sarcocystis neurona* (Phylum: Apicomplexa), was isolated for the first time from the spinal cord of a horse from New York in bovine monocyte cell cultures (Dubey *et al.* 1991). More recently, a similar neurological disease has been described in horses from which Neospora species have been isolated (Daft *et al.* 1997). Even though the organism is recognised by antibodies against *N. caninum*, it has been reported that this organism represents a new species, *N.*

hughesi and differences in the amino acid sequence of 2 immunodominant surface antigens support suggestions that this organism is a new aetiological agent in horses (Marsh *et al*. 1999).

The epidemiology and economic significance of *S. neurona* infection is substantial. In endemic areas in the US, over 40% of horses (45–60%) are seropositive for this protozoa (Granstrom *et al*. 1997; Rossano *et al*. 2001). Of animals clinically affected, 30–40% reportedly fail to respond to current therapy (pyrimethamine/sulfanamides combination) and some of these animals die (Granstrom *et al*. 1997). Additionally, while this combination therapy is successful in many cases, the treatment can be prolonged and the occurrence of relapses after cessation of treatment is not uncommon. Current treatments also carry significant toxicity risks and therefore, safer and more effective and less toxic prophylactic and therapeutic procedures are desirable.

Toltrazuril sulfone (Ponazuril®) and toltrazuril sulfoxide, are the major metobolites of toltrazuril following oral administration as Baycox® at 10 mg/kg (Furr and Kennedy 2000). Toltrazuril sulfone (Ponazuril®) has recently been evaluated for the treatment of EPM (MacKay *et al*. 2000). Toltrazuril sulfone (Marquis®, Bayer Corp., Kansas, USA) has been approved by the Food and Drug Administration (FDA) for the treatment of EPM.

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). On this basis, we elected to develop a highly bioavailable oral formulations of diclazuril namely diclazuril sodium salt. The sodium salt formulation of diclazuril was tested for its ability to increase the bioavailability of orally administered diclazuril for the treatment and prophylaxis of EPM.

Materials and methods

Horses and sample collection

Horses were provided by Saxony Farms, Kentucky, and were maintained on grass hay and feed (12% protein). The feed was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. Horses were not fed for at least 1 h after oral administration of the drug. The animals were vaccinated annually for tetanus and were de-wormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). Horses were kept in a 20-acre field until they were placed in box stalls and provided with water and hay *ad libitum*.

Sodium salt formulation of diclazuril was investigated to determine its use as a treatment for EPM. Four mature Thoroughbred mares weighing 518–564 kg were used to determine the clinical usefulness of oral mucosal administration of diclazuril sodium salt for the treatment of EPM. Diclazuril sodium salt was administered by direct application of 2.2 mg/kg to the oral mucosa. Blood samples were obtained from the right jugular vein for analyses at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168 h into heparinised tubes. All samples were centrifuged at 4°C and 2000 *g* for 15 min, and the plasma aspirated off and stored at -20°C until assayed.

Four mature Thoroughbred mares weighing 495–536 kg were used for determination of the usefulness of diclazuril sodium salt as a feed additive formulation. Diclazuril sodium salt was administered as a feed additive at 2.2 mg/kg. Blood samples were obtained from the right jugular vein for analyses at 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h, plasma samples were prepared and stored as described above.

Four mature Thoroughbred mares weighing 461–576 kg were used for oral administration of diclazuril as Clinacox®, a poultry feed premix imported from Pharmacia-Upjohn, (Ontario, Canada; Dirikolu *et al*. 1999). Clinacox® is 0.5% diclazuril, 99.5% protein carrier. It was administered to horses at a single dose of 5 mg/kg of diclazuril suspended in 6–8 l of water, by nasogastric intubation (as 500 g Clinacox®). Plasma samples were collected, prepared and stored as described above. Additionally, 4 mature Thoroughbred mares weighing 480–556 kg were dosed orally with 2.2 mg/kg diclazuril in DMSO. This solution was prepared by dissolving 100 mg of diclazuril in DMSO. Plasma samples were collected, prepared and stored as described above

Diclazuril analysis

Synthesis of diclazuril sodium salt

Diclazuril powder (100 g) was obtained from New Ace Research Company (Kentucky, USA). To a hot suspension of 20 g of diclazuril in 300 ml absolute ethanol a freshly prepared solution of sodium ethanolate (obtained from 1.18 g, 1.05 mol. Eq. of sodium) in 100 ml absolute ethanol was slowly added keeping the colour of the reaction mixture light brown. After stirring for 1.5 h at 70°C the solvent was evaporated under reduced pressure and the residue was dried under high (less than 1 mmHg) vacuum to obtain 21.2 g (100% yield) of diclazuril sodium salt as amorphous brownish powder. The obtained salt is very soluble in water giving an almost neutral solution.

High pressure liquid chromatography (HPLC)

Sample preparation: Diclazuril was analysed as described by Dirikolu *et al.* (1999). A standard solution of 1 mg diclazuril (Janssen R 64433) was prepared in 1 ml HPLC grade dimethylformamide (DMF) (Sigma-Aldrich 27,054-7). Working standards at 0, 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 µg/ml were prepared by adding specific amounts of the stock diclazuril standard at 0.01, 0.1 and 1 µg/ml in DMF/water (1:1) to 1 ml aliquots of diclazuril free horse plasma. Janssen compound R 62646, a structural analogue of diclazuril, was used as the internal standard. The internal standard was prepared in 1 ml DMF (1 mg/ml) and diluted 1 to 10 in 50% DMF/50% water to yield a 0.1 µg/ml standard solution. Twenty µl of the internal standard solution and 2 ml of 0.1 M potassium phosphate buffer (pH 6.0) were added to 1 ml of plasma sample or standard.

Extraction method: Analytichem C-18 'Mega Bond Elut' columns were placed into 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. Prepared plasma samples were drawn slowly through the column over, at least, a 2 min period. The column was then rinsed sequentially with 2 ml of 0.1 M potassium phosphate buffer (pH 6.0), 2 ml of 1.0 M acetic acid, and 2 ml of hexane. The column was allowed to dry for 5 to 10 min after each rinse. The column was eluted with 4 ml of methanol:HCl (95:1) and the eluent was collected in a silanised glass tube. The solvent was evaporated in tapered bottom tubes under a stream of nitrogen gas at 40°C. The residue was re-suspended first in 100 µl of DMF with moderately vigorous vortexing and sonication. After that, 100 µl of water was added resulting in a 200 µl DMF: water (1:1) solution. This solution was placed into a 300 µl vial for HPLC analysis.

Instrumentation: The HPLC procedure was adapted from that described by Kock *et al.* (1992). The instrument employed was a Beckman System Gold HPLC with 2 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 x 15 cm column. The mobile phase consisted of 46% Solvent A and 54% Solvent B run with a flow rate of 1 ml/min. Solvent A was 80% [0.5% ammonium acetate, 0.01 M tetrabutylammonium hydrogen sulfate (TBAHS) (Sigma # 39684-2) in water] : 20% acetonitrile. Solvent B was 80% methanol : 20% acetonitrile. The diode array detector was set up for single wavelength acquisition at 280 nm with a 12 nm bandwidth. Injections were made with a 20 µl loop.

Pharmacokinetic analysis: Pharmacokinetic analyses were performed, using a non-linear regression programme (Winnonlin, version 3.1) (Pharsight Corporation, North Carolina, USA). The area under the log plasma drug concentrations versus time curve (AUC) was measured by use of a linear trapezoidal approximation with extrapolation to infinity. The slope of the terminal portion (K_{10}) of this curve was determined by the method of least-squares regression (Gibaldi and Perrier 1982).

The compartmental model used is represented by general equation *a* where, Cp is the plasma concentration of compound at any time point (t), A is the Y intercept associated with the terminal elimination phase, K_{01} is the apparent rate constant of absorption, and K_{10} is the apparent rate constant of elimination. The rate constant of absorption (K_{01}) and the absorptive half-life ($t_{1/2}$ K_{01}) were determined, using the method of residuals. 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 (Clo) was calculated by Equation 2

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

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

$$C_{max} = A \times e^{-K10Tmax} - A \times e^{-K01Tmax} \quad (3)$$

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

The relative bioavailabilities (F) of diclazuril as Clinacox®, in DMSO and as a feed additive formulation were calculated from the $AUC_{0\text{-inf}}$ ratio comparison with the diclazuril sodium salt by equation 5.

$$F = AUC_{0\text{-inf}}\ (\text{Diclazuril sodium salt}) / AUC_{0\text{-inf}}\ (\text{Other formulations}) \times \text{Dose (Other formulations)} / \text{Dose (Diclazuril sodium salt)} \quad (5)$$

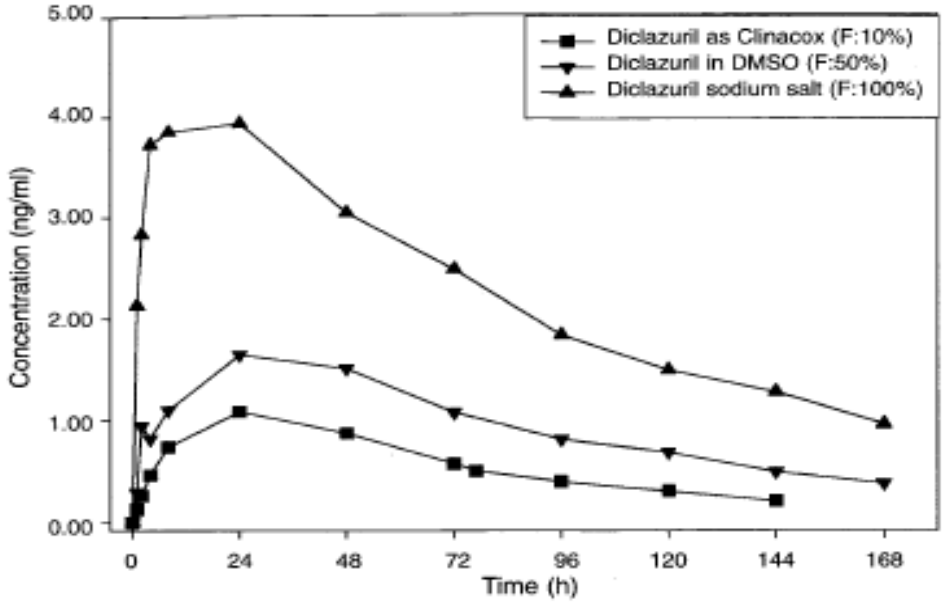

Fig 1: Comparison of mean plasma concentrations of diclazuril following single 2.2 mg/kg oral administration in DMSO (n=4), 5 mg/kg as Clinacox® (n=4) and 2.2 mg/kg oral-mucosal administration of diclazuril sodium salt (n=4).

Results

Analysis of the plasma samples showed rapid absorption of diclazuril following these oral-mucosal applications. Peak plasma (± se) concentrations of 3.93 ± 0.154 mg/ml of diclazuril were observed at about 8–24 h post administration. Thereafter, plasma concentration declined to 0.964 ± 0.058 µg/ml (se) at 168 h after administration with an apparent average elimination half-life of ~ 78 h. Following oral-mucosal administration of diclazuril sodium salt, the observed peak plasma concentrations were in close agreement, ranging from 3.69 µg/ml to 4.37 µg/ml with a mean peak plasma concentration of 3.93 ± 0.154 µg/ml (se) at 24 h post administration.

Figure 1 shows a comparison of the mean plasma concentrations of diclazuril following oral administration of diclazuril as Clinacox® (5 mg/kg; Dirikolu *et al.* 1999), diclazuril in DMSO (2.2 mg/kg) and oral-mucosal administration of diclazuril sodium salt (2.2 mg/kg). The bioavailabilities of diclazuril as Clinacox® ranged from 5% to 14% relative to oral-mucosal administrations of diclazuril sodium salt with a mean bioavailability of about 9.5%. Relative bioavailability of diclazuril in DMSO compared to oral-mucosal administration of diclazuril sodium salt was 50% indicating approximately 1/2 less bioavailability of diclazuril in DMSO following oral administration.

Analysis of the plasma samples showed rapid absorption of diclazuril following oral mucosal administration of diclazuril sodium salt with feed. Peak plasma concentrations of diclazuril were obtained within 4–24 h post administration and ranged from 2 µg/ml to 3.2 µg/ml. Thereafter, the plasma concentration declined to 0.345 ± 0.140 µg/ml (se) at 168 h after administration with an apparent average elimination half-life of ~ 54 ± 3.85 h. The relative oral bioavailabilities of diclazuril sodium salt as a feed additive formulation compared to oral-mucosal administration of diclazuril sodium salt ranged from 34% to 54% with a mean oral bioavailability of 45 ± 4.7% (se).

Discussion and conclusions

In earlier studies (Granstrom *et al.* 1997; Bentz *et al.* 1998; Dirikolu *et al.* 1999), triazine-based antiprotozoal agents were identified as a potentially important therapeutic agent for use in the treatment of EPM. Triazine-based antiprotozoal agents are known for their lipophylic characteristics and they might be expected to be well absorbed following oral administration. Additionally, the absorption of compounds from the gastrointestinal tract depends on the physiochemical properties of the compound, such as lipid solubility and dissociation rate (Houston *et al.* 1974). It is often generalised that an increase in lipid solubility increases the absorption of a drug from the gastrointestinal tract. However, extremely hydrophobic compounds, such as triazine anti-protozoal drugs, have low solubility in gastrointestinal fluids, which results in low absorption and bioavailability (Houston *et al.* 1974). If the compound is a solid and is relatively insoluble in gastrointestinal fluids, it will have limited contact with the gastrointestinal mucosa and, therefore, its rate of absorption will be low.

Bioavailability is an important parameter in clinical trials because the majority of a drug's therapeutic and toxicological effects are proportional to both dose and bioavailability. Additionally, poor oral bioavailabiliy results in variable and poorly controlled plasma concentrations and drug effects. It was therefore, important to maximise oral bioavailability of triazine-based agents with the goal of maximising the ability to control plasma drug concentrations and therefore the clinical efficacy of these agents.

In a previous study, it was suggested that the oral bioavailabilities of triazine-based antiprotozoal agents may vary between individual horses in a clinically significant manner (Dirikolu *et al*. 1999). Three possible solutions to this problem were proposed. The most practical one was the development of a formulation of these drugs that would provide greater oral bioavailablity. The results of this study showed that the sodium salt formulation of diclazuril is well absorbed following oral administration and has the potential to be used as a feed additive.

In conclusion, there is substantial preliminary evidence that sodium salt formulations of diclazuril can be expected to: increase significantly the therapeutic and prophylactic efficacy of a given dose of the active agent as compared to current formulations; improve dosing characteristics by reducing inter- and intra-subject variability in treatment response groups due to very poor and highly variable absorption of current formulations; reduce potential development of drug-resistant strains of disease due to survival, adaptation and selection among parasites in under-treated subjects having lower absorption rates of existing diclazuril treatments; improve our ability to show clinical trial efficacy against a broader range of protozoan-mediated diseases; and enable development of species-specific, easily administered pharmaceutical products, including feed additive formulations, for both treatment and prophylaxis of EPM and various other Apicomplexan-mediated diseases. The findings presented here indicate that additional studies are warranted on the use of these compounds for treatment of EPM.

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