DETECTION, QUANTIFICATION AND PHARMACOKINETICS OF DICLAZURIL IN THE HORSE: A PRELIMINARY REPORT

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ABSTRACT

Diclazuril (4-chlorophenyl [2,6-dichloro-4-(4,5-dihydro-3H-3,5-dioxo-1,2,4-triazin-2-yl)phenyl] acetonitrile) is a benzeneacetonitrile antiprotozoal agent (Janssen Research Compound R 64433) marketed as Clinacox. Diclazuril may have a clinical application in the treatment of equine protozoal myeloencephalitis (EPM), an infectious apicomplexan disease caused by *Sarcocystis neurona*. To evaluate its bioavailability and preliminary pharmacokinetics in the horse we developed a sensitive quantitative high pressure liquid chromatography (HPLC) method for diclazuril in equine biological fluids. Diclazuril was isolated from equine plasma by solid phase extraction with an Analytichem C-18 'Mega Bond Elut' column, eluted with acidified methanol and evaporated. The residue was dissolved in 100 µl N,N-dimethylformamide (DMF) and 100 µl water. A 20 µl aliquot was injected onto a Beckman Ultrasphere ODS column and quantified at 280 nm with a photodiode array detector. Janssen compound R 62646 was used as the internal standard. Peaks for both compounds were symmetrical and the standard curve was linear from 250–10,000 ng/ml. The limit of detection for diclazuril in plasma was 10 ng ml

After administration of a single oral dose of diclazuril at 2.5 g/1,000 lbs (500 g Clinacox), plasma samples from 4 horses showed good oral absorption of diclazuril. Plasma concentrations peaked 24 h after administration with a mean (± se) plasma concentration of 1,077 ± 174 ng/ml and declined to 208 ± 58 ng/ml at 144 h. When the same dose was administered daily for 21 days to 2 horses, a mean steady-state plasma concentration of 7–9 µg/ml was attained. Steady-state levels in the cerebrospinal fluid ranged between 100–250 ng/ml. No parent diclazuril was detected in the urine samples by HPLC. Similarly, routine post race thin layer chromatography (TLC) did not detect diclazuril or any apparent metabolites in urine samples.

INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a chronic infectious neurological disease of horses caused by *Sarcocystis neurona*. Current treatments including pyrimethamine/sulphonamide combinations are successful in many cases, but resistance to treatment or relapse after cessation of treatment is common.

Diclazuril is a benzeneacetonitrile anti-coccidial agent with proven efficacy against intestinal *Eimeria* species in avians, intestinal and hepatic coccidiosis in rabbits and toxoplasmosis in mice (Lindsay *et al.* 1995). Diclazuril has also been evaluated for treatment of isosporiasis and cryptosporidiosis in AIDS patients. Diclazuril and other benzeneacetonitriles may be selectively toxic for apicomplexans (Hackstein *et al.* 1995) and preliminary data suggest that it may have clinical application in the treatment of EPM with significant *in vivo* activity against *S. neurona* (Granstrom *et al.* 1997).

This pilot study describes the development and characterisation of a quantitative HPLC method for diclazuril in equine biological fluids and includes a preliminary evaluation of the pharmacokinetics of this agent in the horse. Because diclazuril may be used in performance horses, we also performed a preliminary evaluation of its detectability in equine urine by HPLC and TLC.

MATERIALS AND METHODS

Horses and sample collection

Four mature Thoroughbred mares weighing 461–576 kg were used for this study. Diclazuril was obtained in small amounts as the authentic standard; the recommended internal standard was also obtained from Janssen Pharmaceuticals (Beerse, Belgium). For administration to horses the only form of diclazuril available to us was Clinacox, a poultry feed pre-mix

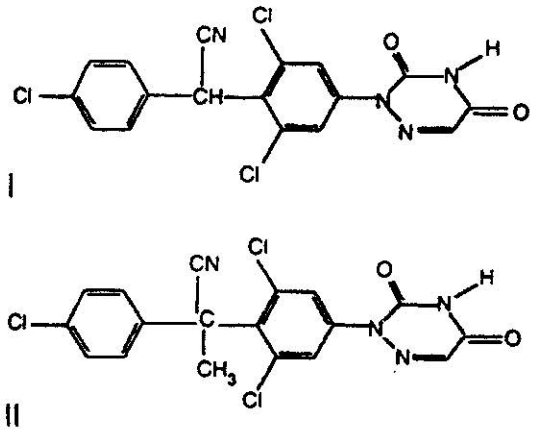

Fig 1: Structures of diclazuril (I) and internal standard Janssen compound R62646 (II)

imported from Canada (Pharmacia-Upjohn, Ontario, Canada), consisting of 0.5% diclazuril, 99.5% protein carrier. It was administered to horses by nasogastric intubation at a dose of 5 mg/kg bwt, suspended in 6–8 l of water. Blood samples were collected into heparinised tubes that were centrifuged at 2,000 rmp for 15 min before the plasma was removed and stored at -20°C until analysed.

In a second experiment, diclazuril (5 mg/kg bwt as Clinacox) was administered daily for 21 days to 2 horses with a presumptive diagnosis of EPM. Blood samples were collected as described above and urine was collected using a Foley catheter with an attached plastic bag. Urine was divided into aliquots and stored at -20°C until assayed. Cerebrospinal fluid (CSF) samples were collected routinely from the lumbosacral space by a licensed veterinarian. If there was no visible evidence of blood contamination, the CSF was retained and stored in serum tubes at -20°C until analysed.

High pressure liquid chromatography

Sample preparation: A standard solution (1 mg/ml) of diclazuril (Janssen R 64433) in HPLC grade N, N-dimethylformamide (DMF [Sigma-Aldrich 27,054-7]) was prepared. Standards were prepared by the addition of a specified amount of diclazuril, in 50% DMF/50% water, to blank plasma sample (1 ml each) over a range from 250–10,000 ng/ml. As described previously, Janssen compound R 62646, a structural analogue of diclazuril, was used as the internal standard. Structures of diclazuril and the internal standard are shown in Figure 1. The internal standard was prepared in 1 ml DMF and diluted 1–10 (100 ng/µl) in 50% DMF/50% water. To each sample, 20 µl of 100 ng/µl internal standard was added, followed by 2 ml of 0.1 M phosphate buffer (pH 6.0). The pH was adjusted to 6 as necessary.

Extraction method: Analytichem C-18 'Mega Bond Elut' columns were placed in an SPS24 VacElut vacuum chamber and treated sequentially with 2 ml HPLC grade methanol and 2 ml 0.1 M phosphate buffer (pH 6.0). The vacuum was turned off as soon as the buffer reached the top of the sorbent bed to prevent the column from drying. The specimen was drawn slowly through the column, taking approximately 2 min in total. The column was rinsed sequentially with 2 ml 0.1 M phosphate buffer (pH 6.0), 2 ml 1.0 M acetic acid and 2 ml hexane. The column was allowed to dry for 5–10 min after each rinse. A labelled silicanised glass conical based tube was placed below the column and an eluate was collected by slowly rinsing the column with 4 ml methanol:HCl (95:5), the solvent was then evaporated under a stream of nitrogen gas at 40°C. The resulting residue was re-suspended in 100 µl of DMF aided by moderately vigorous vortexing and sonication. Finally, 100 µl water was added and the re-suspension procedure was repeated to give a 50:50 (DMF:water) solution. This was placed in a 300 µl insert-containing HPLC vial for injections, ready for analysis.

Instrumentation: The HPLC procedure was a modification of 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 (Beckman, California, USA). The column was a Beckman Ultrasphere ODS, 5 µ particle size and 4.6 mm x 15 cm column size, protected with an Altech C-18 guard 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] in water):20% aceto-nitrile. Solvent B was 80% methanol, 20% acetonitrile. Acetonitrile (Fisher Scientific, New Jersey, USA) and methanol (EM Science, New Jersey, USA) were HPLC grade. After preparation, solvents A and B were de-gassed and filtered through 0.45 µm type HV millipore filtres. The diode array detector was set for single wavelength acquisition at 280 nm with a 12 nm span, using 20 µl injections and 20 µl loop.

Results

The limit for detection of diclazuril in plasma was 10 ng/ml by HPLC. Satisfactory recovery (82%) was obtained for solid phase extraction of diclazuril from equine plasma samples using HPLC. The diclazuril peak eluted at around 13 min (±50 min), and the internal standard peak eluted at 14 min (±50 min). The peaks were symmetrical and the standard curve was linear from 250–10,000 ng/ml

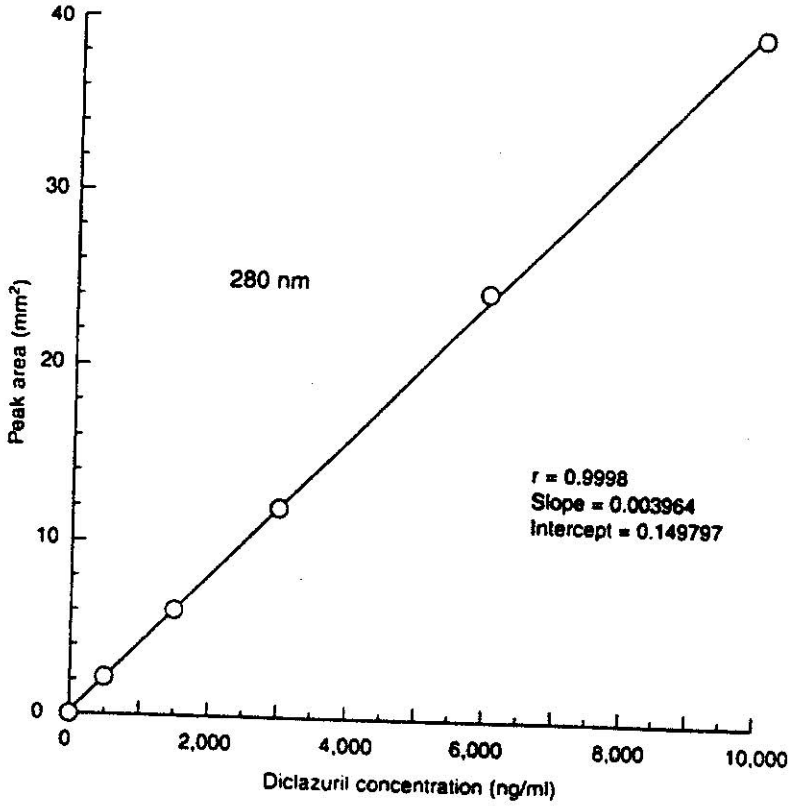

Fig 2: Standard curve of diclazuril concentrations in mobile phase (50% water, 50% DMF) extracted from plasma by HPLC.

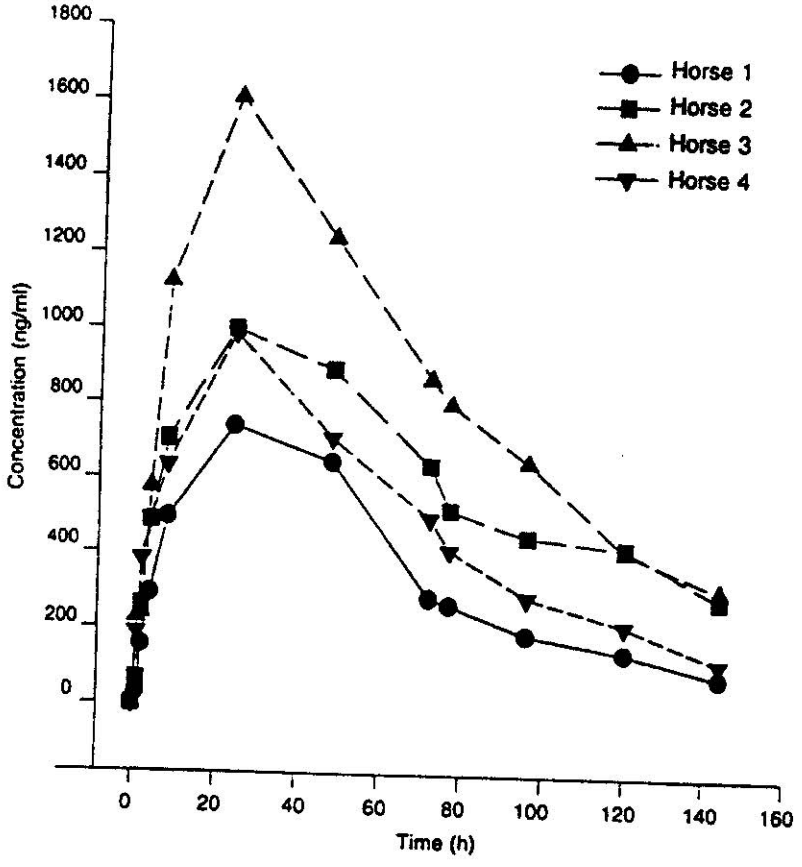

Fig 3: Plasma concentrations of diclazuril in 4 horses following a single oral dose of Clinacox (2.5 g/1,000 lbs).

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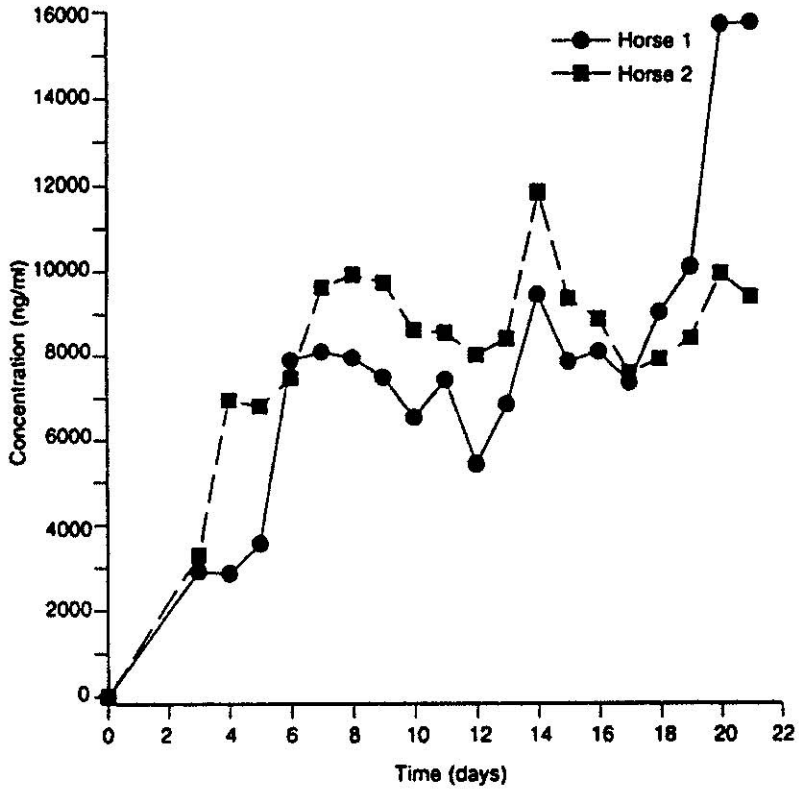

Fig 4 Plasma and steady state plasma concentrations of diclazuril from 2 horses following daily oral administration of Clinacox at 2.5 g/1,000 lbs.

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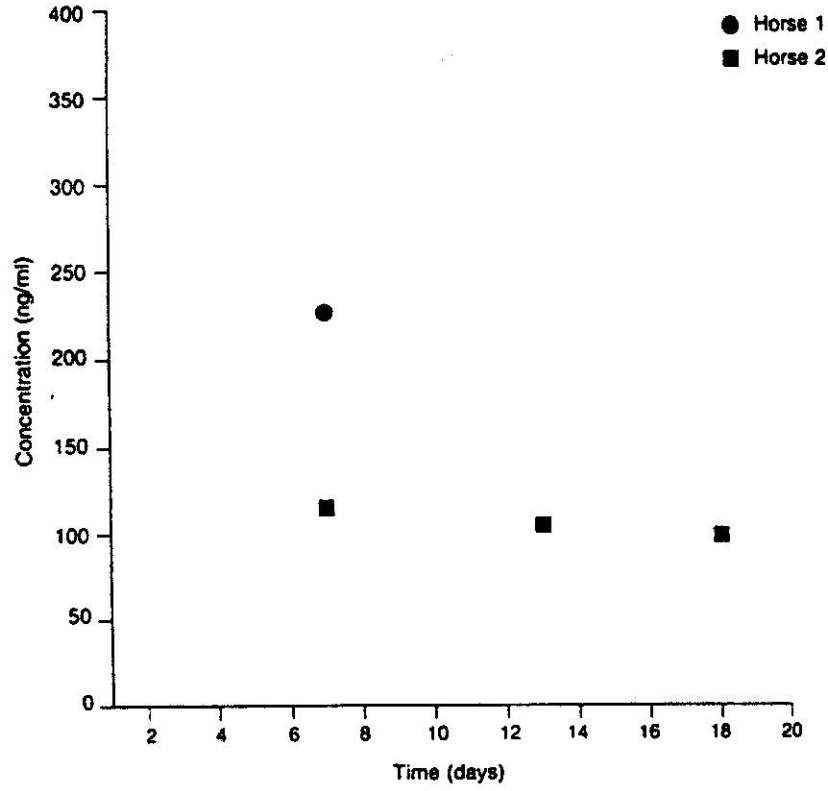

Fig 5: Cerebrospinal fluid concentrations of diclazuril at steady state plasma concentrations of diclazuril from 2 horses following daily oral administration of Clinacox at 2.5 g/1.000 lbs.

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with an r value of 0.9998 (Fig 2). The areas under the curves for diclazuril and the internal standard were recorded; and the internal standard values were used to normalise the diclazuril areas. Integrated peak values were entered into the QuattroPro for Windows programme for statistical analysis of standards and for interpolation of unknown amounts of diclazuril. Standard curves were generated with Sigma Plot for Windows.

After the single oral dose of diclazuril (in the form of Clinacox), analysis of plasma samples showed good oral absorption (Fig 3), with a peak mean (± se) plasma concentration of 1,077 ± 174 ng/ml occuring 24 h after administration. Thereafter, the plasma concentration declined to 208 ± 58 ng/ml at 144 h after administration with an apparent $t_{1/2}$ of approximately 48 h.

Daily administration of Clinacox yielded steady state plasma concentrations of diclazuril at 7–9 µg/ml within approximately 6–8 days (Fig 4). Steady state concentrations of diclazuril in the CSF samples ranged from 100–250 ng/ml (Fig 5). No significant amount of parent diclazuril was detected in urine samples using this HPLC methodology.

In the case of TLC analysis, standard diclazuril was spotted on the TLC plates along with the urine residues. Diclazuril yielded a faint reaction with Dragendorff's reagent followed by cupric chloride and sodium nitrite overspray. Using these TLC methods, we were unable to discern spots associated with Clinacox administration, whether as the parent diclazuril compound or as a metabolite, in post administration samples.

Discussion

The HPLC method reported here was able to detect and quantify diclazuril in equine samples. It is a sensitive analytical method with the limit of detection being 10 ng/ml from extracted plasma samples. In addition, HPLC enabled good recovery (82%) from plasma samples for diclazuril analysis. Chromatograms of both diclazuril and its internal standard demonstrated good linearity using this methodology. Plasma concentrations of diclazuril suggest that it is absorbed well and distributed widely in the horse following oral administration of the compound in its commercially available form, Clinacox, and has a relatively long plasma $t_{1/2}$ (approximately 40–55 h). Following oral administration, the peak plasma concentration of diclazuril was achieved at around 24 h. Oral administration of Clinacox yielded steady state plasma concentrations of 7–9 µg/ml after approximately 8 days.

Diclazuril was also detected in CSF, which suggests that it passes through the blood brain barrier. The fact that the CSF concentrations are much lower than the plasma concentrations implies that it may be highly bound to plasma proteins. On the other hand, diclazuril was present in the spinal cord and brain tissues at detectable concentrations. The lack of detectable levels of parent diclazuril in the urine samples using HPLC and TLC makes it likely that diclazuril is lipophilic and highly metabolised in the horse. Further studies are required to determine the pattern of diclazuril metabolism in the horse. Nevertheless, these results are consistent with the apparent clinical efficacy of diclazuril in the treatment of EPM. To determine specific pharmacokinetic properties, clinical efficacy and the appropriate therapeutic window for diclazuril, further investigations are required.

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