

PHARMACOKINETIC STUDY OF DICLAZURIL IN PRE-RUMINANT AND RUMINANT LAMBS

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ABSTRACT

Diclazuril, 2,6-dichloro- α - (4-chlorophenyl)-4- (4,5-dihydro-3,5-dioxo-1,2,4-triazin-2(3H)yl) benzeneacetonitrile, is an anticoccidial drug, marketed as Vecoxan® for oral use in lambs. Its mechanism of action blocks the excretion of oocysts interrupting the life cycle of the parasite. Because of alleged species specific differences, the aim of the present study is to evaluate the pharmacokinetic of diclazuril in ruminant and pre-ruminant lambs. The drug was orally administrated to ruminant (n=10) and pre-ruminant (n=10) lambs with a dose of 5 mg/kg. Blood samples were collected from 0 to 120 hours after drug administration. Plasma extracts were analyzed using a new HPLC method. The linear concentration range for diclazuril analysis was 0.1-5 μ g/ml ($r^2=0.999$). The plasma concentrations versus time curves were fitted with a non-compartmental method. Significant differences between the groups were found in the concentration versus time curves and also in the pharmacokinetic parameters. This appeared to be related to age differences of the lambs. In conclusion, these findings could be useful to determine a clinical dose of diclazuril in this animal species.

INTRODUCTION

Diclazuril (DCZ), 2,6-dichloro- α -(4-chlorophenyl)-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin-2(3H)yl) benzeneacetonitrile, is an anticoccidial drug (Figure 1). This substance was registered as an anticoccidial feed additive for broilers with a withdrawal period of 5 days in the Council Directive 93/107/EC of 26 November 1993. Since 1996, it has been marked for oral use in lambs (1). The therapeutic dosage is either a single administration of 1-2.5 mg DCZ per kg bodyweight (most commonly from 6-8 weeks of age) or two administrations (the first at 3 to 4 weeks of age and the second about 3 weeks later). Since 2004 its use has been extended to all ruminants and porcine species (2). The indication was for the control of coccidiosis in calves and piglets with a recommended dose regimen of 5 mg/kg bodyweight as a single oral administration. Although the mode of action of DCZ is not completely known, its effect on the asexual or sexual stages blocking the excretion of oocysts induces an interruption of the life cycle of the parasite. Several studies have been published regarding the effectiveness of this drug in different animal species such as chickens (3, 4), turkeys (5), sheep (6), calves (7) and horses (8), but there is a lack of information in the pharmacokinetics of DCZ in sheep. Just a few pharmacokinetic data are reported in EMEA summary reports, showing species specific differences: for instance, DCZ showed a T_{max} of 6 (chicken and turkey), 8 (rat), 12 (calf) and 24 (piglet, lamb, horse) hours, (1, 2, 8, 9). The toxicity of this substance is very low. In acute toxicity studies, oral dosages up to 5000 mg/kg bw caused no mortality

in mice and rats (1). In lambs, DCZ showed a low toxicity and the administration of a dose of DCZ of up to 60 times the therapeutic dose did not induce any adverse effects in this animal species (10).

To best of our knowledge, the pharmacokinetic of DCZ in pre-ruminant and ruminant lambs has not been published to date. The anatomic/physiologic differences in the gastric system between pre-ruminant and ruminant lambs might change the pharmacokinetic values in these animals. The aim of the present study was to evaluate the pharmacokinetics of DCZ in ruminant and pre-ruminant lambs, following the administration of a single oral dose of 5 mg/kg.

MATERIALS AND METHODS

Animals and experimental design

Ten female pre-ruminant lambs belonging to the same flock were selected homogeneously with respect to age (15-21 days) and weight (6-8 kg). Another ten female ruminant lambs, three months of age weighing 20-25 kg, were also used. The animals were housed in a livestock barn and were fed *ad libitum*, only milk to the pre-ruminant lambs and hay to the ruminant lambs. All the subjects were previously determined to be clinically healthy based on physical and haematological examination. The day before the experiment, the animals were individually weighed and randomly marked with a coloured dog-collar for identification. Ruminant and pre-ruminant lambs were orally administered with a dose of 5 mg/kg of DCZ (Vecoxan® Janssen-Cilag S.A., MI, Italy). Blood samples were collected

into heparinized vacutainers from the jugular vein prior DCZ administration (time 0). Further blood collections were carried out at 0.5, 1, 2, 4, 6, 8, 10, 24, 30, 36, 48, 72, 96 and 120 hours after drug administration. Blood was immediately centrifuged and the plasma stored at -20°C until they were analysed. The study protocol was approved by the ethics committee of the University of Pisa (authorization n° 2190) and conveyed to the Italian Ministry of Health.

Animal care and handling were in accordance with the provisions of the European Economic Community Council Directive 86/609 EEC, recognized and adopted by the Italian Government (DL 27/1/1992 n.116).

Analysis equipment and technique

Apparatus

The LC system consisted of a Thermo Finnigan Spectra system P2000 pump, a Thermo Finnigan Spectra system UV 2000 Uv/Vis detector (Waltham, MA, USA) interfaced to a Thermo Finnigan ChromQuest data system, and a Thermo Finnigan Spectra system autosampler/sampleprocessor equipped with a variable 1-100 μ L loop. The LC column was a C₁₈ SunFire, 5 μ m, 4.6 x 250 mm, with guard column of the same packing (Waters, Milford, MA, USA). The LC mobile phases were filtered through 0.22 μ m nylon-66 (N-66) filters with a solvent filtration apparatus. Prior to injection onto the HPLC, sample extracts were filtered through 13 mm 0.2 μ m Acrodisc nylon (Gelman, Ann Arbor, MI) filters.

Reagents and substances

LC grade water was purified in-house with a Milli-Q Plus water system and was used in preparing all solutions. Acetonitrile (ACN), methanol (MeOH), and ethylacetate (EtOAc) were of super HPLC grade from Merk (Darmstadt, Germany). Pure standards of DCZ and clazuril (CZ) internal standard (IS) (Figure 1) were purchased from LGC Promochem (Bologna, Italy). All the other reagents and materials were of analytical grade and supplied from commercial sources. The aqueous and organic components of the mobile phase, degassed with helium under pressure, were mixed by the HPLC.

Standard Solutions

Stock Solutions (1000 μ g/ml).

On the basis of the listed potency or purity of the standard, the amount of DCZ and CZ needed to prepare 100 ml of the individual 1000 μ g/ml standard solutions was calculated. The standard (CZ or DCZ) was weighed to the nearest 0.1 mg (100 mg into a 100 ml volumetric flask) and brought to the mark with MeOH. These solutions were used immediately for the preparation of the fortified solution.

Fortification solution (5 μ g/ml).

Twenty millilitres of each stock standard solution (1000 μ g/ml) were pipetted into a 100 ml volumetric flask and brought to the mark with Milli-Q Plus water: this operation was repeated once to obtain concentrations of 40 μ g/ml. These solutions were successively diluted to reach final concentration of 5 μ g/ml. These solutions were pipetted off into a plastic tube (50 ml), and stored at -80 °C. These solutions remain stable for at

least 12 months.

Calibration Standards

The first dilution of the fortification solution (100 μ g/ml) was again diluted with MeOH, to prepare a five-point standard curve of the drugs at the following concentrations: 25, 50, 75, 100, and 150 μ g/ml (ppm). For levels at or below 20 ppm, a standard curve at 0.05, 0.1, 1 and 5 ppm was prepared instead. These solutions were freshly prepared.

Extraction Procedure

From a disposable 10 ml polypropylene conical tube, 100 μ l of CZ solution (50 μ g/ml) were evaporated under nitrogen flow at 40 °C and a 1 ml of plasma sample was added. The tube was gently vortex-mixed for 1 minute and 3 ml of ACN was added; the sample was then shaken for 10 minutes (150 oscillations/min) by a mechanic oscillator to ensure adequate mixing. The centrifuge tube was centrifuged for 5 min at 3000 x g at 4 °C to effect the phase separation. The clear supernatant was transferred into a glass test tube and evaporated to dryness at 40 °C using N₂. One millilitres of MeOH was added and the sample was vortexed-mixed. The content was transferred into an insulin syringe fitted with a 13 mm Acrodisc nylon filter and filtered into a glass autosampler vial. One hundred microlitres was injected onto the HPLC system within 24 hours after preparation.

Chromatographic Conditions

Plasma extracts were analysed for DCZ using the following isocratic HPLC conditions: mobile phase, consisted of CH₃CN:H₂O (pH 3) 60:40% v/v; flow rate 2.0 ml/min; wavelength, 222 nm; run time, 7 min; column temperature, 25±0.5 °C. One injection of pure CH₃OH was used to equilibrate the LC system. One hundred microlitres of each standard series were injected prior to injecting a sample set. At the end of each day of analysis, the analytical column and guard column were flushed with MeOH/ACN (50:50, v/v).

Quantification

A calibration curve of peak area versus concentration (ng/ml) of each analyte was plotted. Least-squares regression parameters for the calibration curve were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y = peak area and X = concentration of the standard in ng/ml (ppb). Correlation coefficients for each of the calibration curves were routinely >0.99. The method was validated according to EMEA guidelines (11).

Validation procedures

Drug-free blood samples from lambs were screened to ensure the absence of interfering peaks at the retention times of DCZ and IS. Calibration standards were prepared by spiking plasma with DCZ (50, 100, 500, 2000 and 5000 ng/ml). Quality control (QC) samples containing LOQ, low, medium and high concentrations of DCZ (100, 500, 2000 and 5000 ng/ml) were prepared using the same procedure. Linearity of calibration curves based on peak height ratios (DCZ/IS) was assessed by weighted least squares regression analysis (1/y²). Limit of

detection (LOD) and quantification (LOQ) were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10 respectively. Intra- and inter-day precision (expressed as relative standard deviation (R.S.D.)) and accuracy (expressed as percentage of the nominal value) were determined by analysis of replicates (n=4) of LOQ, low, medium and high QC samples on three different days.

Recovery was assessed by comparing extracted solutions with solutions of DCZ at concentrations of 100, 500 and 2000 ng/ml. The same procedure was followed for the IS solution (5 µg/ml).

Stability of stock solutions containing DCZ was tested over 6 hour period at room temperature ($22 \pm 3^\circ\text{C}$) and over 32 days at -20°C . Freeze-thaw stability in plasma was evaluated using spiked samples stored at -20°C and subjected to three freeze-thaw cycles. Long-term stability in plasma was assessed using spiked samples stored in the freezer at -20°C over 22 weeks.

Pharmacokinetics and statistical analysis

Plots of plasma DCZ concentration versus time were constructed for each animal. The pharmacokinetic calculations were carried out using WinNonLin v 5.1.2 (Pharsight Corp, Cary NC, USA). The pharmacokinetic values, the terminal phase rate constant (λ_z), the absorption half-life ($T_{1/2} \lambda_z$), the time of peak concentration (T_{\max}), the peak plasma concentration (C_{\max}), the area under the curve from zero time to LOQ ($AUC_{(0-LOQ)}$), the total body clearance where F is the fraction of dose absorbed (CL F), the volume of distribution based on the terminal phase (Vz F) and the mean resident time (MRT), were calculated using a non-compartmental approach (12).

One way analysis of variance (ANOVA) was applied to the principal pharmacokinetic parameters obtained from the different group of animals. $P<0.05$ was taken as being significant.

RESULTS

The validation procedure included the determination of the following parameters: LOD and LOQ which were 30 and 100 ng/ml, respectively; accuracy, ranged between 2-4%; recovery of the extraction procedures was 75±8%; inter- and intra-day variations were < 6% and 9%, respectively. DCZ and IS were found stable in spiked samples over 22 weeks. The linear concentration range for DCZ analysis was 100-5000 ng/ml ($r^2=0.999$). Blood samples taken from all the animals before DCZ administration (T_0) were found to contain no interference (peaks) from control plasma matrix, haemolysed or not, at the chosen lambda, but after the administration the drug was detected promptly. The average retention time was 3.7 minutes for DCZ and 5.3 minutes for CZ (Figure 2).

No adverse effects following drug administration were observed in lambs. The plasma concentration versus time curves of DCZ in ruminant and pre-ruminant lambs, following a single oral dose of 5 mg/kg, are shown in figure 3. Significant

differences in all the plasma concentrations, except at 96th hour ($p<0.05$), were observed.

After drug administration, plasma level of DCZ was detectable after 1 (n=2), 2 (n=3) and 4 (n=5) hours and up to 72 (n=1), 96 (n=4) and 120 (n=5) hours after administration in 3 months old ruminant lambs, and 30 minutes (n=1) and 1 hour (n=9) and up to 96 hours (n=5) and 120 hours (n=5) in younger non-ruminant animals. DCZ showed slow absorption in both groups: peak plasma concentrations and T_{\max} were of 1321 and 974 ng/ml and of 9.4 and 21.2 hours in the pre-ruminants and ruminants, respectively ($p<0.05$).

The plasma concentration versus time curves fitted with a non-compartmental method for all the animals showed correlation values (r^2) of 0.94 and 0.96 for ruminant and pre-ruminant groups, respectively. Pharmacokinetic parameters are reported in Table 1. Significant differences between the groups were found in $AUC_{(0-LOQ)}$ and T_{\max} ($p<0.05$), suggesting that the fraction of dose reaching the systemic circulation was higher in younger animals. On the other hand, ruminant lambs showed significantly higher values of CL F, Vz F and T_{\max} . The ratio between the average AUCs showed an increase of 25% in younger animals.

DISCUSSION

The new HPLC method was specific, simple, rapid and showed a good linear concentration range (100-5000 ng/ml). The other methods reported in literature are more sensitive (LOQ 10 ng/ml), but the samples' handling/preparation is more expensive and complicated regarding materials and devices employed (13-15).

Specific reports of oral administration and disposition of DCZ in lambs are limited, and no comparisons between pre-ruminant and ruminant lambs, as described in EMEA reports (1, 2), are available. In the present study, although the manufacturer recommends 1 mg DCZ per kg of body weight for therapeutic use in lambs, a 5 times higher than recommended dose was used to obtain a good plasma quantification of the drug.

Concomitant factors have been reported to influence the pharmacokinetic behaviour of drugs, including anatomical, biochemical and functional differences between the adult and the neonate (16). The aim of the present study was to evaluate the pharmacokinetic behaviour of DCZ in field conditions (food and water *ad libitum*). In the present study large intra-group differences in plasma concentrations were obtained in agreement with an earlier study carried out in the horse. Here, the oral bioavailability of DCZ was widely different among the animals (13). It is well recognized that the variations in quality/quantity of feed in the diet, can influence the gastrointestinal transit time of digestion (17). In ruminants it might therefore influence the time available for the absorption of a drug (18).

In pre-ruminant lambs, DCZ disposition was characterised by a more rapid distribution phase, and higher AUC than those obtained in ruminant lambs. This might be attributable to anatomic/physiological changes associated with development of the fore-stomachs and/or to the food content (milk versus

hay) differences (19). Since under normal condition of food intake, (i.e. when food is offered *ad libitum*), the rumen also acts as a storage of feed, in ruminant lamb, DCZ might be bound to the rumen content resulting in small amount absorption through the small intestine (17). In pre-ruminant lambs, the milk's micro fat droplets might aid DCZ absorption (19).

The large inter-species variations reported in DCZ plasma concentrations make pharmacokinetic studies essential for each animal species. Previous studies have been reported the DCZ pharmacokinetics in horses (8, 13), calves (2) and lambs (1). In horses the plasma concentrations were double compared to the lambs (8, 13). In calves (2) plasma concentrations were very low (0.039 µg/ml after 12 hours after administration of 5 mg/kg). Variations in drug absorption due to species between pre-ruminant calves and lambs have already been reported (16). Despite the fact that the dose administered to lambs was different (1), the C_{max} plasma concentrations (normalised to the dose) were in line with the present findings.

In conclusion, for the first time, the pharmacokinetic features of DCZ, following a single oral administration, has been fully investigated in pre-ruminant and ruminant lambs, using a new HPLC-UV detection method. The pharmacokinetic behaviour of the drug appears to be different in the different age groups. Although further pharmacodynamic studies are required, a reduction of DCZ dose (about 25%) in suckling subjects could be considered. The findings of the present study may be useful in determining the clinical dose of DCZ in this animal species.

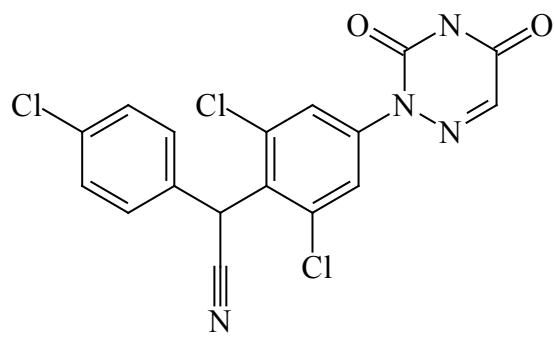
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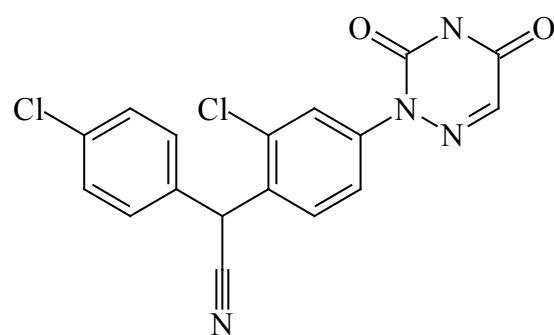
REFERENCES

1. EMEA DICLAZURIL (1996) summary report (1); <http://www.emea.europa.eu/pdfs/vet/mrls/008696en.pdf> (last access: November, 5th 2009).
2. EMEA DICLAZURIL (2004) extension to all ruminants and porcine species, summary report (2); <http://www.emea.europa.eu/pdfs/vet/mrls/089504en.pdf> (last access: November, 5th 2009).
3. Conway, D.P., Mathis, G.F. and Lang, M.: The use of diclazuril in extended withdrawal anticoccidial programs: 1. Efficacy against *Eimeria* spp. in broiler chickens in floor pens. *Poult Sci.* 81:349-352, 2002.
4. Conway, D.P., Mathis, G.F. and Lang, M.: The use of diclazuril in extended withdrawal anticoccidial programs: 2. Immunity to *Eimeria tenella* challenge after drug withdrawal. *Poult Sci.* 81:353-355, 2002.
5. Vanparijs, O., Hermans, L. and Marsboom, R.: Efficacy of diclazuril against turkey coccidiosis in a floor-pen experiment. *Avian Dis.* 33:479-481, 1989.
6. Gradwell, D.: Scouring in lambs following treatment with Vecoxan. *Vet Rec.* 146:591, 2000.
7. Daugschies, A., Agneessens, J., Goossens, L., Mengel, H. and Veys, P.: The effect of a metaphylactic treatment with diclazuril (Vecoxan) on the oocyst excretion and growth performance of calves exposed to a natural *Eimeria* infection. *Vet Parasitol.* 149:199-206, 2007.
8. Dirikolu, L., Karpiesiuk, W., Lehner, A.F., Hughes, C., Woods, W.E., Harkins, J.D., Boyles, J., Atkinson, A., Granstrom, D.E. and Tobin, T.: New therapeutic approaches for equine protozoal myeloencephalitis: pharmacokinetics of diclazuril sodium salts in horses. *Vet Ther.* 7:52-72, 2006.
9. EMEA CLAZURIL (1998) summary report; <http://www.emea.europa.eu/pdfs/vet/mrls/037198en.pdf> (last access: November, 5th 2009)
10. Janssen-Cilag, Vecoxan information, summary of product characteristics. <http://www.vmd.gov.uk/espccsite/documents/141342.doc> (last access: June, 15th 2009).
11. EMEA Note For Guidance On Validation Of Analytical Procedures: Text And Methodology (1995) (CPMP/ICH/381/95); <http://www.emea.europa.eu/pdfs/human/ich/038195en.pdf> (last access: November, 5th 2009).
12. Riviere, J.E. Absorption. In *Comparative Pharmacokinetics. Principles, Techniques, and Applications*. 1st edn. Iowa State University Press, Ames, IA, pp. 21-46, 1999.
13. Dirikolu, L., Lehner, F., Nattrass, C., Bentz, B.G., Woods, W.E., Carter, W.G., Karpiesiuk, W., Jacobs, J., Boyles, J., Harkins, J.D., Granstrom, D.E. and Tobin, T.: Diclazuril in the horse: its identification and detection and preliminary pharmacokinetics. *J Vet Pharmacol Ther.* 22:374-379, 1999.
14. Mortier, L., Daeseleire, E. and Van Peteghem, C.: Liquid chromatographic tandem mass spectrometric determination of five coccidiostats in poultry eggs and feed. *J Chromatogr B Analyt Technol Biomed Life Sci.* 820:261-270, 2005.
15. Mortier, L., Daeseleire, E., Huyghebaert, G., Grijspoor, K. and Van Peteghem, C.: Detection of residues of the coccidiostat diclazuril in poultry tissues by liquid chromatography-tandem mass spectrometry after withdrawal of medicated feed. *J Agric Food Chem.* 53:905-911, 2005.
16. Mengozzi, G., Intorre, L., Bertini, S., Giorgi, M., Secchiari, P.L. and Soldani, G.: A comparative kinetic study of thiamphenicol in pre-ruminant lambs and calves. *Res Vet Sci.* 73:291-295, 2002.
17. Lees, P., Taylor, J.B.O., Maitho, T.E., Millar, J.D. and Higgins, A.J.: Metabolism, excretion, pharmacokinetics and tissue residues of phenylbutazone in the horse. *Cornell Vet.* 77:192-211, 1987.
18. Singh, D., Sanyal, P.K., Swarnkar, C.P., Khan, F.A. and Bhagwan, P.S.K.: Influence of diet type and pretreatment fasting on disposition kinetics of albendazole in sheep. *Vet Res Commun.* 23:229-240, 1999.

19. Watson, A.D., van Gogh, H., van Deurzen, E.J., van Duin, C.T. and van Miert, A.S.: Pharmacokinetics of three sulphonamides in ruminant and preruminant kids. Res Vet Sci. 43:208-216, 1987.



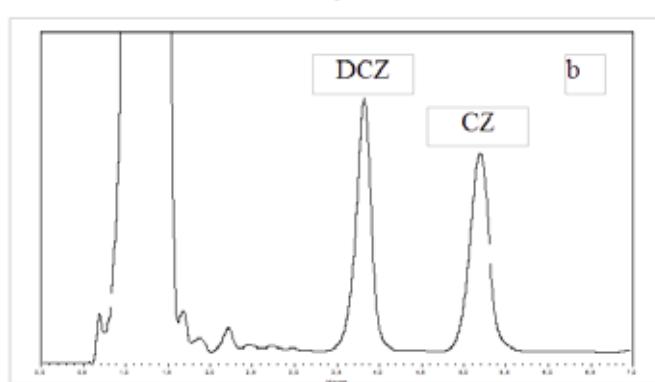
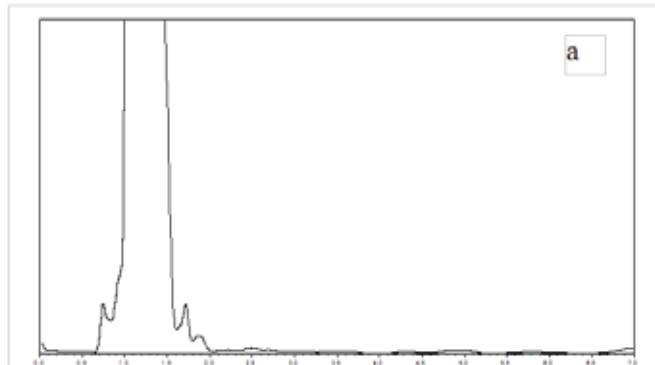
(a)



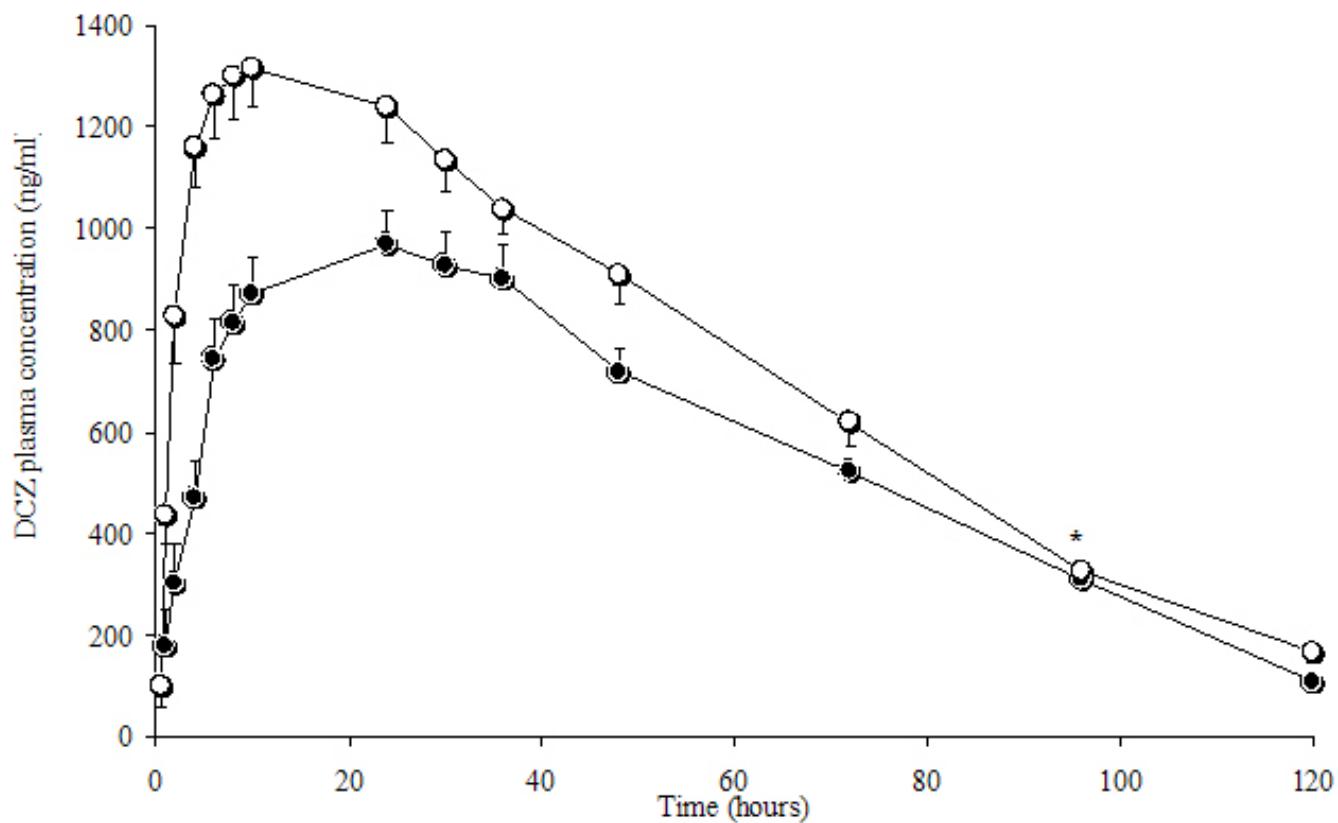
(b)

Fig. 1:

Molecular structure of (a) DCZ and (b) clazuril (internal standard)

**Fig. 2:**

Representative chromatographic curves obtained from (a) DCZ free lamb plasma and (b) lamb plasma following oral administration of 5 mg/kg of DCZ

**Fig. 3:**

Mean plasma concentrations of DCZ following a single oral dose of 5 mg/kg b.w. in pre-ruminant (○) and ruminant lambs (●). Bars represent standard deviation.

Table 1:

Pharmacokinetic parameters, mean (\pm sd), following a single oral administration of DCZ (5 mg/kg) in ruminant (n=10) and pre-ruminant (n=10) lambs.

Parameters	Ruminants		Pre-ruminants	
	mean	sd	mean	sd
r^2	0.94	0.05	0.96	0.04
λz (1/h)	0.029	0.008	0.027	0.01
$T_{1/2} \lambda z$ (h)	25.36	7.12	27.26	9.29
T_{max} (h)	21.2	5.9	9.4*	0.97
C_{max} (ng/ml)	974	251	1321*	268
$AUC_{(0-LOQ)}$ (h μ g/l)	65.85	22.17	87.10*	21.85
CL F (l/h/kg)	7.97	3.57	5.54*	1.3
Vz F (l/kg)	2.86	1.38	2.16*	0.76
MRT (h)	49.83	6.97	48.07	5.36