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Improving the in vitro dissolution rate and pharmacokinetic performance of fenbendazole in sheep using drug nanocrystals

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Abstract

Benzimidazole methylcarbamate anthelmintics, including fenbendazole (FBZ), have only limited water solubility and small differences in drug solubility may have a major influence on their absorption, pharmacokinetic behavior and anthelmintic efficacy. To improve FBZ water solubility and dissolution rate, novel self-dispersible nanocrystals (SDNCs) of FBZ were prepared recently. In this work, the pharmacokinetic behavior of the SDNCs of FBZ and Poloxamer 188 was compared against a physical mixture (PM) of its components. The experiment was conducted following a crossover design with two different experimental phases. In phase I, sheep were treated with the SDNC (n= 3) or the PM (n= 3) formulations by the intraruminal route at the same dose rate (5 mg/kg). The treatment groups were reversed after a 7-days washout period. A non-compartmental analysis of the concentration in plasma versus time results showed that the calculated Cmax and AUC were significantly higher (p < 0.05) for FBZ and its metabolites after the SDNC treatment compared to the PM (for FBZ: Cmax 0.346 µg/mL and AUC0-T 10.1 µg.h/mL after the SDNC vs Cmax 0.157 µg/mL and AUC0-T 5.1 µg.h/mL after the PM treatment). Additionally, population pharmacokinetic parameters of FBZ were estimated for the first time in sheep. In conclusion, the formulation of FBZ as SDNCs is a promising approach to improve FBZ dissolution reaching a higher drug plasma exposure in ruminants.

Keywords: Fenbendazole; nanocrystals; population pharmacokinetics; dissolution rate; bioavailability; sheep

1. Introduction

Gastrointestinal nematode infections are one of the main challenges in small ruminant farming, causing significant health problems which result in serious morbidities and consequently constant economic losses (Charlier et al., 2020; Charlier et al., 2014; Kaplan and Vidyashankar, 2012; Torres-Acosta et al., 2012). Chemotherapy is still the main tool to control these infections today, but the misuse and abuse of anthelmintic drugs has rapidly led to the development of widespread parasitic resistance throughout the world (Jabbar et al., 2006; Papadopoulos, 2008; Rose et al., 2015).
Despite the worldwide phenomenon of resistance to marketed anthelmintics and although some alternative approaches have been recognized to control gastrointestinal nematodes such as vaccines or biological control of infected ruminants resistant to parasites (Hewitson and Maizels, 2014; Waller, 2006; Waller, 2003; Zhan et al., 2014), chemotherapy remains today as the most widely available strategy. Moreover, it will probably continue to be the main strategy used in the near future, integrated with the novel treatments mentioned (Hennessy, 1997). However, since the first reports of parasitic resistance, the development of new classes of anthelmintic drugs, with new mechanisms of action, has been scarce and immediately followed by reports of parasite resistance (Fleming et al., 2006; Kaminsky et al., 2011; Mederos et al., 2016; Mederos et al., 2014; Van den Brom et al., 2015).

In this scenario, a more efficient use of the existing active ingredients is mandatory to sustain the efficacy of parasite treatments. In this sense, re-formulating poorly water-soluble anthelmintic drugs, using innovative delivery systems, is a necessary approach to maximizing drug dissolution of commercial drugs and preserve their efficacy (Hennessy, 1997; Lanusse et al., 2014). Optimizing the drug formulation and therefore its bioavailability may improve its efficacy against parasite resistant strains, since drug concentration and exposure time may be enhanced at the parasite location (Alvarez et al., 2012; Lanusse et al., 2014).

The application of nanotechnology has been recently introduced into medicine, including veterinary, showing a great progress in recent years, where a growth trend predicts that more products will be commercially available over time (Chariou et al., 2020; Meena et al., 2018; Underwood and Van Eps, 2012). Among the strategies used to enhance the dissolution rate of poorly soluble drugs, the formulation of nanocrystals (NCs) has become one of the most preferred techniques due to its flexibility, scalability and high drug loading capacity, which is reflected in the fact that more than 20 products are already available on the market (Mohammad et al., 2019). These formulations consist of solid nano-sized drug particles with crystalline properties that are normally obtained in suspension (nanosuspensions) or in the form of solid self-dispersible nanocrystals (SDNC) (Paredes et al., 2016; Van Eerdenbrugh et al., 2008).
One of the main benefits of formulating crystalline drugs as NCs results from the increased in specific surface produced by the decrease in particle size, which impacts directly on drug dissolution rate and saturation solubility, along with an increased in its mucoadhesiveness (Kesisoglou et al., 2007). In this sense, the oral administration of poorly soluble drug NCs presents many advantages such as improved absorption, rapid action onset and reduced intersubject variability (Shegokar and Müller, 2010). The use of NCs-based formulations in the veterinary field has shown to be an effective approach, increasing the bioavailability and therapeutic response of benzimidazole methylcarbamate (BZM) anthelmintic drugs (Paredes et al., 2018a; Paredes et al., 2020; Paredes et al., 2018b; Pensel et al., 2018).

Fenbendazole (FBZ) belongs to the broad-spectrum anthelmintic family of the BZM and is worldwide used for the treatment of nematode infections in ruminants. Within the Biopharmaceutical Classification System, FBZ is classified as a class II drug (low solubility/high permeability), which means that the \textit{in vivo} dissolution rate has a major impact on the oral bioavailability (Campbell, 1990; Lanusse and Prichard, 1993). Crucially, an increase in the dissolution rate of such drugs may lead to an enhanced bioavailability and, consequently, to an increased therapeutic efficacy (Alvarez et al., 1999; Lanusse et al., 1993; Pensel et al., 2018; Pensel et al., 2015). In this sense, it was previously communicated the preparation and characterization of FBZ self-dispersible nanocrystals (FBZ SDNC) with enhanced \textit{in vitro} dissolution performance (Melian et al., 2020). However, the pharmacokinetic performance of this novel formulation, and of NCs in general in ruminants still remains unexplored.

In this work, the comparative plasma pharmacokinetic behavior of FBZ (and its metabolites) intraruminally (i.r.) administered to sheep as either an SDNC or a PM formulation was assessed for the first time. A parent-metabolite joint population pharmacokinetic (PopPK) model was developed to characterize the absorption and disposition of FBZ and its active metabolite fenbendazole sulfoxide (FBZ-SO) in sheep, focusing on quantifying the effect of SDNC formulation on systemic drug exposure.

\textbf{2. Materials and methods}
2.1. Materials

FBZ and flubendazole (FLU) of pharmaceutical grade were kindly provided by Laboratorio Uruguay S.A. (LUSA, Montevideo, Uruguay). Poloxamer 188 (P188) was provided by BASF (Ludwigshafen, Germany). Pure reference standards of FBZ-SO and fenbendazole sulphone (FBZ-SO2) were synthesized and purified in-house according to Soria-Arteche, et al., 2005, with minor modifications (Soria-Arteche et al., 2005). HPLC grade solvents (acetonitrile and methanol) were bought from Baker, Mallinckrodt (Baker, 119 Phillipsburg, 120 USA).

2.2. Methods

2.2.1. Preparation of SDNCs of FBZ and in vitro dissolution assay

The methodology of preparation of the SDNCs was already reported in a previous work along with the formulations characterization (Melian et al., 2020). Briefly, the preparation of the FBZ SDNCs used in this work included a step for reducing FBZ particle size by wet bead milling using a NanoDisp® laboratory-scale mill (NanoDisp, Córdoba, Argentina) followed by spray-drying. The FBZ SDNC formulation selected to use in the in vivo studies consisted of FBZ and Poloxamer 188, as a stabilizer, in 1:1 proportion.

A control consisting of a physical mixture (PM) of the components of the SDNCs (FBZ and P188 in 1:1 proportion) was prepared by manually mixing the materials in a mortar.

The dissolution tests performed for the pure drug, FBZ SDNC and the corresponding PM were under SINK conditions (equivalent to 5 mg of FBZ), in a USP dissolution apparatus 2 (SR6 SR11-6-Flask Dissolution Test Station, Hanson Research), using 900 mL of HCl 0.1 N as dissolution medium, stirred at 50 rpm (37.0°C). The filtered (0.45 µm) were collected at 3, 5, 10, 15, 30, and 60 minutes, filtered again through 0.22 µm pore membranes and analyzed for drug content using a UV-visible spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific) at 289.5 nm.
2.2.3. In vivo pharmacokinetic study

Experimental animals

Six (6) parasite-free Corriedale male sheep (average weight: 21.0 ± 2.3 kg) were used in this experiment. During the experiment and for 20 days before, animals were kept indoors and fed with a commercial balanced concentrate diet and supplied with water ad libitum.

Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar).

Experimental design, treatment and sampling

The study was conducted following a single-dose, 2-period, 2-sequence, 2-treatment crossover design in 6 parasite-free Corridale male sheep. Animals were randomly assigned to a treatment administration sequence, receiving 5 mg/kg of both FBZ SDNC and FBZ PM 2% w/v suspensions by the i.r. route separated by a washout period of 7 days. The 2% w/v formulation suspensions were prepared in situ, right before the administration by adding the proper amount of water to the previously weighted solid formulation and agitating vigorously by 5 min.

Blood was collected prior to treatment and at 2, 4, 6, 8, 10, 12, 18, 24, 28, 32, 48, 60 and 72 h post-treatment. Immediately after collection, plasma was separated by centrifugation at 3000 x g for 15 minutes. Plasma samples were placed into plastic vials and stored at −20°C until HPLC analysis.

Plasma samples extraction

Plasma samples (1 mL) were spiked with 20 μL of FLU (stock solution 26 μg/mL) as internal standard. FBZ and its metabolites were extracted by solid-phase extraction using disposable cartridges (Strata C18-T, Phenomenex, CA, USA) previously conditioned with 1 mL of methanol, followed by 1 mL of ultrapure water. All samples were injected into cartridges and then sequentially washed with 1 mL of
ultrapure water and eluted with 2 mL of HPLC grade methanol. The eluent was evaporated to dryness at 40 °C under N₂ flow (TurboVap LV Evaporator, Zymark), then reconstituted with 250 μL of methanol and analyzed by HPLC.

**HPLC analysis**

The liquid chromatography analysis was performed using a Shimadzu LC-20AT HPLC System (Kyoto, Japan), with loop of injection of 50 μL, an auto sampler (SIL-10AF Schimadzu, Kyoto, Japan) and a UV-VIS detector (SPD-10A, Shimadzu, Kyoto, Japan). Data and chromatograms were collected and analyzed using the Class LC10 software (SPD-10A, Shimadzu Corporation, Kyoto, Japan). A reverse phase C18 separation column was used (Zorbax Eclipse XBD, Agilent, 4.6 mm× 150 mm, 5 μm), with detection at 289 nm. A mobile phase with a mixture of acetic acid (0.5% v/v)-acetonitrile (80:20), at a flow rate of 1mL/min was used to elute the analytes and at 35 °C. Initial condition (80:20) was maintained for 3 min, and then changed in 7 min to 30:70, modified to 80:20 in 2 minutes, and finally maintained for 3 minutes. The retention times under these chromatographic conditions were: 8.9, 10.9, 11.7 and 12.9 minutes for FBZ-SO, FBZ-SO₂, FLU and FBZ respectively.

A calibration curve was performed by mean of spiked plasma with known concentrations of pure standards of FBZ and its metabolites (fortified samples). The range of calibration curves was between 0.05 to 1.00 µg/mL. This showed good linearity with correlation coefficients greater than 0.999. Recovery of the three molecules under study was estimated by comparison of the peak areas from spiked plasma, resulting from direct injections of standards in methanol. The absolute recovery for FBZ-SO, FBZ-SO₂, FLU and FBZ ranged between 77 and 91% with coefficients of variation (CV) ≤ 6%. The limit of quantification was defined as the lowest measured concentration of the calibration curve with a CV <20%, accuracy of ±20% and absolute recovery ≥ 70%. The statistical analysis was performed using GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, California USA).

**Population pharmacokinetics modeling**
A nonlinear mixed-effects model was developed to characterize the pharmacokinetics of FBZ and FBZ-SO in blood plasma with a population approach and to assess the formulation effect on drug bioavailability and disposition. Parameter estimation was conducted in the MonolixSuite® 2019 R2 (Lixoft, SimulationsPlus) using the Stochastic Approximation Expectation Maximization (SAEM) algorithm (Savic et al., 2011). A simultaneous parent-metabolite fit was performed during model building to reach the base model, given that rich data was collected for both compounds. FBZ-SO2 was not considered in this analysis given its null contribution to the pharmacodynamic outcome. Model development was guided with both metrics and graphical diagnostics. The Akaike Information Criterion (AIC) was used as the main metric to assess the overall fit weighting by the complexity of the model and thus following the principle of parsimony. Uncertainty in parameter estimation and biological plausibility of typical pharmacokinetic parameter estimates for the parent drug and the metabolite were also considered to guide the analysis. Goodness of fit plots based on parameter estimates such as the distribution of individual weighted residuals (IWRES) and the population weighted residuals (PWRES), IWRES and PWRES versus concentration and versus time plots, observation versus individual and population predictions, and correlation of random effects were evaluated. Finally, the Visual Predictive Check (VPC) was implemented as a key simulation-based diagnostic plot (Mo, 2007).

One- and two-compartment pharmacokinetic models were evaluated to describe FBZ and FBZ-SO disposition. All quantified drug transference processes were assumed to follow first-order kinetics. To ensure parameter identifiability, the fraction of metabolized FBZ leading to FBZ-SO was assumed to be 1. Different models were evaluated to characterize FBZ oral absorption: immediate zero-order absorption; immediate first-order absorption; lagged first-order absorption; and transit model absorption. Between-subject variability (BSV) and between occasion variability (BOV) were assessed in all pharmacokinetic parameters assuming a log-normal distribution in the random effects (i.e., the discrepancies between the individual and the population estimate), as described below:

\[ \theta_i = \theta_{pop} \cdot e^{\eta_i} \]  

Equation 1
Where $\theta_i$ is the parameter estimate for the i-th subject, $\theta_{pop}$ the typical value for the population and $\eta_i$ the subject discrepancy from $\theta$ assumed to be normally distributed with mean zero and variance $\omega^2$. BSV and BOV were expressed as coefficient of variation (CV), estimated from the respective variance as:

$$CV = 100\sqrt{e^{\omega^2} - 1} \quad \text{Equation 2}$$

The effect of body weight on pharmacokinetic disposition parameters was included at the beginning of the analysis according to the allometric model. Hence, the population estimates ($\theta_{pop}$) for the elimination clearance (CL) and the volume of distribution (Vd) of both FBZ and FBZ-SO were parametrized as follows:

$$\theta_{pop} = \theta_{pop20} \cdot (\frac{WT_i}{20})^b \quad \text{Equation 3}$$

being $\theta_{pop20}$ the typical estimate for a 20 kg animal, $WT_i$ the individual bodyweight and $b$ the allometrics scaling parameter, which was fixed at 0.75 and 1 for CL and Vd, respectively.

Residual variability was described for both compounds using a combined error model:

$$Y_{ij} = C_{ij}(1 + e_{prop_{ij}}) + e_{add_{ij}} \quad \text{Equation 4}$$

Where $Y_{ij}$ stands for the observed concentration and $C_{ij}$ denotes the predicted concentration for the i-th subject at time j. Residual error for each observation has therefore an additive ($e_{add_{ij}}$) and a proportional ($e_{prop_{ij}}$) component which are normally distributed with mean of 0 and variances $\sigma_{add}^2$ and $\sigma_{prop}^2$, respectively.

Once the base model was defined, the formulation effect on pharmacokinetic population parameters was evaluated using the following parametrization:

$$\theta_{SDNC} = \theta_{PM} \cdot e^{\beta_{SDNC}} \quad \text{Equation 5}$$
being $\theta_{SDNC}$ and $\theta_{PM}$ the parameter estimates for the FBZ SDNC and PM formulations, respectively, and $\beta_{SDNC}$ the formulation effect linked to the SDNC formulation (fixed to 1 for the PM formulation). To assess the statistical significance of a formulation effect on a given parameter, the fit improvement measured with the objective function value (OFV) provided by Monolix was considered. This metric corresponds to the twice negative logarithm of the log-likelihood function. Assuming a chi-squared distribution for the difference in OFV, a single covariate effect was regarded significant ($P < 0.05$) when the OFV was reduced in at least 3.84 units (log-likelihood ratio test). In addition, the significance in the magnitude of each covariate effect ($\beta_{SDNC}$) was assessed through a Wald Test. Significant covariate-parameter relationships were kept and included in a full model. Then, these effects were evaluated again by backward deletion, confirming the significance of each formulation-parameter relationship when an increase in the OFV of at least 10.9 units ($P < 0.01$) was observed after its removal from the full model. The final model was re-evaluated using goodness-of-fit plots and other relevant metrics, as mentioned above.

3. Results

3.1. In vitro dissolution performance of FBZ formulations

FBZ SDNC formulation containing 50% of P188 was selected to be evaluated in vivo among other FBZ formulations reported in previous works since it presented an excellent in vitro dissolution performance and the highest formulation process yields (Melian et al., 2018; Melian et al., 2020). This formulation presented a FBZ mean particle size of 266 nm and was successfully obtained by a relatively simple wet bead milling process followed by a spray-drying step. The in vitro dissolution profiles of the FBZ SDNCs, its corresponding PM and pure FBZ are depicted in Figure 1. This figure shows how during the first 15 min of the assay, the amount of drug dissolved from pure FBZ could not be quantified, while FBZ dissolved from the SDNCs reached around 85% of the total amount of assayed drug. Furthermore, the amount of drug dissolved from the SDNCs was significantly higher than the amount dissolved from the PM during the complete assay. FBZ SDNCs presented higher values of dissolution efficiency (DE)
compared to the PM (data not shown) (Melian et al., 2020) for the different dissolution times evaluated (5, 30 and 60 minutes). For the SDNCs the DE was estimated in 61% during the first 5 minutes of the dissolution assay and 92% at 60 minutes, while for the PM the DE values were 14% and 47%, respectively.

3.2. *In vivo* pharmacokinetic study

FBZ is extensively metabolized in the ruminant host. FBZ main metabolite, FBZ-SO, retains anthelmintic activity and is also a commercialized compound. The comparative mean plasma concentration profiles of FBZ (a), FBZ-SO (b) and FBZ-SO2 (c) quantified after the i.r. administration of FBZ in the form of a SDNC or PM suspensions are depicted in Figure 2. For both experimental groups (SDNC or PM), FBZ-SO was the main analyte recovered in plasma, followed by the parent drug (FBZ) and finally the inactive sulphone metabolite (FBZ-SO2) was quantified in lower concentrations.

The results of mean pharmacokinetics metrics after performing a non-compartmental analysis of the data for FBZ and FBZ-SO are shown in Table 1. The estimated AUC and Cmax were statistically higher ($p < 0.05$) for the SDNC compared to the PM formulation. Both FBZ and FBZ-SO reach a peak concentration significantly earlier ($p < 0.05$) when FBZ was administered as SDNC compared to the PM formulation.

The plasma pharmacokinetic behavior of both, FBZ and FBZ-SO, were best described by one-compartment disposition models parametrized with the apparent volume of distribution (Vd) and the elimination clearance (CL). FBZ absorption was characterized by first-order kinetics (ka) with a lag time (Tlag). BSV was estimated for FBZ Vd and CL, while a significant BOV was quantified in the absorption parameters ka, Tlag and the extent of absorbed FBZ (F). All parameters were estimated with acceptable precision. Parameter estimates are shown in Table 2. A VPC stratified by formulation is included in Figure 3 to illustrate the goodness of fit.
Formulation differences were found to be significant in FBZ bioavailability and lag time. In fact, the bioavailability achieved by FBZ SDNC was found to be 1.92 times higher than after the FBZ PM formulation, and the lag time was significantly lower (0.54 versus 3.3 hours).

4. Discussion

As it was discussed in Melian et al. (2020), the SDNCs presented many advantages that contributed to their improved dissolution rate: the enlarged surface area produced by the nanometrization process, an increased saturation solubility of the drug and a highly homogeneous distribution of FBZ in the polymeric matrix, corroborated by Confocal Raman Microscopy. The results obtained in the pharmacokinetic study correlate with the in vitro dissolution profiles obtained and the DE values estimated. The DE parameter obtained from in vitro data is theoretically related to in vivo data, since it reflects the amount of drug that persist dissolved during a certain period of the dissolution assay (Khan and KA, 1975). In this sense, the absorption of a drug is proportional to its concentration and the time it remains dissolved in a certain region of the gastrointestinal tract, both variables included in the DE calculation. The results demonstrate an improved absorption of FBZ after the administration of the SDNC-based formulation. In addition, disposition parameters of both FBZ and FBZ-SO were not affected by the formulation. The pharmacokinetic population model allowed the estimation of the absorption half-life for FBZ (18.6 h) and elimination half-lives for FBZ (2.8 h) and FBZ-SO (4.3 h). The relatively faster disposition process of FBZ in contrast to the estimated absorption rate indicates the presence of flip-flop pharmacokinetics after oral administration in sheep, a phenomenon previously observed in studies assessing FBZ exposure after oral and intravenous administration in pigs and alpacas (Lakritz et al., 2015; Petersen and Friis, 2000). Both studies reported an increase in the mean terminal FBZ half-life after oral administration: 2.59 to 8.38 hours in pigs; and 5.9 to 23 hours in alpacas. In accordance with these estimations, the decline in both FBZ and FBZ-SO concentrations at the terminal phase of the concentration versus time curve in sheep would be mainly driven by FBZ absorption rate. This behavior could be explained by a limited in vivo drug dissolution rate at the gastrointestinal tract. However, the magnitude of the absorption half-life
estimated in this study (18.6 h) plus the fact that no formulation-related differences were found in this aspect, suggest the presence of other processes taking place at a pre-systemic level. Enteric reabsorption has been previously demonstrated for both FBZ and FBZ-SO (Hennessy et al., 1993). In addition, the metabolic conversion of FBZ to FBZ-SO is reversible by reductive metabolism within the rumen of sheep and cattle (Lakritz et al., 2015). Taken together, the enterohepatic cycling of FBZ-SO, produced in a higher proportion by pre-systemic metabolism after oral administration, and its conversion to FBZ, could lead to a sustained input of the latter to the systemic circulation. This effect might be masking formulation-related differences in FBZ in vivo dissolution rate, only evidenced in the lower lag time of the SNDC formulation. Nonetheless, the higher extent of absorbed FBZ shown by this formulation is likely achieved by a more efficient in vivo dissolution profile.

As previously mentioned, BZMs as FBZ are potent anthelmintic molecules with very low water-solubility. This limitation affects directly their dissolution and absorption process in both monogastric and ruminant organisms and, consequently, restrains their systemic bioavailability and clinical efficacy, contributing to the resistance phenomenon (Lanusse and Prichard, 1993). Even though, several technological approaches have been developed to increase the bioavailability of poorly water-soluble drugs in monogastric organisms by improving aqueous drug solubility and in vitro dissolution, these approaches may not necessarily ensure improved drug bioavailability in ruminants. For instance, the use of cyclodextrins which results successful to increase BZM bioavailability in monogastric organisms such as mice, may not be an optimal formulation for ruminants due to cyclodextrin ruminal degradation (Ceballos et al., 2012). For the SDNC preparation, the dissolution improvement correlates with an enhanced plasma drug exposure, confirming the potential of nanotechnology applications on the rational delivery of therapeutic compounds with limitations of aqueous solubility.

Even though work has been done in order to look for alternative pharmaceutical strategies to improve the systemic availability of poorly water-soluble anthelmintics for use in ruminant species, generally these
compounds are commercially available for veterinary use as suspensions for oral administration. In a previous work (Melian et al., 2020), it was also studied along with the FBZ SDNC and PM a commercial suspension of FBZ. *In vitro* dissolution tests were performed and DE estimated for the three formulations at different dissolution times. As proposed in this previous report, the higher DE values achieved with the SDNC were strongly related to an enhanced drug bioavailability compared with the results of the PM, and even though the commercial suspension was not tested *in vivo* in this work, its dissolution profile and calculated DE were strongly similar to those obtained for the PM (Melian et al., 2020), suggesting that the *in vivo* performance of the SDNC formulation would probably be significantly superior compared to this marketed suspension.

It was previously demonstrated for albendazole (ABZ), another compound of the BZM family, that a higher systemic exposure to the drug, achieved with increasing doses of ABZ, was correlated with a significant increase in the efficacy of the drug, in lambs infected with an *Haemonchus contortus* resistant isolate (Alvarez et al., 2012). In addition, Barrere et al. evaluated the presence of single nucleotide polymorphisms (SNPs) in the β-tubulin gene of *H. contortus* after an ABZ treatment at three dose rates (5, 15 and 45 mg/kg) (Barrère et al., 2012). This investigation corroborated a strong relation between the presence of SNPs at codons 167 and 200 in the isotype 1 of the β-tubulin and the survival of *H. contortus* individuals at a high doses of ABZ, showing that heterozygosity at both codons 167 and 200 conferred resistance with treatments of up to three times the recommended ABZ dose rate (Barrère et al., 2012). These discoveries could suggest that an increase in FBZ bioavailability achieved with the SDNC formulation could be promising for treating certain BZM resistant populations of *H. contortus*.

Finally, as part of an ongoing research project to search for new anthelmintics, several novel valerolactam-benzimidazole hybrids compounds were reported (Munguía et al., 2013; Munguía et al., 2015) with low water solubility as their commercial benzimidazole precursors. The results obtained in
this work using FBZ as a drug model, encourage us to confirm the suitability of the SDNC formulation also for the new hybrid molecules in new in vivo studies.

4. Conclusion

It was possible to achieve a significant bioavailability enhancement for FBZ and its metabolites in sheep by improving the in vitro dissolution rate of the parent drug, formulated as a SDNC. Additionally, for the first time, FBZ and FBZ-SO population pharmacokinetics parameters were estimated in sheep, indicating a flip-flop kinetic for FBZ. In fact, the relatively shorter elimination process of FBZ in contrast to its absorption suggests a flip-flop kinetics of this compound, therefore the ability of the formulation to achieve greater and longer systemic exposure of the drug could be essential in attaining a greater effect.

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Lakritz, J., Linden, D., Anderson, D.E., Specht, T.A., 2015. Plasma concentrations of fenbendazole (FBZ) and oxfendazole in alpacas (Lama pacos) after single intravenous and oral dosing of FBZ. Veterinary Medicine: Research and Reports 6, 71.


Waller, P.J., 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control.


**Figure captions**

Figure 1. Dissolution profile of fenbendazole spray dried nanocrystals (FBZ SDNC), physical mixture (PM) and pure fenbendazole (FBZ). FBZ was not quantifiable during the assay of the pure drug before 60 minutes.

Figure 2. Fenbendazole and its metabolites plasma concentrations. Mean (±SD) plasma concentration profiles (n = 6) for a) fenbendazole (FBZ); b) fenbendazole sulfoxide (FBZ-SO) and c) fenbendazole sulphone (FBZ-SO2), after intraruminal administration of a 5 mg/kg FBZ dose as either fenbendazole spray dried nanocrystals (FBZ SDNC) or physical mixture (PM) suspension to sheep.
Figure 3. Visual predictive check (VPC) plots for fenbendazole (FBZ) stratified by formulation type.

Table 1. Plasma pharmacokinetic metrics (mean ± SD) for FBZ and FBZ-SO, obtained after the intraruminal administration of FBZ (5 mg/kg, n=6) formulated as SDNC or PM to sheep.

<table>
<thead>
<tr>
<th>PK METRIC</th>
<th>FBZ SDNC</th>
<th>FBZ PM</th>
<th>FBZ SO SDNC</th>
<th>FBZ SO PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg/mL)</td>
<td>0.35 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>8.3 ± 2.3</td>
<td>17.0 ± 9.4</td>
<td>20.3 ± 7.8</td>
<td>27.7 ± 10.5</td>
</tr>
<tr>
<td>AUC 0-T (µg.h/mL)</td>
<td>10.1 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.1 ± 0.5</td>
<td>25.9 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.1 ± 1.2</td>
</tr>
<tr>
<td>T½el (h)</td>
<td>14.0 ± 8.2</td>
<td>13.1 ± 6.4</td>
<td>18.2 ± 6.4</td>
<td>17.1 ± 4.1</td>
</tr>
</tbody>
</table>

Pharmacokinetic metrics with different superscript letters are statistically different at p < 0.05. FBZ: fenbendazole; FBZ-SO: fenbendazole sulfoxide; SDNC: self-dispersible nanocrystal; PM: physical mixture; Cmax: peak plasma concentration; Tmax: time to the Cmax; AUC 0-T: area under the plasma concentration vs. time curve from 0 up to the quantification time; T½el: elimination half-life (obtained by noncompartmental analysis of the data).
Table 2. FBZ and FBZ-SO population pharmacokinetic parameters

<table>
<thead>
<tr>
<th>PARAMETERS (unit)</th>
<th>FINAL ESTIMATES (RSE%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ka (h⁻¹)</td>
<td>0.0373 (9.6)</td>
</tr>
<tr>
<td>Tlag (h)</td>
<td>3.3 (29.2)</td>
</tr>
<tr>
<td>Vd (L)</td>
<td>75.0 (22.3)</td>
</tr>
<tr>
<td>CL (L.h⁻¹)</td>
<td>18.5 (8.7)</td>
</tr>
<tr>
<td>Vdm (L)</td>
<td>43.0 (9.7)</td>
</tr>
<tr>
<td>CLm (L.h⁻¹)</td>
<td>6.77 (7.9)</td>
</tr>
<tr>
<td>Formulation effect on Tlag (βSDNC-Tlag)</td>
<td>-1.800 (25.4)</td>
</tr>
<tr>
<td>Formulation effect on F (βSDNC-F)</td>
<td>0.66 (16.7)</td>
</tr>
<tr>
<td>BSV Vd (%)</td>
<td>48.66 (33.5)</td>
</tr>
<tr>
<td>BSV CL (%)</td>
<td>7.77 (42.7)</td>
</tr>
<tr>
<td>BOV ka (%)</td>
<td>31.33 (22.7)</td>
</tr>
<tr>
<td>BOV Tlag (%)</td>
<td>80.81 (23.7)</td>
</tr>
<tr>
<td>BOV F (%)</td>
<td>18.24 (22.5)</td>
</tr>
<tr>
<td>a₁ (µg/mL)</td>
<td>0.0094 (21.3)</td>
</tr>
<tr>
<td>b₁</td>
<td>0.1290 (10.8)</td>
</tr>
<tr>
<td>a₂ (µg/m³)</td>
<td>0.0103 (8.9)</td>
</tr>
<tr>
<td>b₂</td>
<td>0.1180 (2.0)</td>
</tr>
</tbody>
</table>

Estimates for the FBZ and FBZ-SO population pharmacokinetic parameters and the formulation effects on Tlag (βSDNC-Tlag) and F (βSDNC-F) are reported as mean (RSE %). The RSE% accounts for the estimation of uncertainty. Between-subject variability (BSV) and between occasion variability (BOV) are reported as coefficient of variation. a₁ and b₁: estimates of the additive and proportional parameters of the residual error model for FBZ, respectively; a₂ and b₂: estimates of the additive and proportional parameters of the residual error model for FBZ-SO, respectively; FBZ: fenbendazole; FBZ-SO: fenbendazole sulfoxide; ka: FBZ first-order absorption rate; CL: FBZ elimination clearance for a 20 kg sheep; CLm: FBZ-SO elimination clearance for a 20 kg sheep; Vd: FBZ apparent volume of distribution for a 20 kg sheep; Vdm: FBZ-SO apparent volume of distribution for a 20 kg sheep; Tlag: lag time in FBZ absorption; F: FBZ bioavailability.
Graphical abstract

Highlights

- In vitro dissolution of fenbendazole was improved by its formulation as nanocrystal
- Nanocrystals of fenbendazole presented an improved bioavailability in sheep
- Pharmacokinetic parameters of fenbendazole were estimated in sheep
Figure 1

% FBZ Dissolved vs. Time (min)

- FBZ SDNC
- FBZ PM
- Pure FBZ

Figure 1
Figure 2

(a) FBZ

(b) FBZ-SO

(c) FBZ-SO2

Plasma concentration (μg/mL) vs. Time (h)