EFFECTS OF FEEDING ON THE PLASMA DISPOSITION KINETICS OF THE ANTHELMINTIC ALBENDAZOLE IN LAYING HENS

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ABSTRACT

1.To optimize the potential use of ABZ as an anthelmintic in poultry, the effects of different feed correlated factors (fasting and type of diet) on the plasma disposition kinetics of ABZ and its metabolites in laying hens were evaluated.

2.Experiment-I: Twelve hens were distributed into two groups: Fed-group, hens fed *ad libitum* with commercial food were orally treated with ABZ (10 mg/kg); Fasted-group, animals receiving the same food were fasted over a 12 h period before ABZ treatment.

3.Experiment-II: Twelve hens were distributed into two groups: Pelleted-group, hens fed *ad libitum* with pelleted commercial food were orally treated with ABZ (10 mg/kg); Grain-group, hens fed *ad libitum* with a diet based in grains (wheat, corn) received the same treatment.

4.Blood samples were taken at different times post-treatment and plasma analysed by HPLC. ABZ and its metabolites ABZSO and $ABZSO_2$ were recovered in plasma after the oral administration in all the groups.

5.The 12 h fasting period did not modify the disposition kinetics of ABZ, ABZSO and $ABZSO_2$ in hens. In both groups ABZSO was measured in plasma up to 24 h post-treatment with similar C_{max} and T_{max} values.

6.The type of feed affected ABZ kinetics. The mean ABZSO concentration profile were higher and detected longer in the hens fed on grain compared to the fed on pelleted ration. Athough higher metabolite concentration profile was measured in the Grain-group, the $AUC_{0-\infty}$ values did not reach statistical differences between groups. Meanwhile, other pharmacokinetic parameters ($T_{1/2for}$ and $T_{1/2el}$) demonstrated the influence of diet on ABZ disposition.

7.Those feeding related factors affecting ABZ kinetic behaviour should be considered to optimise its use in parasite control in poultry. The knowledge on drug kinetic behaviour is

crucial to ensure the adequate and sustainable use of the limited available anthelmintic therapeutic tools in avian parasite control.

INTRODUCTION

Aviary and deep-litter systems with or without access to a free-range area are increasingly used in European egg production units. This production system has the advantage to promote natural behaviour and increase animal welfare with the inconvenient that a increased exposure to helminth parasites with a fecal–oral route of transmission has been reported (Papini and Cacciuttolo, 2008; Jansson *et al.* 2010; Höglund and Jansson, 2011; Kaufmann *et al*. 2011) Among the poultry helminths, *Ascaridia galli* and *Heterakis gallinarum* are the most common species (Permin *et al*. 1999). They can cause weight depression (Kilpinen *et al*. 2005) and damage the intestinal mucosa, sometimes leading to haemorrhages, anaemia and severe diarrhoea. Heavy *A. galli* infections can obstruct the small intestine and cause death (Ramadan and Znada, 1991) of affected animals. Although quite a large number of organic layer farmers used homeopathic, phytotherapeutic or other alternative medicines, the use of chemotherapeutics is currently inevitable to prevent animal suffering or distress in organic husbandry (Van Der Meulen *et al*. 2007). However, the current situation is similar to that of more than a decade ago (Ruff, 1999) with very few drugs available for treatment of poultry helminth infections because the size of the market and severity of the problem does not justify the escalating cost of developing and obtaining regulatory approval for a new drug.

The benzimidazoles (BZD) are anthelmintic drugs (albendazole (ABZ), fenbendazole (FBZ), flubendazole (FLBZ), triclabendazole (TCBZ), etc.) widely used in veterinary and human medicine, being FLBZ the only BZD registered for poultry in many countries around the world (EMEA, 2006).

The characterisation of the BZD plasma disposition kinetics can be used to predict its anthelmintic efficacy. In fact, there is a direct relation between the concentration profiles of BZD compounds measured in the bloodstream and those recovered within target parasites (Alvarez *et al*. 1999; Alvarez *et al*. 2000), as well as BZD systemic exposure and clinical efficacy (Hennessy *et al.* 1995; Moreno *et al.* 2004; Entrocasso *et al.* 2008; Alvarez *et al.* 2012; Barrère *et al*. 2012). The knowledge of the pharmacokinetic and metabolic patterns of BZD anthelmintic in the different mammalian species as ruminant (Lanusse and Prichard, 1993a), dogs (McKellar *et al*. 1993; Sánchez *et al.* 2000), pigs (Alvarez *et al*. 1996) and human (Edwards and Breckenridge, 1988) has been widely studied. However, the available information on the kinetics of these compounds in avian species is scarce (Csiko *et al*. 1996; De Ruyck *et al*. 2001). Since the anthelmintic efficacy of ABZ has been demonstrated in chickens (Tucker *et al*. 2007), and there are several evidences that ABZ is being extra-labelled used in avian production, we recently studied the plasma disposition kinetics of ABZ in laying hens (Bistoletti *et al*. 2013).

It has been demonstrated that several host-related factors may affect the pharmacokinetics and the resultant clinical efficacy of BZD compounds in different mammalian species. The gastrointestinal digesta passage rate is affected by alteration in the quality and quantity of the feed consumed, which confers variable absorption time affecting bioavailability of orally administered BZD anthelmintics (Lifschitz *et al*. 1997; Sánchez *et al.* 1999; McKellar *et al*. 2002; Gokbulut *et al*. 2010).

The gastrointestinal anatomy and physiology in birds differs significantly from mammalian species. Unlike mammals, the avian have no teeth in the buccal cavity (no grinding); there is no sharp distinction between the pharynx and mouth (oropharynx). Salivary glands are well developed in poultry and the oesophagus is expanded to form a crop. This

may influence the pharmacokinetic processes of most drugs, mainly when oral therapy accounts for more than 90% of total drug administration in poultry (Vermeulen *et al*. 2002).

To improve the efficacy and safety of BZD anthelmintics, it is necessary to characterize all the factors that may affect their pharmacokinetics. The knowledge of the differences on drug behaviour and drug efficacy among animal species and the identification of other factors affecting drug biotransformation pathways are relevant to achieve the optimal parasite control and to limit the selection of drug-resistant parasites (Křížová-Forstová *et al*. 2011). To optimize the potential use of ABZ in poultry to a better parasite control, the aim of the current study was to evaluate the effects of feeding related factors (fasting and diet composition) on the plasma disposition kinetics of ABZ and its metabolites, the active albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$), after oral administration to laying hens.

MATERIALS AND METHODS

Animals

A total of twenty four (24) six month old *Plymouth* Rock Barrada laying hens with an average body weight of 2.2 ± 0.3 kg were used in the current experimental work. Animals were monitored daily for 2 weeks during acclimatization period, in which no clinical sign of disease were observed. The hens were under uniform conditions of housing and feeding, according to their specific requirements, with water and feed available *ad libitium*. Before the experiments the hens had not been medicated with any antiparasitic drug. Animal procedures and management protocols were approved by 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 (htpp:// www.vet.unicen.edu.ar).

Drug formulation

Drug formulations used in the experiment were prepared in our laboratory. The ABZ 2% suspension used for single oral administration was prepared weighting carefully 0.5 g of pure ABZ standard and adding 25 ml of HPLC water and 0.125 g of carboxymethylcelulose.

Experimental design, treatments and sampling

Experiment I: effect of fasting

Twelve (12) hens were randomly distributed into two (2) groups of six (6) animals each as follow: Fed group, hens fed *ad libitum* with balanced commercial food were treated with ABZ (10 mg/kg, 2 % suspension) by the oral route through a 25-cm length plastic cannula; Fasted group, hens were fasted over 12 h before to the ABZ treatment, which was performed as previously described for the Fed group.

Experiment II: effect of diet composition

Twelve (12) hens were randomly distributed into two (2) groups of six (6) animals each as follow: Pelleted group, hens were fed *ad libitum* with pelleted commercial food and treated with ABZ (10 mg/kg, 2 % suspension) by the oral route through a 25-cm length plastic cannula; Grain group, hens were fed *ad libitum* with a diet based in crushed grains (wheat and corn) and treated with ABZ as previously described for the Group pelleted.

After drug administration (both experiments), blood samples (1 ml) were taken by an intravenous catheter previously placed into the left wing vein at 1, 2, 3, 6, 9, 12, 15, 24, 30 and 48 h post-treatment. The volume of blood taken in each sample was replaced by the i.v. infusion (1 ml) of sterile physiological saline solution. Blood samples collected in heparinized tubes were centrifuged at 2000 *x g* for 10 min, and the supernatant plasma collected was frozen at -20 ºC to be later analysed by high performance liquid chromatography (HPLC).

Reagents and Chemicals

Pure reference standards (97-99% purity) of ABZ and its metabolites, ABZSO, $ABZSO_2$ and the internal standard (IS) oxibendazole (OBZ) were provided by Toronto Research Chemicals Inc. (Toronto, Canada). Acetonitrile solvent used for the extraction and drug analysis were HPLC grade and purchased from Sintorgan® S.A. (Buenos Aires, Argentina). Ammonium acetate (HPLC grade) was from Baker (Phillipsburg, USA). Water was double distilled and deionized using a water purification system (Simplicity® Millipore, São Paulo, Brazil).

Plasma sample analysis

Following the methodology previously described, plasma samples (0.5 ml) were extracted in order to ABZ/metabolites quantification. Plasma samples were analysed using a HPLC method previously validated (Bistoletti *et al*. 2011). The analytical method was developed using a Shimadzu Chromatography system (Shimadzu Corporation, Kyoto, Japan). The equipment was composed for two LC-10AS solvent pumps, an automatic sample injector (SIL-10A), an ultraviolet visible spectophotometric detector (UV) (SPD-10A), a column oven (Eppendorf TC-45. Eppendorf, Madison, WI, USA) set at 35°C, and a CBM-10A data integrator. Data and chromatograms were collected and analysed using the Class LC10 software (SPD-10A. Shimadzu Corporation. Kyoto. Japan). A C_{18} reversed-phase column (Kromasil® Eka Chemicals AB, NY, USA) of 250 x 4.6 mm with 5 µm particle size was used for analyte separation. The detection of drugs/metabolites was done at a wavelength of 292 nm. The calibration curves for each analyte were constructed by least squares linear regression analysis, showed good linearity with correlation coefficients greater than 0.9964. The absolute recoveries for ABZ, ABZSO and $ABZSO_2$ ranged between 81.8 and 98.2%. Precision (intra- and inter- assay) (CV) was lower than 7.79%. The limits of quantification (LOQ) for ABZ and its metabolites ranged between 0.05 and 0.125 µg/µl.

Pharmacokinetic analysis

The pharmacokinetic analysis of the plasma concentration *vs*. time curves for ABZ and its metabolites for each animal was carried out using the PKsolution 2.0 software (Summit Research Services, Ashland, USA). The following equation was used to describe the biexponential plasma concentration-time curves for ABZSO and $ABZSO_2$ obtained after the oral administration of ABZ (Notari, 1987):

$$C_p = Be^{-\beta t} - Be^{-Kt}$$

where C_p = concentration at time t after administration ($\mu g \cdot ml^{-1}$); B = concentration at time zero extrapolated from the elimination phase ($\mu g \cdot ml^{-1}$); e= base of the natural logarithm; β= terminal slope (h^{-1}) and K is the rapid slope obtained by feathering which represents the first order metabolite formation rate constant (K_{for}) (h^{-1}). The elimination half-life ($T_{1/2el}$) was calculated as ln 2/β. The peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration-time curve for each analyte. The area under the concentration-time curve for ABZ/metabolites in plasma fluid was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity ($AUC_{0-\infty}$) by dividing the last experimental concentration by the terminal slope (β). Statistical moment theory was applied to calculate the mean residence time (MRT) for ABZ and metabolites in plasma as follows (Gibaldi and Perrier, 1982).

$$MRT = AUMC/AUC$$

Where AUC is defined previously and AUMC is the area under the curve of the product of time and the plasma drug concentration *vs*. time from 0 to ∞ (Gibaldi and Perrier, 1982).

Statistical analysis of the data

The pharmacokinetic parameters and concentration data are reported as mean ± SEM. The time-based parameters (MRT, $T_{½for}$, $T_{½el}$) are expressed as harmonic means. Student t and Mann–

200 Whitney Test were used to compare parameters between groups. A value of $P< 0.05$ was
201 considered statistically significant.
202
203 **RESULTS**
204 **Effect of fasting**
205 ABZ parent drug and theirs ABZSO and $ABZSO_2$ metabolites were detected in plasma after the
206 oral administration of ABZ in fed and fasted groups. The comparative ABZ, ABZSO and
207 $ABZSO_2$ plasma profiles obtained for both groups are shown in Figure 1. The 12 h fasting
208 period did not modify the overall plasma drug disposition of ABZ/metabolites, resulting in
209 similar ($P> 0.05$) pharmacokinetic parameters obtained in fed and fasted animals. The plasma
210 pharmacokinetic parameters calculated for ABZSO and $ABZSO_2$ in fed and fasted groups are
211 presented in Table 1. The low ABZ concentrations detected during a short time period precluded
212 the development of a complete pharmacokinetic analysis of the data obtained for this molecule
213 after its oral administration. The statistical comparison between groups did not shown differences
214 in any case. In both experimental groups the ABZ C_{max} was reached at 2 h, with values ranging
215 from 0.11 and 0.21 µg/ml for fed and fasted groups, respectively. ABZ parent drug was rapidly
216 biotransformed to ABZSO and $ABZSO_2$. In both groups, ABZSO was measured in plasma till
217 24 h post-treatment with similar C_{max} and T_{max} values. $ABZSO_2$ plasma drug exposure was lower
218 compared to that observed for ABZSO, being quantified in plasma from 2 to 30 h post-treatment.
219 The $ABZSO_2$ metabolite reached the C_{max} (0.47 µg/ml) at 8 h (T_{max}) post-treatment in fed
220 animals, values which resulted similar in fasted hens (C_{max}= 0.42 µg/ml; T_{max}= 9 h).
221 **Effect of diet composition**
222 The mean plasma concentration profiles for ABZ and its metabolites after its oral
223 administration at 10 mg/kg to hens fed with pelleted commercial balanced (Pelleted group) or
224 crushed grains (Grain group) diets are shown in Figure 2. Table 2 summarises the plasma

pharmacokinetic parameters for ABZSO and $ABZSO_2$ obtained after the oral administration of ABZ to hens from Pelleted or Grain groups. The type of feed affected ABZ kinetics. Both ABZ and metabolites mean concentration were higher and detected longer in the Grain group compared to that observed in the Pelleted group. Once again, the scarce ABZ concentration quantified in plasma precluded a complete pharmacokinetic study. There was a general upward trend in the plasma concentration profiles of ABZSO in the Grain group. In fact, an increment of ABZSO AUC (64%) was observed in hens fed with grain compared to that obtained in the Pelleted group. However, the high variability in these concentrations at the different sampling times did not permit to obtain statistically significant differences between groups. Meanwhile, statistical differences for $T_{1/2el}$ and $T_{1/2for}$ for ABZSO, and $T_{1/2el}$ and MRT for $ABZSO_2$ between groups, demonstrated the influence of diet on ABZ disposition.

DISCUSSION

New tendencies in animal production regarding the sustainability and welfare has led to the ban of the conventional cages for laying hens in Germany since 2010 and the European Union after 2012 (1999/74/EC, Anonymous, 1999). In accordance with this legislation, a progressively increasing number of farmers have adopted breeding programs on soil; as a consequence, a high prevalence of helminth infections has been reported. In Germany, almost all hens (99.6%, N=737) harboured at least one helminth species. The most prevalent species were the nematodes *Heterakis gallinarum* (98%) followed by *Ascaridia galli* (88%) and *Capillaria* spp. (75.3%). The overall prevalence of the cestodes was 24.9%. The vast majority of the hens are subclinically infected with helminth species. The results indicate that it is essential to adopt alternative control strategies in order to lower infection risks in organic production systems which are gaining popularity (Kaufmann *et al*. 2011). Although quite a large number of organic layer farmers used homeopathic, phytotherapeutic, or other

alternative medicines, the use of chemotherapeutics is currently inevitable to prevent animal suffering or distress in organic husbandry (Van Der Meulen *et al*. 2007).

The close relationship between pharmacokinetics and clinical efficacy for anthelmintic compounds has been well documented. The characterization of the plasma disposition kinetics of anthelmintics (parent drug and/or its metabolites) can be used to predict and optimize its antiparasitic efficacy (Lanusse and Prichard, 1993a).

We recently studied the plasma disposition kinetics of ABZ in laying hens (Bistoletti *et al*. 2013) to know the pharmacokinetic behavior of ABZ in this species as the first step to evaluate its potential as an anthelmintic tool for use in this species. In order to optimize its use to a better parasite control, in the current study the effect of different feed related factors, such as fasting and diet composition, on the plasma disposition kinetics of ABZ and its metabolites were assessed for the first time after oral administration to laying hens.

The limited water solubility of BZD anthelmintics allows them to be formulated only as suspensions, pastes or granules for oral administration. In these experiments ABZ was administered to the hens as a micronized suspension. The dissolution of the drug particles is a crucial step that precedes the gastrointestinal absorption of a drug formulated as a suspension. The aqueous solubility of ABZ is markedly higher at low pH values (McKellar and Scott, 1990). Different factors affecting the plasma disposition kinetics of BZD anthelmintics, may affect the time over which the parasites are exposed to toxic concentrations. It has been reported that feeding restriction significantly modifies the plasma disposition kinetics of ABZ and its metabolites in different mammalian species (Singh *et al*. 1999; Sánchez *et al*. 2000; Gokbulut *et al*. 2010). In the present work we evaluated the effect of 12 h fasting period on ABZ plasma disposition after oral administration to laying hens. Unlike observations what happens in other animal species, fasting pre-treatment did not induced marked changes in the ABZ/metabolites plasma drug exposure in laying hen. ABZSO and $ABZSO_2$ plasma exposure

(expressed as $AUC_{0-\infty}$) did not differ between hens fasted before treatment compared to those obtained in control (unfasted) animals, indicating that the 12 h fasting period was not enough to significantly modify the plasma disposition kinetics of ABZ/metabolites in hens.

On the other hand, numerous studies have reported the way in which the type of diet affects the kinetics of the different BZD anthelmintic drugs in mammalian species (Alvarez *et al*. 1996; Knox and Steel, 1997; Virkel *et al*. 1999; Gokbulut *et al*. 2007). Different feeds may induce changes in pH and microbial population at intestinal level. Consequently, drug biotransformation and absorption through biological membranes could be modified. The accumulated data appears to indicate that the effect of diet composition on the pharmacokinetics of BZD anthelmintics differs between monogastric and ruminants species. Some effects of diet composition on ABZ pharmacokinetics in hens were observed in the current study. The observed effect, mainly focused in the rate of absorption/formation of ABZ/ABZSO, could be related with changes on gastrointestinal anatomy/structure induced by the food. It has been reported that consumption of diets with different characteristics may have a direct effect on the morphological structure of the digestive system of the birds, such that any alteration in the structure of the feed might have a significant effect on performance by restricting or making some nutrients and/or drugs unavailable (Macari *et al*. 1994). For example, birds that eat fiber and/or coarse food tent to have a longer gastrointestinal tract (Denbow, 2000). On the other hand, both gizzard atrophy (Nir *et al*. 1994a; Nir *et al*. 1995) and a discrete intestinal hypertrophy (Nir *et al*. 1994a) have been observed when finely ground food was fed to the birds. In the current experiment, we compare ABZ pharmacokinetics in hens fed with cereals (wheat and corn) or pelleted food and differences were found between groups. ABZ and metabolite concentrations were higher in grain fed hens than in pelleting fed group. However, these differences were not as evident as observed in ruminants, since statistical differences were not found for C_{max} and $AUC_{0-\infty}$, probably due to

300 the high variability of the data, which has been associated with the eating habits of the hens
301 (Bistoletti *et al*. 2013). Interestingly, while in other animal species such as pigs (Alvarez *et al*.
302 1996), dogs (Sánchez *et al*. 2001) and ruminants (Prichard *et al.* 1985; Hennessy *et al.* 1993)
303 ABZ is not detected in plasma after its enteral administration, the parent drug was quantified
304 in hens. This finding has been previously associated to a fast absorption process and/or a slow
305 metabolic rate in hens compared with other species (Csiko *et al*. 1996; Bistoletti *et al*. 2013).
306 Additionally, the irrigation of the hen gastrointestinal tract may help to explain the obtained
307 result. While drug absorbed at proventriculus, gizzard or intestines reach the liver drained by
308 the venous portal system, drug absorbed at the crop reach the systemic circulation through the
309 yugular vein (Sisson & Grossman, 1982) avoiding the liver "first pass" effect.

310 Gastrointestinal absorption of ABZ is limited by its poor water solubility, which is
311 markedly improved at low pH values. While in ruminants the abomasum play an important
312 role in ABZ dissolution, in avian species this function is associated to the gizzard (muscular
313 stomach), which is a powerful triturating machine with low physiological pH (between 2-3.5)
314 (Vermeulen *et al*. 2002). When hens were fed with different diets based in grains or pellets,
315 the ABZ dissolution and absorption processes may have been different. It has been
316 demonstrated that ingredients with larger particle size have lower rate of passage through the
317 gastrointestinal tract (Amerah *et al*. 2007), which results in a greater contact between the
318 food/gastrointestinal content and the intestinal mucosa. In addition to this, pelleting also
319 seems to directly affect the gastrointestinal tract structure. Nir *et al*. (1995) observed that
320 pelleting resulted in a decrease in the weight and contents of the proventriculus, gizzard and
321 small intestine, as well as a decrease in the small intestine length, without changing the pH of
322 these segments (Nir *et al*. 1994a; Nir *et al*. 1995). Considering these aspects, hens fed with
323 corn or wheel grains need longer digestion time compared to that fed with pellets. The
324 extended time for grinding cereal grains in the gizzard, produced a greater decrease of pH

value, thus promoting a better and longer dissolution process of ABZ particles and the subsequent higher absorption of the drug from the gut, explaining the higher plasma concentrations (ABZ/metabolites), longer $T_{1/2for}$ (related to a delayed ABZ absorption) and $T_{1/2el}$ for both ABZSO (active) and $ABZSO_2$ metabolites in the Grain group. Meanwhile, since the pellet dissolves within the proventriculus (Nir *et al*. 1994b), the digestion time in the gizzard and contact of food/drug with the acidic media is shorter than that for grain diet, explaining the lower ABZ/metabolites plasma disposition and shorter $T_{1/2for}$ and $T_{1/2el}$ found for this group.

In conclusion, different factors affecting the ABZ plasma pharmacokinetics in laying hens were evaluated. A 12 h fasting period did not produce any effect on ABZ disposition in laying hens. The type of feed affected the peak plasma concentrations of ABZ and its metabolites and their persistence on the blood stream, which may be related to a better dissolution and gastro-intestinal absorption of ABZ in grain fed hens. Overall, those feeding related factors affecting ABZ kinetic behaviour should be considered to optimise its use in parasite control in poultry. The knowledge on drug kinetic behaviour is crucial to ensure the adequate and sustainable use of the limited available anthelmintic therapeutic tools in avian parasite control.

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500 **Figure legends**

501

502 Figure 1. Mean (±SEM) albendazole (ABZ), albendazole sulphoxide (ABZSO) and
503 albendazole sulphone ($ABZSO_2$) plasma concentration profiles obtained after oral
504 administration of ABZ (10 mg/kg) to laying hens either fed *ad libitum* or fasted over 12 h
505 prior to treatment.

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508 Figure 2. Mean (±SEM) albendazole (ABZ), albendazole sulphoxide (ABZSO) and
509 albendazole sulphone ($ABZSO_2$) plasma concentration profiles measured after oral
510 administration of ABZ (10 mg/kg) to laying hens either fed on grains based or pelleted diet.

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