Received: 27 November 2017 | Revised: 7 March 2018 | Accepted: 14 May 2018

DOI: 10.1111/jvp.12675

ORIGINAL ARTICLE

WILEY JOURNAL OF Veterinary Pharmacology and Therapeutics

Albendazole treatment in laying hens: Egg residues and its effects on fertility and hatchability

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Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, Grant/Award Number: 11220080102184

Abstract

This work characterized the egg residual concentrations of albendazole (ABZ) and its sulphoxide (ABZSO) and sulphone ($ABZSO_2$) metabolites and evaluated their effect on egg fertility and hatchability after ABZ treatments to laying hens. Seventy hens were allocated in groups: Group-1 was the control without treatment; Group-2 received a single ABZ oral dose (10 mg/kg); Group-3, -4 and -5 were treated with ABZ in medicated feed over 7 days at 10, 40, or 80 mg kg^{-1} day^{-1}, respectively. Eggs were analyzed to determine the ABZ/metabolite level by HPLC or subjected to incubation to evaluate the fertility and hatchability. Only ABZSO and $ABZSO_2$ metabolites were quantified in egg after ABZ single oral administration with maximum concentrations of 0.47 ± 0.08 and 0.30 ± 0.07 μg/ml, respectively. ABZ and its metabolites were found in eggs after 7-day ABZ treatments. The egg residue exposure estimated as AUCs (areas under the concentration *vs.* time curve) were 100.5 (ABZ), 56.3 (ABZSO) and 141.3 μg hr g^{-1} ($ABZSO_2$). ABZ administration did not affect the egg fertility at any dosages. Egg hatchability was not affected by ABZ treatment at 10 mg/kg in medicated feed, but it decreased when the dose was 4–8 times higher. These results should be considered when ABZ is used for deworming laying hens.

KEYWORDS
albendazole, egg fertility, egg hatchability, egg residues, laying hens

1 | INTRODUCTION

Benzimidazole (BZD) anthelmintics are widely used in veterinary medicine. They have broad spectrum against gastrointestinal roundworms, gapeworms and tapeworms. Endoparasites are a common and frequently underestimated problem in avian farms (Tucker, Yazwinski, Reynolds, Johnson, & Keating, 2007). In addition, according to European animal welfare legislation, there is an upward trend in production systems based on alternative poultry programs (soil and open field management), with the resulting increase in the prevalence of infections by helminths (Kaufmann, Das, Sohnrey, & Gauly, 2011). This trend demonstrates the need to have the necessary therapeutic tools to control these parasites. Two BZDs, flubendazole (FLBZ) and more recently fenbendazole (FBZ) (EMA/42178/2014), are the only BZDs licensed for use in poultry in many countries. As a consequence, maximum residue limits (MRLs) have been established for these molecules in eggs. Albendazole (ABZ) has been reported to be effective in the treatment of *Capillaria*, *Ascaridia*, *Heterakis*, and tape worms in chickens (Oryan, Sadjjadi, & Azizi, 2009; Tucker et al., 2007). It has been labelled only for cattle and sheep. However, ABZ is currently being extra-label used in avian production systems. Toxicological studies in both farm and laboratory animals have shown ABZ and its active sulphoxide metabolite (ABZSO) to be embryotoxic/teratogenic (Delatour, Garnier, Benoit, & Longin, 1984; Teruel, García, & Catalano, 2009). The pharmacokinetic behaviour and tissue residue depletion of these anthelmintics have been well reported in ruminant species. However, the available information on BZD kinetics and tissue residue profiles in poultry is more limited.

The plasma pharmacokinetic behaviour of ABZ after administration in laying hens was recently evaluated (Bistoletti, Alvarez, Lanusse, & Moreno, 2013; Bistoletti, Moreno, Alvarez, & Lanusse, 2011). In order to know the impact of potential ABZ residues in feed after its therapeutic use in avian production, the aims of this work were (a) To characterize the residual concentrations of ABZ and its sulphoxide (ABZSO) and sulphone ($ABZSO_2$) metabolites in eggs after ABZ treatment to laying hens; (b) To evaluate the effect of ABZ/metabolites on the fertility and hatchability of eggs collected from treated laying hens.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

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

2.2 | ABZ treatments

Drug formulations administered to hens in this work were prepared in our lab, following the methodology previously reported (Bistoletti et al., 2013). The formulations were as follows: ABZ 2% suspension for oral administration; ABZ medicated feed at three levels of concentrations (concentration checked: 133.5, 583.3 and 1083.3 ppm) were prepared to administer approximately 10, 40 and 80 mg/kg dose levels. Calculations were made considering the daily dose to be administered, the mean daily food intake (150 g) and the mean body weight of hens.

2.3 | Experimental design

All the experiments were carried out following ethical guidelines of the Animal Welfare Committee of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (Internal Protocol: 01/2014; Approval date: 28,04,2014).

Seventy (70) *Plymouth Rock Barrada* adult laying hens (10–12 months old) and 2.5 ± 0.3 kg body weight were used in the experiment. The hens were housed and acclimated for 2-weeks with water and balanced commercial food ad libitum. They did not receive any medication before the experiments. They were allocated in five groups: Group 1 (n = 15) was the control without treatment; Group 2 (n = 10) received a single ABZ oral dose (10 mg/kg); Groups 3, 4 and 5 (n = 15 each) were treated with ABZ in medicated feed over 7 days at 10, 40 or 80 mg kg^{-1} day^{-1}, respectively. Eggs were collected during treatment and 2 days post-treatment from all groups. Egg from Group 2 were collected and frozen to be later analyzed to determine the ABZ/metabolite egg concentrations by HPLC. Some eggs (n = 6) daily collected from Groups 3, 4 and 5 were frozen and analyzed to determine the ABZ/metabolite level reached. The remaining eggs (Groups 1, 3, 4 and 5) were identified and subjected to incubation as described in the following subsection. To know ABZ and metabolite plasma levels, a blood sample was taken (Groups 1, 3, 4 and 5) from the left wing vein 1 day after the end of the treatment. Blood samples were taken in heparinized tubes and centrifuged at 1000 *g* for 10 min. The plasma collected was placed into plastic tubes and frozen at −20°C to be analyzed later by HPLC.

2.4 | Incubation process: Egg fertility and hatchability evaluation

Eggs were disinfected with quaternary ammonium and preincubated (2 hr at 38°C followed by cooling to room temperature). The incubation process was carried out in incubator at controlled conditions of humidity (55%–60%), temperature (37–38°C) and appropriate air ventilation. The eggs were manually rotated 90 degrees five times per day. Eggs were examined by ovoscopy at 4 and 18 days of incubation to determine fertility. The eggs that were not viable at 4 days of incubation were removed and counted to evaluate fertility. Humidity and temperature were changed to 70%–75% and 36–37°C at 18 days of incubation. From this day, egg were not rotated anymore. Chicks were born at 21 days. Hatchability was evaluated according to the number of chicks born. Fertility and hatchability percentages in each group were calculated according to the following formulas (Ricaurte, 2005):

$$\text{Fertility} = \frac{\text{Number of fertile eggs}}{\text{Initial number of eggs}} \times 100$$

$$\text{Hatchability} = \frac{\text{Number of chicks born}}{\text{Number of fertile eggs}} \times 100$$

2.5 | Egg and plasma sample analysis

Experimental or fortified egg and plasma samples were processed and analyzed by HPLC following exactly the methodology previously published (Bistoletti et al., 2011). Plasma samples (0.5 ml) spiked with the IS (20 µl, 20 µg/ml) were extracted by addition of 1 ml acetonitrile (twice) under a high-speed vortexing shaker over 5 min. After centrifugation (2000 *g*, 10 min, 10°C), the clear supernatant was evaporated (40°C) to dryness (Speed-Vac®, Savant, Los Angeles, CA, USA) and then reconstituted with 200 µl of mobile phase. A 50 µl aliquot was injected into the HPLC system. Homogenized egg sample aliquots (0.5 g) fortified with the IS (10 µl, 50 µg/ml) were mixed with 0.5 ml of NaOH (1 N) and extracted by addition of 1 ml acetonitrile (three times) under high speed vortexing. After centrifugation, the supernatants were collected, evaporated to dryness and reconstituted with 200 µl of mobile phase, of which 50 µl

were injected into the Shimadzu Chromatography HPLC system. The HPLC method to quantify ABZ and its metabolites in egg and plasma was previously validated (Bistoletti et al., 2011). The calibration curves for each analyte in both matrices were constructed by least squares linear regression analysis, showing good linearity with correlation coefficients between 0.995–0.999. The absolute recoveries for ABZ, ABZSO and $ABZSO_2$ ranged between 77.4% and 98.2%. Precision (intra- and inter-assay) (CV) was lower than 9.75%. The limits of quantification (LOQ) were 0.05 (ABZ), 0.125 (ABZSO) and 0.05 ($ABZSO_2$) μg/ml for plasma; and 0.10 (ABZ), 0.25 (ABZSO) and 0.25 ($ABZSO_2$) μg/g for egg.

2.6 | Pharmacokinetic and statistical analysis

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 (AUC) for ABZ/metabolites residues in egg was calculated by the trapezoidal rule (Gibaldi & Perrier, 1982) using the PK Solution 2.0 software (Summit 10 Research Services, CO, USA). The pharmacokinetic parameters and concentration data are reported as mean ± *SD*. Statistical analysis of the fertility and hatchability results was carried out by a Contingency table using the Instat 3.0 Software (Graph Pad Software, San Diego, CA). The chi-square test was used to indicate the order of significance. A value of $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | ABZ single oral administration

Egg residues (μg/g) (mean ± *SD*) after ABZ single oral administration (10 mg/kg) to laying hens are shown in Table 1. Although ABZ residues were not detected in egg, ABZSO and $ABZSO_2$ metabolites were quantified up to 2 days after administration with maximum concentrations of 0.47 ± 0.08 and 0.30 ± 0.07 μg/g, respectively.

3.2 | ABZ feed medication at 10, 40, or 80 mg kg^{-1} day^{-1}

After ABZ administration at therapeutic level (10 mg/kg) in feed for 7 days, residue of both parent drug and its metabolites were found (Figure 1). ABZ, ABZSO and $ABZSO_2$ residual concentrations were quantified in eggs from 2 up to 9 days after the start of treatment. The highest egg residual profile was measured for $ABZSO_2$ with a maximum mean concentration (1.35 ± 0.01 μg/g) at day 7 after the start of treatment. ABZ egg residues were quantified from day 3, reaching a maximum at day 7 (C_{max}:1.03 ± 0.01 μg/g) and decreasing till day 9 after the start of treatment. ABZSO residues were the lowest found in eggs from day 2 to day 10 after the start of treatment, reaching a C_{max} (0.57 ± 0.01 μg/g) at day 7. The egg residue exposure estimated as AUCs (areas under the concentration *vs.* time curve) were 100.5 (ABZ), 56.3 (ABZSO) and 141.3 μg hr g^{-1} ($ABZSO_2$).

TABLE 1 Egg residues (μg/g, mean ± *SD*) of albendazole (ABZ), albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$) after ABZ single oral administration (10 mg/kg) to laying hens

	Day 1	Day 2
ABZ	ND	ND
ABZSO	0.25 ± 0.15	0.47 ± 0.08
$ABZSO_2$	0.20 ± 0.02	0.30 ± 0.07

Notes. ND: No detected.

Figure 1 shows plasma (adapted from Bistoletti et al., 2013) and egg AUC comparison after ABZ administration at 10 mg/kg in feed to hens for 7 days.

Figure 2 shows total egg residues (ABZ+ABZSO+$ABZSO_2$) after ABZ administration (10 mg kg^{-1} day^{-1}) in feed to laying hens. Taking into account the maximum residue limits (MRLs) stated for FLBZ (0.4 μg/g) and fenbendazole (1.3 μg/g) in eggs (EMA, 2006; EMEA, 2014), ABZ residues were above this MRLs from the 3rd treatment day till two days post-treatment, and from 4th till 7th treatment day, respectively.

When ABZ was administered at higher than therapeutic level (40 and 80 mg/kg) in feed to laying hens, residues of ABZ and metabolites were also quantified in egg. As shown in Figure 3, residues found after 40 and 80 mg/kg doses were much higher than those found after therapeutic administration (10 mg/kg). However, the increases observed in the egg drug residues were not proportional to the increase in the administered dose in feed.

Plasma drug levels were checked at the end of treatments in all groups. As shown in Figure 5, only the ABZ metabolites were quantified in plasma. ABZSO plasma concentrations were higher than $ABZSO_2$, but both were correlated to the administered dose level.

3.3 | Effect on fertility and hatchability

Fertility and hatchability values obtained after incubation of eggs collected in the different groups are shown in Figure 4. The statistical comparison of fertility results between control and each ABZ treated group did not show any differences. On the other hand, while the comparison of hatchability between the control and the treated group at the lower dose (10 mg/kg) showed no statistical differences, significant differences were found when the control was compared with groups treated with the highest doses (40 and 80 mg/kg).

4 | DISCUSSION

To know the ABZ pharmacokinetic behaviour in poultry, plasma disposition after ABZ administration to laying hens was previously described. After ABZ single administration by intravenous or oral route, both ABZ and metabolites concentrations were quantified in plasma. However, ABZ was not measured in plasma after ABZ administration at 10 mg kg^{-1} day^{-1} for 7 days in feed to laying hens

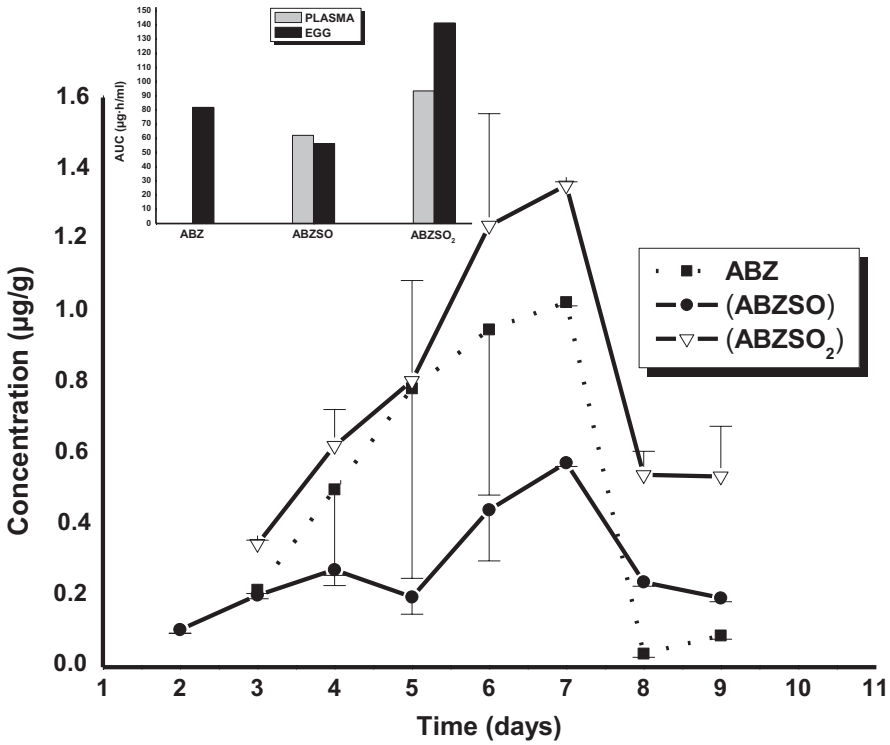

FIGURE 1 Mean (±*SD*) albendazole (ABZ), albendazole sulphoxide (ABZSO), and albendazole sulphone ($ABZSO_2$) egg residue profile vs. time obtained after ABZ medicated feed administration (10 mg kg^{-1} day^{-1}) for 7 days to laying hens. The inserted graph represents the AUC value comparison between egg and plasma for ABZ, ABZSO and $ABZSO_2$ after the same treatment

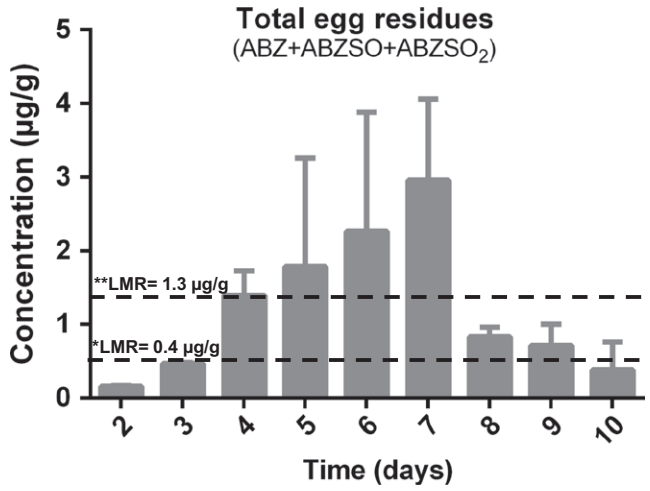

FIGURE 2 Mean (±*SD*) egg total residues, sum of albendazole (ABZ), albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$), after ABZ administration in feed (10 mg kg^{-1} day^{-1}) for 7 days to laying hens. Maximum Residue Limits (MRL) stablished for *flubendazole (FLBZ) and **fenbendazole (FBZ) in egg (EMA, 2014; EMEA, 2006)

(Bistoletti et al., 2013). Drug residue profiles in egg after ABZ therapeutic administration in feed to laying hens have been reported for the first time in the present work. Only ABZSO and $ABZSO_2$ metabolite residues were quantified in egg after ABZ single oral administration. As ABZ is metabolized in the liver and only low concentrations were quantified in plasma for three hours (discussed in Bistoletti et al., 2013), these were insufficient to accumulate in the egg. It is interesting that after ABZ therapeutic administration at 10 mg kg^{-1} day^{-1} for 7 days, high disposition of this molecule was found in egg, although ABZ plasma exposure was reported to be null (Figure 1). After its administration for several days in feed, small amounts of ABZ below the limit of quantification would have reached the plasma. ABZ through the plasma would arrive and accumulate in the yolk (developing egg in the ovary). This process made it possible to quantify ABZ residues in eggs collected at day 3 after the start of treatment. These residues increased progressively in the egg, reaching its maximum value on the last day of treatment. High ABZSO and $ABZSO_2$ residue levels were also quantified in egg. While ABZSO relative bioavailability was similar in both plasma and egg, $ABZSO_2$ egg concentrations were higher than that found in plasma. Therefore, ABZSO and $ABZSO_2$ metabolites were also accumulated in egg reaching maximum values at the end of treatment. As previously reported in plasma after ABZ treatment for several days in feed (Bistoletti et al., 2013), we observed that egg $ABZSO_2$ residue profile was also higher than that for ABZSO. Remarkably, after single oral ABZ administration, the ratio between metabolite profiles in egg changed, ABZSO C_{max} being higher than $ABZSO_2$, in agreement with the results reported for ABZ metabolites in plasma after the same administration (Bistoletti et al., 2013).

As ABZ has not been licensed for use in avian production, no MRLs have been established for this molecule in eggs. On the contrary, MRLs have been determined for FLBZ (400 µg/kg) and FBZ (1300 µg/kg) in egg. The available data about drug residues in egg after BZD administration are scarce. Csiko et al. (1996) reported drug egg residues after ABZ single administration in water (10 mg/kg) to laying hens. Consistent with the present work after single administration, ABZ parent drug was not found in the eggs, while ABZSO and $ABZSO_2$ were quantified at similar range levels (Csiko et al., 1996). FLBZ and metabolites were reported in egg collected

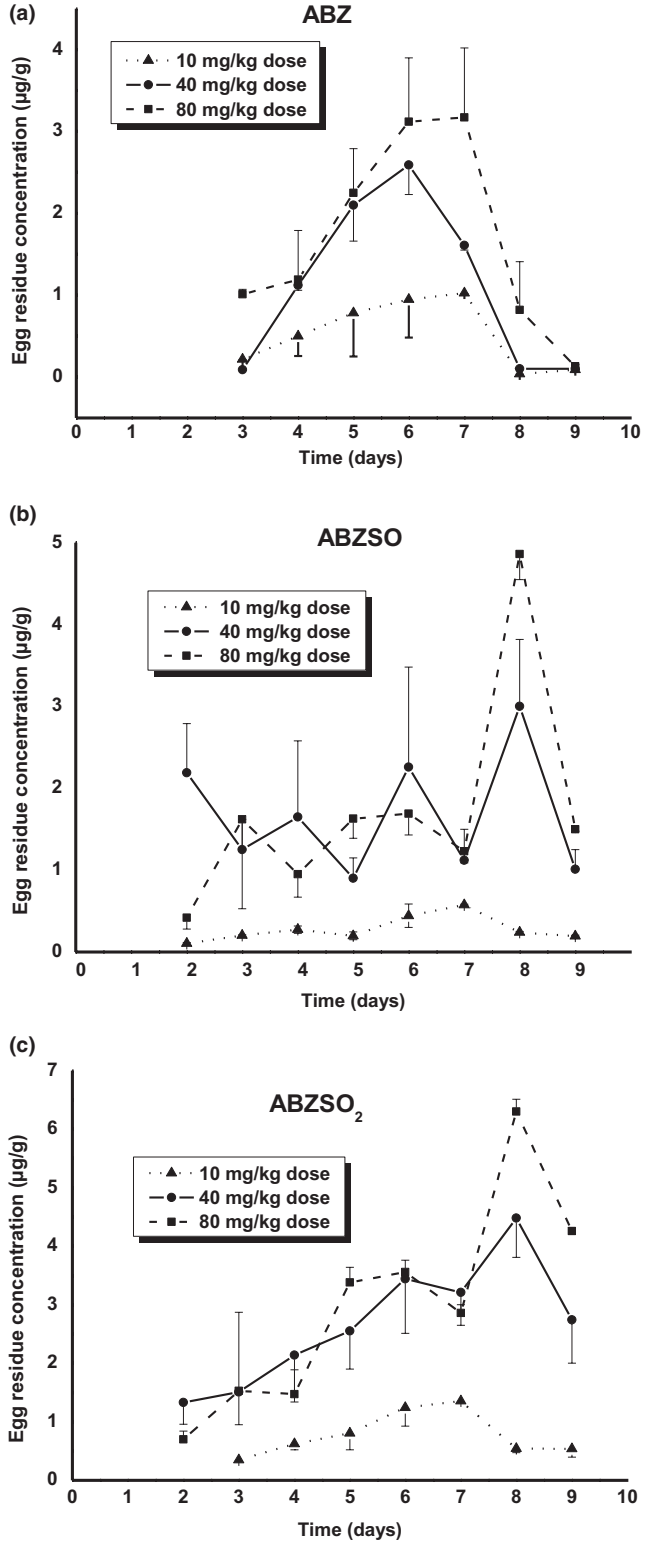

FIGURE 3 Mean (±*SD*) egg residue profiles vs. time for albendazole (ABZ) (a), albendazole sulphoxide (ABZSO) (b), and albendazole sulphone ($ABZSO_2$) (c), obtained after ABZ medicated feed administration at 10, 40 or 80 mg kg^{-1} day^{-1} for 7 days to laying hens

from laying hens treated with FLBZ for 21 (3, 10 or 30 mg/kg) (Kan, Keukens, & Tomassen, 1998) or 7 days (10 mg/kg) (Van Leemput, Agneessens, & Vlaminck, 2006), and were below MRLs allowed.

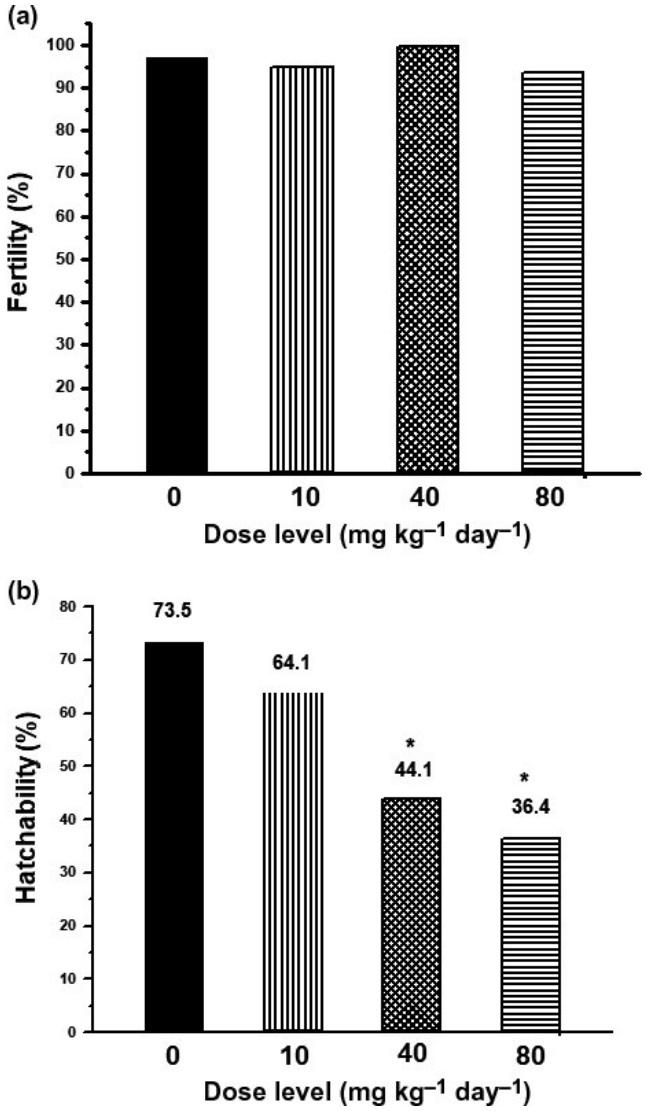

FIGURE 4 Fertility (a) and hatchability (b) percentages obtained for eggs collected from laying hens treated with ABZ at 0 (control), 10, 40 or 80 mg kg^{-1} day^{-1} for 7 days. *Statistically different ($p < 0.05$) to the untreated control group

In fact, label information of commercial FLBZ recommends 0-day withdrawal time in egg. Oxfendazole sulphone residues were also detected in eggs from hens treated with FBZ at 5 (over 3 days) (EMA, 2013) or 1 mg/kg (for 5 consecutive days) (EMA, 2014) as medicated water and all residue levels were below MRL at all-time points during and after treatment. According to the last residue study, a withdrawal period of zero day for eggs was established for FBZ oral suspension (Panacur AquaSol® 200 mg/mL) for use in drinking water for hens (EMA/42178/2014). In the present work, we found residues in egg after both single and therapeutic ABZ administration. The total residues after therapeutic administration were over the BZD MRL mentioned as references (Figure 2). According to these values, it is evident that after ABZ administration to laying hens it would be necessary to establish a withdrawal time or to suspend the consumption of eggs during the treatment

to guarantee the health of the consumer. However, the specific MRLs for ABZ in eggs should be determined in advance.

The embryotoxic and teratogenic effects of ABZ when administered to pregnant animals have been demonstrated. ABZ has been shown to produce increased resorption, decreased birth weight, teratogenic effects, such as external, skeletal and vascular abnormalities, when administered during gestation in mice, rats, rabbits, sheep and zebrafish (Capece et al., 2003; Carlsson, Patring, Erik Ullerås, & Oskarsson, 2011; Cristòfol et al., 1997; Navarro, Cristòfol, Carretero, Arboix, & Ruberte, 1998; Teruel, Felipe, Solana, Sallovitz, & Lanusse, 2003; Teruel et al., 2009). Most of the ABZ toxicity studies have been carried out in mammals, with few data in birds. A single study about the effect of FBZ on hatchability of eggs concluded that this is not affected by the treatment (Taylor, Kenny, Houston, & Hewitt, 1993).

In the present work, the ABZ effect on the fertility and hatchability of the eggs, when administered as a treatment in hens, was evaluated. The results obtained clearly demonstrate that as the dose of ABZ increases, there is a proportional increase in plasma concentrations (Figure 5). In contrast, egg residues did not experience a dose-proportional increase. Although the lowest egg residue levels were measured after treatment with the lowest dose of 10 mg/kg, similar residue levels were obtained after 40 and 80 mg/kg dose treatments. Fertility was not affected at any dose level, but there was a significant decrease in hatchability at the highest doses (40 and 80 mg/kg). Fertility refers to the number of embryonated eggs in relation to the number of eggs placed in the incubator, once the clear eggs have been discarded after the first mirage. In other words, fertility shows the bonding ability of the spermatozoon and the ovule; therefore, a poor fertility can only be imputable to the breeding stock (Ricaurte, 2005).

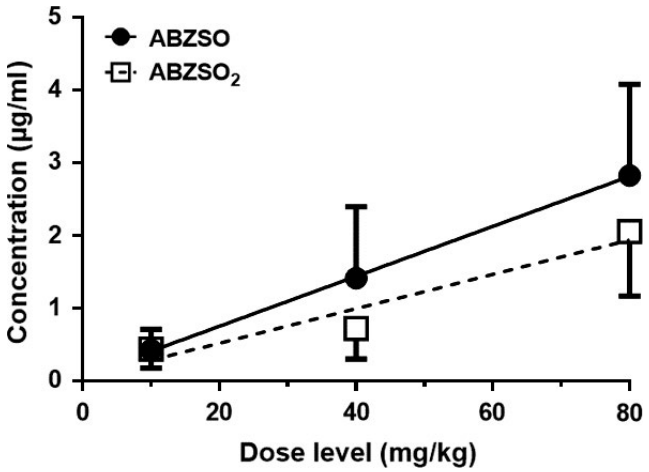

FIGURE 5 Albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$) plasma concentration (µg/ml) at day 8 after the start of 7 days treatment with albendazole at 10, 40 or 80 mg kg^{-1} day^{-1} dose level in feed to laying hens. A high correlation was observed between level dose administered and ABZSO (*r*:0.999) and $ABZSO_2$ (*r*:0.961) plasma concentration at the end of treatment

In our study, both broods were exposed to different doses of ABZ for seven days. As shown by the results obtained, both metabolites were measured in plasma of laying hens at 7 days. When ABZ and metabolites reach plasma, they are distributed throughout the body and in the laying hen it also reaches ovary (with developing follicles) and oviduct (where egg white is formed and secreted). Some studies consider egg yolk as the main egg compartment where drug residues are deposited (Anhalt, 1977; Hafez, 1991), whereas other authors report a higher level of residues in the white than in the yolk (Blom, 1975).

Taking into account the egg formation process, the ABZ and metabolite egg residues after treatment would have reached the egg at different formation stages through the plasma (Kan & Petz, 2000): yolk formation stage, with the fast-growing follicles (which will be ovulated within 10 days), along with lipoproteins from the liver; white formation stage in the magnum (approx. 3 hr), together with soluble proteins from the liver; white hydration stage in the uterus (approx. 6 hr). In fact, the transfer of drugs during the white formation has been demonstrated, which explains that drugs administered in the afternoon lead to the presence of residues in eggs laid the next day (Donoghue & Hairston, 2000). The processes mentioned would explain the progressive increase in residues found in eggs throughout the ABZ treatments in the present assay. We could also assume that the arrival of more water-soluble metabolites would occur through the white, although not exclusively. However, we should have analyzed white and yolk separately to corroborate this hypothesis.

Studies carried out on cell cultures of rat embryos showed that ABZSO produces a cytotoxic effect at the concentration of 4.2 µg/ml (Whittaker & Faustman, 1991). ABZSO also inhibits tubulin polymerization at the concentration of 1.95 µg/ml (Lacey & Watson, 1985) and inhibits bovine embryo development at 0.5 µg/ml (Piscopo & Smoak, 1997). In the current study, the ABZSO residue levels quantified in the eggs after ABZ treatments with 40 and 80 mg/kg doses, widely exceeded the reported cytotoxic concentrations. However, these concentrations did not affect the fertility, the union of the ovum and the spermatozoid. Consistent with our results, a study carried out on the fertility of eggs collected from FBZ treated hens showed no effect, albeit egg production decreased (Taylor et al., 1993). On the other hand, a decrease has been reported in the semen quality of male turkeys when they were treated with FBZ with the consequent decrease in fertility (Bakst, Kramer, & Long, 2006). In the present work, the males received the same treatments (feed medicated with ABZ) as the hens, when coexisting in the same yard for reproductive purposes, but this did not affect their fertility. Failure of rooster fertility could have occurred as the presence of ABZ/metabolite residues has been reported at significant levels in the sperm and seminal fluid of sheep treated with oral ABZ (Batzias, Theodosiadou, & Delis, 2004).

On the other hand, according to a previous report (Piscopo & Smoak, 1997), the residues that reached the egg after the treatments with ABZ at high doses of 40 and 80 mg/kg in the feed evidently altered the embryonic development, leading to a decrease in egg hatchability.

To conclude, the aim of the present work was to contribute to the knowledge of ABZ behaviuor in laying hens previously reported (Bistoletti et al., 2013). The results stated here provide useful information on egg residue profile of ABZ after oral administration in feed to laying hens. Residue levels in eggs were higher than those in plasma, and therefore residues accumulated in eggs. Total egg residue level after ABZ treatment for seven days was higher than MRLs established for FLBZ or FBZ. This fact showed the need to determine MRL for ABZ in eggs. ABZ administration to laying hens did not affect the egg fertility at the assessed dosages. Although egg hatchability during ABZ treatment at the therapeutic dose (10 mg/kg) was not affected, it decreased when the dose was 4–8 times higher than that required to obtain satisfactory efficacy. As synthetic anthelmintics still remain the most important tool of internal parasite control in poultry, these results should be considered to use ABZ rationally in laying hens.

ACKNOWLEDGMENTS

This study was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina (PIP N° 11220080102184). The experimental animals and facilities provided by Unidad Académica Dr. Ramón Santamarina, Escuela de Educación Agropecuaria N°1 (Tandil, Buenos Aires, Argentina), is acknowledged. The authors would like to thank the farmer for collaborating with this study.

CONFLICT OF INTEREST

There is no potential conflict of interests associated with this study.

AUTHORS' CONTRIBUTION

L. Moreno has contributed to the design, organization and experimental development of the work. She has also been in charge of the analysis of results and writing of the manuscript. She has read and approved the final manuscript. M. Bistoletti has participated in the method validations for sample analysis, the analysis of the samples, the control of the egg incubation process, and in the experimental development of the work. She has read and approved the final manuscript. H. Fernández has participated in the design and control of the egg incubation process, and the egg evaluation to determine fertility and hatchability. He has read and approved the final manuscript. L. Cantón has contributed to the sampling, and samples preparation and storage for further analysis. She has read and approved the final manuscript. L. Ceballos has contributed to the validation of the analysis methods, the preparation, extraction and analysis of the samples. She has read and approved the final manuscript. C. Cantón has contributed to the care of the animals, preparing the medicated food, administering the medication, and taking samples. She has read and approved the final manuscript. C. Lanusse has participated in the data analysis and correction of the manuscript. He has read and approved the final manuscript. L. Álvarez has contributed to the design of the work, the treatment administration, sampling, and correction of the manuscript. He has read and approved the final manuscript.

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How to cite this article: Moreno L, Bistoletti M, Fernández H, et al. Albendazole treatment in laying hens: Egg residues and its effects on fertility and hatchability. *J vet Pharmacol Therap*. 2018;00:1–8. https://doi.org/10.1111/jvp.12675