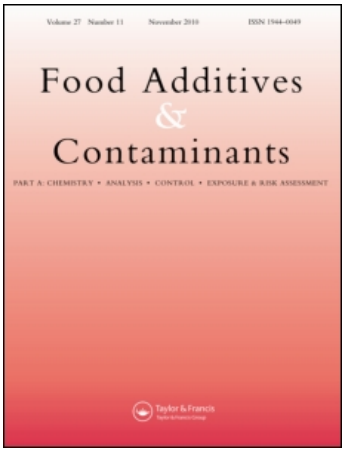


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# Residual concentrations of the flukicidal compound triclabendazole in dairy cows' milk and cheese

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Triclabendazole (TCBZ) is a flukicidal halogenated benzimidazole compound extensively used in veterinary medicine. Liver fluke control in lactating dairy cattle is difficult because treatment should be implemented only during the dry period to avoid milk residues. However, control in endemic areas is usually implemented as regular treatments three to four times a year, even during the lactating period. Thus, information on TCBZ milk excretion and the risk of the presence of drug residues in fluid milk and milk-derivate products is essential. The experimental aims were to evaluate the comparative disposition kinetics of TCBZ and its sulpho-metabolites in plasma and milk in lactating dairy cattle after the oral administration (12 mg $kg^{-1}$) of TCBZ and to assess the pattern of residues in cheese made with milk from treated dairy cows. Both TCBZ sulphoxide and sulphone metabolites but not TCBZ were detected in milk (up to 36 and 144 h, respectively) and plasma (up to 144 h) after oral administration of TCBZ. Residual concentrations of TCBZ sulpho-metabolites were found in cheese made with milk from treated animals. The total average residual concentration in fresh cheese was 13.0-fold higher than that obtained in milk used for its elaboration. The high concentrations of TCBZ sulpho-metabolites recovered in fresh cheese should be seriously considered before milk from treated cows is used for making dairy products.

**Keywords:** high-performance liquid chromatography; veterinary drug residues; milk; cheese



## Introduction

Triclabendazole (TCBZ) is a flukicidal halogenated benzimidazole thiol derivative. It was introduced in the early 1980s for the treatment of fasciolosis in animals. In fact, *Fasciola hepatica* is a liver trematode parasite that infects humans and a wide variety of domestic and wild mammals. TCBZ has been the drug of choice for treating liver fluke infections in livestock for over 20 years, and more recently it has been used successfully to treat human cases of fasciolosis (Esteban et al. 1998; Ortiz et al. 2000; Curtale 2008) due to its high activity against both adult and juvenile flukes (Boray et al. 1983; Smeal and Hall 1983).

After a relatively short period of time following its introduction in to the pharmaceutical market, fluke strains resistant to TCBZ were observed in farm animals in Australia in the mid-1990s (Overend and Bowen 1995) and, soon after that, resistant flukes were reported in a number of European countries (Ireland, the UK, the Netherlands and Spain) (Fairweather 2005; Alvarez-Sánchez et al. 2006). Parallel to the spread of TCBZ resistance, a critical increase in the prevalence of fasciolosis was observed, which was mainly attributed to environmental changes (Mas-Coma et al. 2005, 2009). Consequently, farmers dismissed the use of TCBZ in favour of older fasciolicide compounds; however, none of these is as active against the damaging immature stages of liver fluke as TCBZ.

The Cajamarca Valley in Peru in South America, where the present experimental trial was performed, is an endemic area with a high prevalence of fasciolosis in humans (15–30%) (Knobloch 1985; Ortiz et al. 2000; Esteban et al. 2002), the latter being in agreement with a high animal infection (78% and 97% in cattle and sheep, respectively) (Claxton et al. 1997). The rural population (almost 8 million people) in the Andean countries, mainly Bolivia and Peru, where the climatic

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characteristics of a high altitude (2000–4200 m above sea level) and the contamination of pastures and water sources by infected animals, is estimated to be at risk levels (World Health Organization (WHO) 1995; Fuentes et al. 1999; Mas-Coma et al. 2005). In livestock, *F. hepatica* infection is an important cause of production losses worldwide and it usually lacks visible clinical signs. However, even when infections are subclinical, they may have a major economic impact as reductions in weight gain (Elitok et al. 2006), fertility (Loyacano et al. 2002) and milk yield (Schweizer et al. 2005).

The control of fasciolosis in livestock is mainly based on the administration of anthelmintics, but the effective control of fasciolosis is difficult, particularly in lactating dairy cattle, which can be treated only during the dry-off periods to avoid drug residues in milk intended for human consumption. However, in endemic areas as Cajamarca (Peru) and many other regions worldwide where the fasciolosis is recognised as a major problem in dairy cattle, parasite control programmes are implemented as regular antihelmintic treatments three to four times a year, even during the lactating period. The milk produced (without any withdrawal time) is destined for human consumption as fluid milk or cheese. Currently, some benzimidazole anthelmintics such as oxfendazole, fenbendazole and thiabendazole are approved in dairy animals by federal authorities in different countries but a withdrawal time after treatment is required to avoid residual concentration above of the maximum residue limits (MRLs) established by appropriate regulatory agencies. The MRLs for TCBZ in edible tissues, expressed as the marker residue, keto-triclabendazole (keto-TCBZ) has been determined (European Agency for the Evaluation of Medical Products (EMEA) 2005; WHO 2009); however, the MRL in milk has not been established and, therefore, the MRL permitted in milk should be zero.

Several studies on the plasma pharmacokinetic profile of TCBZ (parent drug and its metabolites), its metabolism and tissue residues (Wongtavatchai and MacNeil 2006; Reeves and Swan 2009) have been performed in ruminant species, including sheep (Hennessy et al. 1987; Mestorino et al. 2008; Reeves and Swan 2009; Virkel et al. 2009), goats (Kinabo and Bogan 1988) and cattle (Sanyal 1995; Mestorino et al. 2008; Reeves and Swan 2009). However, there are no data available on the plasma disposition of TCBZ and/or its metabolites in lactating dairy cattle and its milk excretion.

The work reported here was designed to evaluate the concentration profiles of TCBZ and its sulpho-metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$), in plasma and milk from lactating dairy cows orally treated with TCBZ and to assess the pattern of residues in cheese made with milk from TCBZ-treated cows.

## Material and methods

### *Chemicals*

Reference standards (99% purity) of TCBZ and its sulpho-metabolites (TCBZSO and $TCBZSO_2$) were provided by Novartis Animal Health (Basel, Switzerland). Stock solutions ($1000\,\mu g\,ml^{-1}$) were prepared in methanol HPLC grade. Ammonium acetate and solvents used for chemical extraction and chromatographic analyses (HPLC grade) were purchase from Baker, Inc. (Phillipsburg, NJ, USA). TCBZ commercial formulation (10%, Fasinex®, Novartis) was obtained from the local market.

### *Experimental animals, treatment and sampling*

The study was carried out on a dairy farm situated in Cajamarca, Peru. It is located at 2750 meters above sea level. Seven female Holstein dairy cows weighing between 490 and 630 kg were used. They were kept under field conditions, grazing on pasture and with free access to drinking water during the whole experimental period. The health of the animals was closely monitored prior to and throughout the trial period. Dairy cattle were milked twice a day (every 12 h) and the whole milk production was measured throughout the trial. The average milk production during the trial period was $121 \cdot day^{-1}$ per animal. Each animal was orally treated with a single dose of TCBZ (Fasinex®, TCBZ 10%, Novartis) at a dose rate of $12\,mg\,kg^{-1}$ of body weight.

Blood samples were taken from the jugular vein in heparinised vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) prior to treatment and at 6, 12, 24, 36, 48, 60, 72, 84, 96, 120 and 144 h post-treatment. Moreover, at each collection time, a milk sample (approximately 50 ml) was collected from the whole milking yielded by each cow, after homogenisation. The blood samples were centrifuged at 2000 *g* for 20 min, and the recovered plasma was transferred to vials. Milk and plasma samples were frozen at −18°C until analysed. The whole milk production of all experimental animals collected at 12, 24, 36, 48, 60 and 120 h post-treatment was pooled and processed according to the creamy cheese production protocol used at the dairy unit where the current experimental work took placed. Creamy cheese is a fresh cheese typical from Cajamarca. Cheese samples were taken after 3 days of cheese production. The cheese slices (15 g) were minced, mixed and kept as a pooled sample in appropriate vials. Samples were frozen at −18°C until analysed. All blank samples were taken before treatment from the same experimental animals.

### *Analytical procedures*

#### *Sample clean-up/extraction*

Pure reference standards (99%) of TCBZ and its sulpho-metabolites (TCBZSO and $TCBZSO_2$) were used to validate the HPLC method. Standard solutions of TCBZ and its sulpho-metabolites were prepared by successive dilutions in methanol from stock solutions ($1000\,\mu g\,ml^{-1}$) and stored at $-18^{\circ}C$. The fortified and experimental samples (plasma, milk and cheese) were added with oxibendazole (OBZ) (as internal standard, 99.2% purity).

The extraction procedures to quantify TCBZ and its sulpho-metabolites in fortified and experimental samples (plasma, milk and cheese) were carried out following modifications of previously described methods (Moreno et al. 2005; Virkel et al. 2009). Aliquots of the collected plasma (0.5 ml), milk (0.5 ml) and cheese (0.5 g) were supplemented with OBZ and then mixed with 1 ml acetonitrile. Cheese samples were previously alkalinised with sodium hydroxide 5 N (20 µl). The samples were shaken in a multivortex for 20 min, sonicated in a ultrasonic bath for 8 min and then centrifuged at 2500 *g* for 15 min. The supernatants obtained from milk, plasma and cheese samples, after solid extraction with C18 cartridge (Strata, Phenomenex, CA, USA), were evaporated to dryness using a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, CA, USA). The dry residue was dissolved in 500 µl of mobile phase.

#### *Chromatographic conditions: drug/metabolites analysis samples*

A total of 50 µl of each extracted sample were injected through an autosampler (Shimadzu SIL 10 A Automatic Sample Injector) into a Shimadzu 20 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Kromasil C18 (5 µm, 250 mm × 4.60 mm) reverse-phase column (Eka Chemicals, NY, USA) at 30°C and a UV detector (Shimadzu, SPD-20A UV detector) reading at 300 nm. The mobile phase was composed of acetonitrile/ammonium acetate (0.025 M, pH 6.6) and pumped at $1.2\,ml\,min^{-1}$ as an elution gradient (0–5 min: 64/36; 6–9 min: 52/48; 10–12 min: 64/36; 13–20 min: 52/48). The analytes were identified with the retention times of 99% pure reference standards. Chromatographic peak areas of each molecule were measured using the integrator software (Class LC 10, Shimadzu Corporation, Kyoto, Japan) of the HPLC system.

#### *Method validation*

A complete validation of the analytical procedures for the extraction and quantification of TCBZ and its sulpho-metabolites in each matrix was performed before the analysis of experimental samples from the specific trial was begun. Calibration lines in the ranges of $0.1–20\,\mu g\,ml^{-1}$ (plasma and milk) or $\mu g\,g^{-1}$ (cheese) for TCBZ and its metabolites were plotted using the peak area ratios between each analyte and the internal standard. The data were analysed for linearity using a least-squares linear regression analysis, and using the Run test and ANOVA to determine if the data differed from a straight line. The absolute recovery of the drugs under study was measured by comparison of the peak areas from spiked samples with the peak areas resulting from direct injections of standards in mobile phase. The recoveries of TCBZ, TCBZSO and $TCBZSO_2$ from cow plasma, milk and cheese were obtained in the ranges of $0.1–20\,\mu g\,ml^{-1}$ or $\mu g\,g^{-1}$, using three replicates for each drug concentration. The inter-day precision of the extraction and chromatographic procedures was evaluated by processing four replicate aliquots of pooled liquid and solid samples containing known amounts of TCBZ and its sulpho-metabolites ($0.5, 5, 15\,\mu g\,ml^{-1}$ or $\mu g\,g^{-1}$) on different working days. The stability of TCBZ and its sulpho-metabolites in spiked milk and cheese samples ($0.5, 5, 15\,\mu g\,ml^{-1}$ or $\mu g\,g^{-1}$) during the storage in freezer was evaluated. The accuracy of the analytical method was estimated by the differences between observed and calculated concentrations, and it is expressed as the percentage of relative error (% RE). The accuracy was estimated for all of the matrices under study at TCBZ and its sulpho-metabolites concentrations of $0.5, 5, 15\,\mu g\,ml^{-1}$ or $\mu g\,g^{-1}$ with three determinations for each concentration value. The coefficient of variation (CV) for recovery and inter-day precision of the method were calculated (Bolton 1984). The limit of quantification (LOQ) was defined as the lowest concentration that can be measured with acceptable precision ($CV < 20\%$) and accuracy (20%) (Snyder et al. 1997).

#### *Drug quantification and pharmacokinetic and statistical analyses of the data*

Drug concentrations in experimental samples were determined by HPLC, calculating the ratio between the areas under the peaks of TCBZ or its sulpho-metabolites and OBZ (internal standard) using the CR10 software and interpolating these areas on the calibration lines prepared for each biological matrix. The statistical program (Instat 3.0, Graph Pad Software, Inc., San Diego, CA, USA) was used for linear regression analyses and linearity tests. The milk and plasma concentration versus time curves obtained after treatment in each individual animal was analysed with the PK Solution 2.0 (Ashland, OH, USA) computer program.

The TCBZSO and $TCBZSO_2$ kinetic variables estimated in plasma and milk are reported as mean ± standard deviation (SD). The Student's *t*-test and Mann–Whitney *U*-test were used to estimate the

differences between kinetic parameters obtained in milk and plasma. Values lower than $p<0.05$ were considered to be significant.

## Results and discussion

A complete validation of the analytical method used to measure TCBZ, its sulpho-metabolites and OBZ (as internal standard) in plasma, milk and cheese was performed. The regression lines showed a high correlation coefficient ($r=0.999$) for each concentration range and the departure from linearity was not statistically significant. The concentrations in the experimental samples were determined by interpolation using the standard lines. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked samples (plasma, milk and cheese) and those of direct standards prepared in mobile phase (concentration range between 0.1 and 20 $\mu g\,ml^{-1}$). TCBZ and its sulpho-metabolites absolute recovery ranges in different matrices were: 74–88% (plasma), 66–80% (milk) and 67–89% (cheese). The coefficients of variation (CVs) of inter-assay precision on different working days were <8.0% and relative error (accuracy) values were <4.0% for all analysed samples. The LOQ was $0.1\,\mu g\,ml^{-1}$ or $\mu g\,g^{-1}$ for all matrices. The thermal stability of TCBZ and its sulpho-metabolites in milk and cheese samples was also established with no evidence of degradation during sample processing (room temperature) and after 3-month freezer storage. The results obtained in this validation procedure ensured the reliability of the method for detecting TCBZ and its sulpho-metabolites residues in plasma, milk and cheese. The validated method was successfully applied to quantify the TCBZ sulpho-metabolites in plasma, milk and cheese made with milk from treated cows with TCBZ by oral route.

After the oral administration of TCBZ in dairy cattle, its sulpho-metabolites (TCBZSO and $TCBZSO_2$) were detected in plasma. The absence of TCBZ and the presence of its sulpho-metabolites in plasma of dairy cows indicate, as previously reported (Hennessy et al. 1987; Sanyal 1995), that TCBZ was mainly removed from portal blood and extensively metabolised pre-systemically. This phenomenon is explained by the fact that TCBZ, during absorption from the digestive tract, can be metabolised either in the intestine mucose or liver (Hennessy et al. 1987; Virkel et al. 2006). Overall, TCBZ is oxidised to form the primary metabolites, TCBZSO and $TCBZSO_2$. The sulpho-metabolites present in the systemic circulation exhibited an extensive diffusion to different tissues such as mammary gland and, consequently, TCBZSO and $TCBZSO_2$ were recovered in milk after oral treatment.

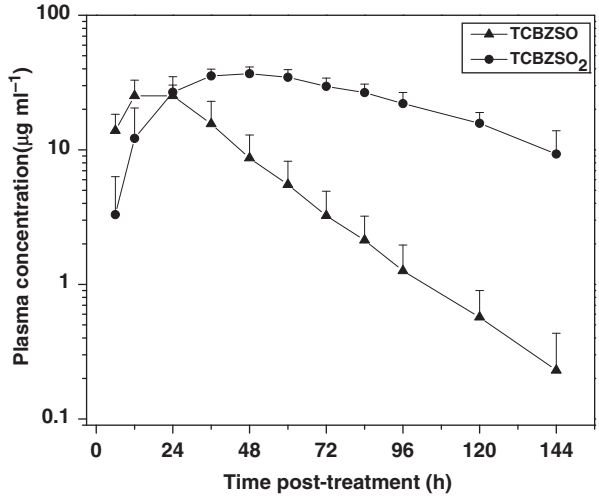


Figure 1. Plasma concentration profiles (mean ± SD) of triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$) measured after oral administration of triclabendazole ($12\,mg\,kg^{-1}$) to dairy cows. Triclabendazole was not detected at any time post-administration.

Figures 1 and 2 depict the concentration versus time profiles of both sulpho-metabolites of TCBZ detected in plasma and milk after TCBZ oral administration in dairy cattle. TCBZSO and $TCBZSO_2$ concentration profiles in plasma were higher than those measured in milk. Plasma concentration increased progressively to reach peak concentrations of $26.8\,\mu g\,ml^{-1}$ (TCBZSO) and $37.9\,\mu g\,ml^{-1}$ ($TCBZSO_2$) at 16 and 51 h post-treatment, respectively. The same metabolites were found in milk but at lower concentration levels. Milk residues increased progressively to reach peak concentrations of $2.4\,\mu g\,ml^{-1}$ ($TCBZSO_2$) at 48 h post-treatment. Although TCBZSO was detected in milk from 6 and up to 36 h post-administration, the pharmacokinetic analysis was not possible because insufficient data points were obtained with the sampling design employed. $TCBZSO_2$ was the metabolite recovered at the highest concentrations along the experimental time both in the blood stream and milk.

The pharmacokinetic parameters summarising the disposition of TCBZSO and $TCBZSO_2$ in plasma and milk are shown in Table 1. $TCBZSO_2$ plasma AUC values after TCBZ oral administration were 3.0-fold higher than those of TCBZSO ($p<0.001$). This significant difference between metabolites plasma availability obtained in cows after oral treatment was not observed in goats as reported elsewhere (Kinabo and Bogan 1988). This and other differences among animal species are not surprising considering the number of factors known to affect the disposition kinetics of antiparasitic drugs in ruminants (McKellar and Scott 1990).

The plasma kinetic analysis showed that the time to reach the peak concentration ($T_{max}$), elimination

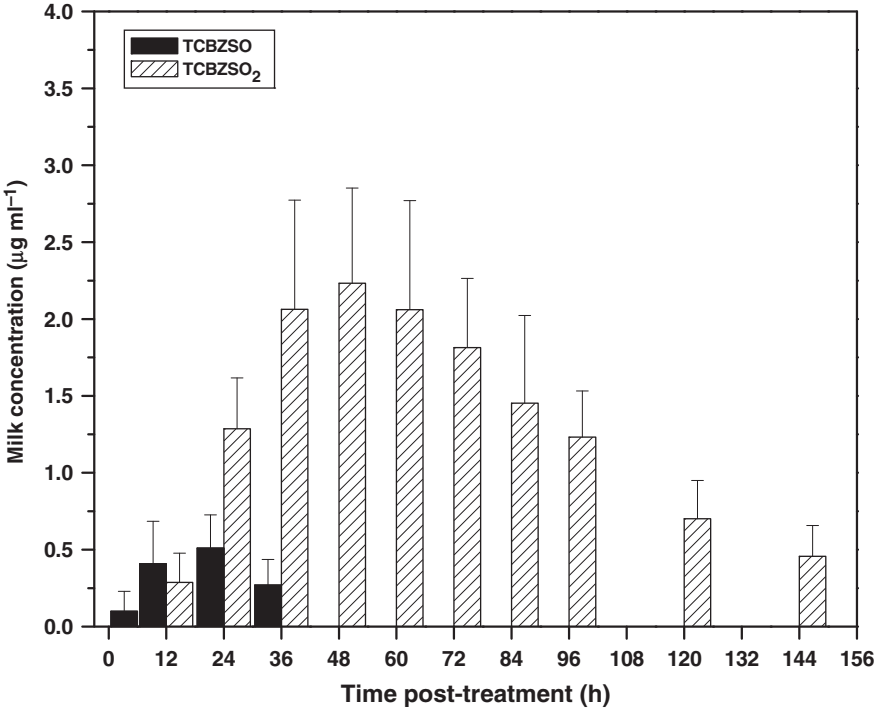


Figure 2. Milk concentration profiles (mean ± SD) of triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$) measured after oral administration of triclabendazole (12 mg kg$^{-1}$) to dairy cows. Triclabendazole was not detected at any time post-administration.

Table 1. Mean (± SD) of the kinetic parameters describing the disposition of triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$) in plasma and milk of dairy cows after oral administration of triclabendazole (12 mg kg$^{-1}$) ($n = 7$).

| Kinetic parameter | TCBZSO | $TCBZSO_2$ |
|---|---|---|
| *Plasma* | | |
| $C_{max}$ (μg ml$^{-1}$) | 26.8 ± 8.9 | 37.9 ± 4.4 (**) |
| $T_{max}$ (h) | 16.3 ± 7.5 | 51.4 ± 11.4 (***) |
| $T_{½el}$ (h) | 17.8 ± 2.9 | 49.2 ± 8.4 (***) |
| $AUC_{0-t}$ (μg h ml$^{-1}$) | 1073.1 ± 381.2 | 3266.8 ± 377.4 (***) |
| MRT (h) | 33.3 ± 4.9 | 95.8 ± 12.4 (***) |
| $AUC_{plasma/milk}$ ratio | | 19.4 ± 4.4 |
| *Milk* | | |
| $C_{max}$ (μg ml$^{-1}$) | n.a. | 2.41 ± 0.67 |
| $T_{max}$ (h) | n.a. | 48 ± 9.8 |
| $T_{½el}$ (h) | n.a. | 34.0 ± 6.7 |
| $AUC_{0-t}$ (μg·h ml$^{-1}$) | n.a. | 177.6 ± 50.2 |
| MRT (h) | n.a. | 81.8 ± 7.5 |
| Percentage dose recovered | 0.11 ± 0.07 | 1.3 ± 0.42 (***) |

Notes: Mean kinetic variables obtained for TCBZSO are statistically different at $p < 0.01$ (**) or $p < 0.001$ (***) from those obtained for $TCBZSO_2$ after oral triclabendazole administration. $C_{max}$, peak milk or plasma concentration; $T_{max}$, time to peak concentration; $T_{½el}$, elimination half-life; AUC, area under the concentration versus time curve; MRT, mean residence time; and $AUC_{plasma/milk}$ ratio, the ratio between the AUC values obtained in plasma and milk where the drug was measured.
n.a., Not applicable.

half-life and mean residence time (MRT) observed for TCBZSO were shorter ($p < 0.001$) than those of $TCBZSO_2$. This corroborates that the metabolic pathway for TCBZ in lactating dairy cattle was the same to that reported previously for other ruminant species such as sheep (Hennessy et al. 1987; Mestorino et al. 2008), goat (Kinabo and Bogan 1988) and buffalo (Sanyal 1995). The previously described long persistence of TCBZ sulpho-metabolites in plasma, as consequence of a high binding to plasma proteins (90–95%) along with a slow release from rumen (Hennessy et al. 1987; Sanyal 1995; Mestorino et al. 2008) was evidenced in the current work by longer plasma residence times and elimination half-lives compared with other benzimidazole anthelmintics such as albendazole, fenbendazole and their metabolites (Lanusse et al. 1995; Sánchez et al. 1996).

Reflecting the observation in the bloodstream, TCBZ parent drug was not recovered in milk. Concentrations of TCBZSO and $TCBZSO_2$ in milk were attained in parallel with those found in plasma. The major milk residue was represented by $TCBZSO_2$, as reported previously in goats (Kinabo and Bogan 1988). It was detected from 12 h and increased progressively to reach a peak concentration of 2.4 μg ml$^{-1}$ at 48 h post-treatment.

The maximum milk residual concentrations of TCBZSO and $TCBZSO_2$ were smaller (approximately 50- and 17-fold, respectively) when compared with

plasma maximum concentrations. These differences were lower than those reported in goats, although in the current experimental work, the detection time of $TCBZSO_2$ in milk (with values higher than $0.1\,\mu g\,ml^{-1}$) was longer than those reported in goats (Kinabo and Bogan 1988).

Consistently with the plasma kinetic results, $TCBZSO_2$ showed the highest dose percentage excreted in milk. The total percentage of dose recovered in milk for $TCBZSO_2$ was significantly higher ($p < 0.001$) than that observed for TCBZSO (Table 1). Results reported here show that even though a limited distribution of the sulpho-metabolites from the bloodstream to the milk (clearly reflected in the AUC ratios between plasma and milk) (Table 1), the percentage of $TCBZSO_2$ recovered in milk was 1.3% of the administered dose.

The percentage of the sulphone-metabolite (1.3%) recovered in milk was similar to that reported in cows by Counotte et al. (1990) but higher than that reported for other benzimidazole anthelmintic compounds such as albendazole (approximately 0.1%). However, the percentage of sulphoxide-metabolite excreted was similar to that previously reported for albendazole (Moreno et al. 2005). As a consequence of a high binding to plasma proteins (90–95%) and a lower lipophilicity of TCBZ sulpho-metabolites compared with other antiparasitic drugs such as ivermectin (macrocyclic lactone compound), the percentage of $TCBZSO_2$ excreted in milk was lower than ivermectin (5%) (Toutain et al. 1988).

Residual concentration of TCBZSO (from 6 to 36 h) and $TCBZSO_2$ (from 12 to 144 h) were found in pooled milk after the oral administration of TCBZ. This milk collected from treated cows was used in the cheese-making phase. The sum of total residues (TCBZSO and $TCBZSO_2$) in pooled milk was between 0.6 and $2\,\mu g\,ml^{-1}$ at different days of cheese elaboration.

Once the cheese-making process was completed, cheese samples were collected on day 3 of the ripening period. Sampled cheeses were fresh and the whey was drained without pressure. The total residual concentrations in cheese (between 1.1 and $20.0\,\mu g\,g^{-1}$) were higher than those measured in the milk used for their production. The mean ratio between the total residual concentration values measured in cheese and milk used for its production was 13.4-fold higher in cheese (Figure 3). The concentration of both metabolites increased (ranging between 2.4- and 26.1-fold) compared with their residual concentrations in the milk used to make that cheese. The increment of solid contents in the cheese may have accounted for the residual concentration of both metabolites found in cheese. On the other hand, the high variation in ratio values at different days of production could be due to the artisanal cheese making process carried out in the trial reported here, where TCBZSO and $TCBZSO_2$ residues could have been lost in the whey (renneting and ripening period) in dissimilar proportions on different days of production. This could explain why residual concentration in the milk (sum of TCBZSO and $TCBZSO_2$) used for cheese making was not correlated with the residual concentration in the elaborated cheese. Unfortunately, whey samples were not collected during the cheese making process (renneting time) and ripening period (3 days).

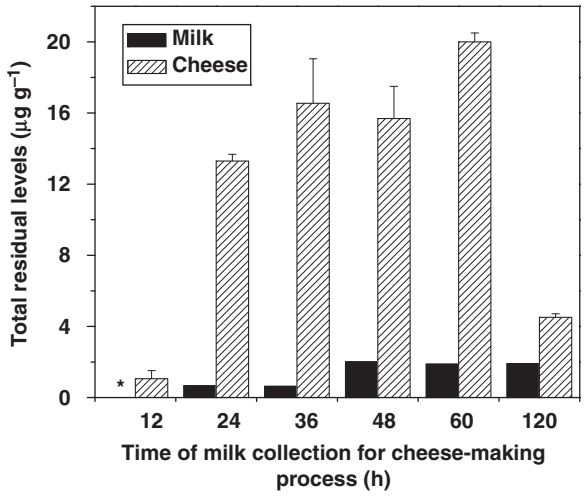


Figure 3. Comparison of the total residual levels (sum of triclabendazole sulphoxide and triclabendazole sulphone concentrations) measured in pooled milk and cheese elaborated with milk collected from treated cows ($n = 7$) at different time post-treatment. *Value below the limit of quantification of the validated method. Triclabendazole was not detected at any time post-treatment in either matrix.

Similar studies on albendazole reported a higher concentration of its sulpho-metabolites in whey compared with that found in semi-hard cheese, which was attributed to the polarity of these metabolites (Fletouris et al. 1998; De Liguoro et al. 1996). Unfortunately, a follow-up during cheese making (renneting time and consistence of the curd) and ripening period was not done. However, no major differences in the cheese-making process were observed after using milk from either TCBZ-treated or untreated dairy cows. As shown in a previous experimental work in our laboratory, the residual concentrations of TCBZ sulpho-metabolites were stable during thermal milk processing (Iezzi et al. 2009). The large variation in the cheese/milk concentration ratios in different days of production could be a consequence of the erratic whey losses during the cheese making process. Hence, the residual concentrations of TCBZ sulpho-metabolites in fresh cheese could be higher and more variable due to differences in whey retention by cheese.

In conclusion, the pharmacokinetic results reported here show that TCBZ sulpho-metabolites are excreted by milk after TCBZ oral administration in lactating dairy cows, being $TCBZSO_2$ the major TCBZ metabolite excreted in milk (1.3% of the dose). The percentage of $TCBZSO_2$ excreted in milk is lower compared with other more lipophilic antiparasitic drugs such as ivermectin and moxidectin. However, it is important to highlight that residual concentrations of TCBZ sulpho-metabolites found in fresh cheese are 13-fold (between 2.4- and 26.1-fold) higher than those obtained in the milk utilised for cheese making.

Keto-TCBZ has been proposed as a marker residue, since all the extractable TCBZ-related residual molecules can be easily oxidised to form this keto-TCBZ derivative. Besides, some factors accounting for conversion of keto-TCBZ to total TCBZ residues were established for different edible tissues (WHO 2006). However, residue limits for neither TCBZ nor any of its metabolites were established for milk or milk derived products. In the current trial, keto-TCBZ was not determined due to the lack of the analytical standard, but it can be expected that the high concentrations of the TCBZ sulpho-metabolites measured may also correspond to high concentrations of the marker residue (i.e. keto-TCBZ). Regardless the analyte being measured in milk, drug residues are retained in cheese curd with an increment in residual concentrations occurring due to cheese dehydration (whey losses). Therefore, the high concentrations of TCBZ sulpho-metabolites recovered in fresh cheese should be seriously considered before issuing any recommendation on the manufacturing of milk from treated cows (i.e. cheese elaboration).

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