Pattern of Eprinomectin Milk Excretion in Dairy Sheep Unaffected by Lactation Stage: Comparative Residual Profiles in Dairy Products

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ABSTRACT

Eprinomectin (EPM) is a broad-spectrum endectocide compound approved for use in dairy cattle with a zero milk-withdrawal period, but has not been registered for use in lactating dairy sheep. The pattern of EPM excretion in milk was comparatively characterized following its pour-on administration (500 μg/kg) to lactating dairy sheep at two different stages of lactation. The relationship between milk excretion and plasma disposition kinetics of EPM was characterized. Residual EPM concentrations were assessed during cheese making (whey and curd) and ripening (cheese) by high-performance liquid chromatography and fluorescence detection. EPM was poorly distributed from the bloodstream to the mammary gland and low concentrations were excreted in milk. The level of milk production (early-mid and mid-late lactation) did not affect either the plasma-milk distribution or the pattern of residual concentrations in milk. During cheese making, the highest residual concentrations of EPM were measured in the curd, which increased during cheese ripening, reaching a maximum after 40 days. However, these residual concentrations were below the maximum residue limit of 20 ng/ml established for EPM in bovine’s milk. Therefore, these dairy products could be considered safe for consumers after the EPM antiparasitic pour-on treatment (500 μg/kg) in lactating dairy sheep.

EPM applied to goats at a cattle standard dose rate of 500 μg/kg presented a rather poor efficacy against the dose-limiting species Trichostrongylus colubriformis (6). Limited efficacy against this specific intestinal nematode in goats may be related to lower systemic availability of EPM in this animal species (9). However, EPM topically applied to sheep at 500 μg/kg was an effective and safe anthelmintic against most sheep gastrointestinal nematodes but less effective against Trichostrongylus spp. due to either a sub-optimal dosage or possible resistance of Trichostrongylus spp. (7). Additionally, Hoste et al. (12) demonstrated that EPM pour-on (500 μg/kg) was effective (between 99.5 and 100%) against three main species of gastrointestinal nematodes in sheep and the nasal botfly, Oestrus ovis. Although further studies are necessary to establish optimal EPM dosages in sheep, its topical administration at 500 μg/kg seems to cover a broad spectrum of antiparasitic activity.

Previous pharmacokinetics studies showed that EPM, due to its plasma-milk partitioning profile (2), is an endectocide compound approved for use in dairy cattle with a zero milk-withdrawal period, but it has not been registered for use in lactating dairy sheep. However, there is considerable evidence that EPM, like other antiparasitic drugs, is used as an extra-label medication in the ovine species. Although different management strategies are used to prevent or minimize production losses, the use of antiparasitic drugs is still the main control measure available against parasitism...
in dairy animals during lactation, which has been correlated with enhanced milk production (11, 15, 17, 18). However, only pour-on formulations of EPM and moxidectin (MXD) are currently approved for use in dairy cattle without a required withdrawal time for milk.

The plasma and milk kinetic behaviors of EPM have been investigated in lactating cattle (2), goats (9), and non-lactating goats (1). Some differences should be considered in the elimination kinetics for this endectocide compound in dairy sheep from the available information on EPM disposition in other animal species so it seems necessary to evaluate its pattern of milk excretion in dairy sheep.

The presence of drug residues in milk supplies may have public health implications and is perceived by consumers as undesirable. Moreover, some active substances may have no affect on milk processing (cheese making) although their residual concentrations found in cheese are markedly higher than in milk. Recent work in our laboratory (13) demonstrated that the milk residual concentrations of IVM and MXD do not affect milk processing (cheese making) but a high residual proportion of both molecules are found in the curd and cheese after 40 days of maturation.

The chemical differences between EPM and other closely related endectocide compounds and the lack of information on its pattern of milk excretion in dairy sheep motivated the current work. To assess EPM’s potential for use in lactating dairy sheep, the goals of the trials reported here included i) evaluation of the relationship between plasma disposition and milk excretion at two different stages of lactation following topical administration of EPM to dairy sheep, and ii) assessment of the EPM residual concentrations in milk, whey, curd, and cheese at different times during the ripening period.

MATERIALS AND METHODS

Experimental animals, treatment, and sampling. Twelve female Pampinta (three-quarters East Friesian and one-quarter Corriedale) dairy sheep between 2 and 4 years old with a mean body weight of 86 kg were used. The experimental animals were clinically healthy, free of parasites, and kept under field conditions, grazing on pasture 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. Ewes were allocated into two groups of six animals (n = 6) according to milk yield (stage of lactation). Dairy sheep were milked once a day with a milking machine and milk production was measured prior to and throughout the trial. Average milk production during the trial was 978 ± 42.0 ml/day for group A (early-mid stage of lactation) and 622 ± 68.5 ml/day for group B (mid-late stage of lactation).

Each animal of each group was treated with an EPM formulation immediately after milking was completed. The formulation (0.5% solution, lot H969240, Ivomec, Eprinex, Merial S.A., Argentina) was applied topically (pour-on) with a syringe on the skin along the dorsal midline of the back from the withers to the tail. This formulation is commercially available for lactating dairy cattle and it was given at a dosage of 500 µg/kg. Neither pain nor irritation was observed at the site of application at any time after treatment. Blood samples were taken from the jugular vein in heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, N.J.) before treatment, and at 8 and 12 h and 1, 2, 3, 4, 5, 7, 9, 11, 15, 20, 25, 30, and 35 days after treatment. Milk samples were collected before treatment, and at 1, 4, 8, 12 h and 1, 2, 3, 4, 5, 7, 9, 11, 15, 20, 25, 30, and 35 days after treatment. At each sampling point, an aliquot of 50 ml of milk was collected by hand milking, discarding the first 30 to 50 ml and before mechanical milking of each sheep. Blood samples were centrifuged at 2,000 g for 20 min and the recovered plasma was transferred to vials. Milk and plasma samples were stored at −20°C until analyzed.

On days 1, 3, 5, 10, 15, and 25 posttreatment, all of the milk from each animal of experimental group A (early-mid stage of lactation) was pooled and processed into a semihard cheese using the protocol of the INTA Anguil Dairy Sheep Experimental Unit (Anguil, Argentina) (22). The raw milk obtained from each treated and untreated group was Pasteurized at 65°C for 30 min, cooled to 32 to 35°C, and supplemented with CaCl₂ and lactic starter (lyophilized) before cheese making. After adding adult bovine rennet, the curd was cut, stirred slowly while heating to 38°C, drained, and then pressed into molds, salted in brine, and stored at 10 to 12°C and 80% relative humidity for 40 days. During cheese making, curd and whey samples were collected in vials and stored at −20°C until analyzed. Cheese samples were taken after 1, 20, and 40 days of ripening. Cheeses were round with a diameter of 9 cm and a height of 4 to 6 cm at the center, and weighed between 300 and 400 g. Samples were taken from the cheese center, halfway from the center to the ring, and next to the rind portion. The cheese slices (30 g) were minced, mixed, pooled in appropriate vials, and stored at −20°C until analyzed.

Analytical procedures. The extraction procedures and chromatographic conditions to quantify EPM in fortified and experimental samples (plasma, milk, whey, curd, and cheese) were performed as described below.

Drug extraction and derivatization. EPM and abamectin (ABM) reference standards were used to validate the high-performance liquid chromatography (HPLC) method. Standard solutions of EPM were prepared by successive dilutions in acetonitrile from the parent stock solution (1 mg/ml) and stored at 4°C. The fortified and experimental samples (plasma, milk, whey, curd, and cheese) were added to 100 µl of ABM as internal standards (100 ng/ml).

Liquid (plasma, milk, and whey) and solid (curd and cheese) samples were extracted using the analytical method described for ivermectin (13). The precipitates obtained from the cheese samples were re-extracted once with acetonitrile. Water (a volume equal to that of acetonitrile) was mixed with the supernatant obtained from samples. All supernatants were then subjected to a conditioned Strata C18-T cartridge (Phenomenex, Torrance, Calif.) and the solid phase extraction was started. The eluate obtained was evaporated to dryness under a nitrogen stream at 60°C in a water bath, and the dry residue was derivatized as previously described (8).

Chromatographic conditions. Concentrations of EPM were determined using a Shimadzu LC-10ATVP HPLC system (Shimadzu Corporation, Kyoto, Japan), which included a fluorescence detector set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. The mobile phase of deionized water, methanol, triethylamine, phosphoric acid, and acetonitrile (6:25:0.2:0.2:6.86, vol/vol/vol/vol/vol) was pumped at a flow rate of 1.0 ml/min through a BDS C18 reverse-phase column (5 µm, 250 by 4.60 mm; Thermo Quest, Hypersil Division, Needham, Mass.) kept in an oven at 30°C. EPM was identified by comparison with the retention time of a pure reference standard. The peak areas
were calculated using the integrator software (Class LC 10 Software, version 1.2, Shimadzu Corporation) of the HPLC system.

**Method validation.** A complete validation of the analytical procedures for extraction and quantification of EPM in each matrix was performed before analyzing the experimental samples. The method validation was as previously described (13). The coefficient of variation (CV) for recovery and interday precision of the method was calculated (4). The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with acceptable precision (CV < 20%) (21).

**Drug quantification and pharmacokinetic and statistical analyses of the data.** Drug concentrations in experimental samples (liquid and solid) were determined by HPLC by calculating the ratio between the peak areas corresponding to EPM and ABM using the Class LC 10 Software, version 1.2, 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, Calif.) was used for linear regression analyses and linearity tests. The milk and plasma concentration versus time curves obtained after treatment of each individual animal were analyzed with the PK Solution 2.0 pharmacokinetic program (Ashland, Ohio). Pharmacokinetic variables were determined using a noncompartmental method as previously described (13).

The Mann-Whitney test was used to estimate differences between kinetic parameters obtained in milk and plasma and the volume of milk produced by each experimental group; probabilities of $\alpha < 0.05$ were considered significant. The Student’s $t$ test was used to estimate differences between experimental groups in the fat content and to compare the residual concentrations of EPM between milk and curd during cheese making.

Tukey’s range test was used to indicate the order of significance when a significant $F$ value was obtained among cheeses at different maturation times. A value of $\alpha < 0.05$ was considered statistically significant.

**RESULTS AND DISCUSSION**

A complete validation of the analytical method used to measure EPM and ABM (as internal standard) in plasma, milk, and dairy products was performed. The regression lines showed a high correlation coefficient for each concentration range ($r = 0.997$ to 0.999) and the departure from linearity was not statistically significant ($P < 0.05$). High recoveries were obtained using this simple method based on liquid- and solid-phase extraction. The mean absolute extraction recoveries from different matrices (plasma, milk, whey, curd, and cheese) were between 86 and 92%. Interday precision of the analytical procedures, obtained after HPLC analysis of EPM-spiked blank samples (2 and 20 ng/ml or ng/g) on different working days, showed a CV between 2.1 and 7.2% for EPM in the different matrices. The LOQ was 0.1 ng/ml or ng/g for all matrices except for whey, where the LOQ was 0.25 ng/g. Results obtained in this validation procedure ensured the reliability of the method for detecting EPM residues in milk and dairy products.

The plasma and milk concentration profiles measured after pour-on administration of EPM in both experimental groups are compared in Figures 1 and 2. In both experimental groups, EPM was detected in plasma between 8 h and 15 days posttreatment and in milk between 4 h and 15 days after its pour-on administration to lactating dairy sheep. The highest concentrations of EPM were measured in plasma (for both lactation periods).

The kinetic variables summarizing the disposition of EPM in plasma and milk are presented in tables as inserts in Figures 1 and 2, respectively. The plasma concentrations increased progressively to a maximum concentration ($C_{\text{max}}$) of 2.3 ng/ml at day 3 posttreatment. Systemic exposure measured as the plasma area under the drug concentration-time curve from time zero to $t$ ($\text{AUC}(0-t)$) values for EPM was 16 ng-day/ml, which was between the values described for dairy cattle (239 ng-day/ml) (2) and lactating dairy goats (8.24 ng-day/ml) (9) after the pour-on treatment at the same dose rate. These differences among species could be attributed to differences in metabolism, skin morphology, physiology, and the drug’s capacity to pass through the skin layers.

EPM concentrations in milk were lower than those in plasma. The milk residues increased progressively to reach a $C_{\text{max}}$ of 1.5 ± 0.32 ng/ml (group A) and 1.8 ± 0.41 ng/ml (group B) at day 3 posttreatment. The low affinity of EPM for sheep milk observed in both experimental groups was depicted by the AUC milk/plasma ratio values (Fig. 2). These ratios were below 1.0 compared with the value obtained for other endectocide compounds (13).

After pour-on administration, a smaller fraction of EPM (between 0.03 and 0.06% of the total dose) was excreted in milk compared to other endectocide compounds such as IVM and MXD in dairy ewes (13). Although significant differences in milk yield ($P = 0.0001$) and fat content ($P = 0.0248$) were observed between animals in the early-mid (978 ml/day and 6.5% fat content) and mid-late (622 ml/day and 8.0% fat content) lactation stages, no statistical differences were observed in the kinetic parameters evaluated.

The results reported here indicate that milk excretion was not an important route of elimination for EPM unlike other endectocide drugs such as IVM and, particularly, MXD (5, 13). The low distribution of EPM from the bloodstream to the milk and its low residual concentrations in milk were clearly reflected in the residue values measured in the dairy products under evaluation.

EPM milk residual concentrations were detected at 1, 3, 5, 10, and 15 days of sampling in the milk collected from experimental group (group A) derived for cheese making. During milk processing, the highest proportion of EPM was found in the curd, which agrees with the higher fat (30%) and total solid (50%) content for this cheese. Statistical differences were observed between EPM residues in milk and curd (Fig. 3). The EPM residues in whey cannot be measured because these residue values were below the LOQ of the analytical method used (0.25 ng/g).

Although the EPM concentration values obtained in pooled milk were lower than those obtained for other endectocide compounds such as IVM and MXD in lactating dairy sheep (13), the average ratio between EPM concentrations measured in curd and milk (3.4 ± 0.24) at different days of elaboration were similar to the ratios obtained for both IVM and MXD (13). During cheese making with milk obtained from treated ewes, the stability of EPM was...
checked after heating at 65°C during 30 min. No metabolic degradation products were observed in the chromatograms. No statistical differences were observed between concentrations of EPM in milk without thermal treatment and samples heated at 65°C for 30 min. Therefore, this antiparasitic molecule was stable at this temperature in the milk during processing.

Although residual concentrations of EPM were found in milk, the acidity value of the raw milk was similar to that of milk obtained from untreated ewes. Unfortunately, an exhaustive follow-up of the parameters during cheese making (renneting time, rate of firmness and consistency of the curd) was not done. However, no major differences in the cheese-making procedure were observed after using milk from either EPM-treated or untreated dairy sheep. This is in accordance with results previously reported, where high concentrations of other endectocide compounds such as IVM and MXD present in bovine milk did not affect the acid fermentation process in vitro (14).

At different sampling days (days 1, 20, and 40) during ripening of the semihard cheese, the proportion of EPM gradually increased (from 3.7- to 5.1-fold) compared with their residual concentrations in the milk used for cheese making. The highest residual concentration for EPM was obtained after 40 days of cheese ripening, which was statistically different \((P < 0.05)\) to that measured at day 1 of maturation, except for cheeses prepared from milk obtained 1 day after treatment (Fig. 4). A decrease in cheese yield product of 20 and 26% was observed during ripening. This water loss and the corresponding increase in total solid (between 51 and 66%) and fat (between 28 and 40%) content in the ripened cheese may have accounted for the high concentrations of EPM found in cheeses after 40 days of maturation. A linear correlation between the percentages of water loss, total solid and fat contents, and EPM concentrations during maturation was observed \((r > 0.90)\). This is in accordance with results previously reported for cheeses prepared with milk obtained from treated goats, where the highest EPM concentrations were detected in ripened cheeses (40 days) (3).

This same pattern was observed in our previous work where IVM was administrated subcutaneously to dairy sheep at 200 \(\mu\)g/kg (13). IVM milk excretion was higher than that observed for EPM given at 500 \(\mu\)g/kg. The highest IVM residual concentrations were recovered in cheese at 40 days of maturation (Fig. 5). Comparison of the milk
FIGURE 2. Comparative mean (n = 6) concentration profiles of eprinomectin (EPM) in milk after its pour-on administration (500 μg/kg) to lactating dairy sheep at different stages of lactation. The insert (table) shows milk kinetic parameters obtained for EPM. The level of milk production at the early-mid lactation (978 ml/day) was significantly higher (P = 0.0001) than the mid-late lactation stage (622 ml/day).

FIGURE 3. Distribution of eprinomectin (EPM) residues in milk and curd during cheese making process. Values are expressed as mean drug concentrations (nanograms per gram) ± SEM. Mean concentration values obtained in curd are significantly different at α = 0.001 from those obtained in milk collected at different times posttreatment.

FIGURE 4. Level of eprinomectin (EPM) residual concentrations in cheese (prepared with milk collected at different days post-treatment) in different stages of the ripening period. Mean EPM residual concentration values (nanograms per gram) ± SEM obtained in cheese after 40 days of maturation are statistically different at α = 0.05 (*) or α = 0.001 (****) from those obtained in cheese after 1 day of maturation.
excretion profiles for these avermectin-type compounds is a very good example of how a minor chemical modification (EPM compare to IVM) may substantially modify the plasma-milk partitioning coefficients. Conclusively, the lower milk excretion observed for EPM compared with IVM was clearly reflected in lower residual concentrations recovered in dairy products.

Although the highest EPM concentrations were found in cheese after 40 days of maturation (5-fold higher than milk), the maximum levels of EPM (marker residue) detected in sheep milk and cheese were below the maximum residual level (MRL) (20 μg/kg) allowed for bovine milk (10). From the results of the present work, it can be concluded that the treatment of lactating dairy sheep with topical EPM (500 μg/kg) at different lactation stages will produce drug residues in both milk and cheese below the accepted MRL for human consumption. This information regarding the pattern of EPM milk excretion in sheep will be statistically different at α = 0.001 from those obtained for EPM.

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