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Licking induced changes to the pattern of moxidectin milk elimination after topical treatment in dairy cows

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Pour-on administration of the macrocyclic lactones anti-parasitic compounds in beef and dairy cattle is now worldwide accepted. However, the information available on their milk excretion pattern, after topical administration is rather limited. Additionally, the cattle licking behaviour has been proven to affect the kinetics of these anti-parasitic compounds. The purpose of this study was to investigate the influence of the natural licking behaviour on the plasma and milk disposition of moxidectin (MXD), topically administered (500 μg/kg) in lactating dairy cows. Ten lactating Holstein dairy cows (705 kg body weight) were allocated into two experimental groups (n = 5). The licking was prevented during 5 days postadministration in animals in group I, and the remaining cows (group II) were allowed to lick freely. MXD concentrations profiles were measured in plasma and milk over 15 days posttreatment. The licking restriction period caused marked changes in MXD disposition kinetics both in plasma and milk. Both plasma and milk MXD concentrations (partial *AUC* 0–5 days) were significantly lower ($P < 0.05$) in licking-restricted cows. After the 5-day of restriction period, the animals were allowed to lick freely, which permitted the oral ingestion of MXD, situation clearly reflected both in plasma profile and milk excretion pattern. Despite the enhanced MXD milk concentrations measured in free-licking cows, drug concentrations did not reach the maximum MXD residues limit.

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INTRODUCTION

Moxidectin (MXD) is a macrocyclic lactone from the milbemycin family of endectocide compounds, which is routinely administered to achieve systemic anti-parasitic effects in livestock animals. Currently, endectocide compounds are extensively used for broad-spectrum parasite control, and their topical administration in cattle is widespread. MXD is marketed as injectable formulations for use in beef cattle and as topical (pour-on) formulations for use in beef and dairy cattle. As for other macrocyclic lactones such as ivermectin (IVM), doramectin (DRM) and eprinomectin (EPM), the topical 'pour-on' formulation of MXD has displaced the conventional injectable formulation, because it offers considerable practical and pharmacological advantages compared with other administration routes, e.g. avoidance of liver first-pass effect and no drug residue at the injection site (Baggot & Brown, 1998).

The advantages of persistent anthelmintic efficacy of the injectable formulations of IVM, DRM and MXD in strategical programs for controlling gastrointestinal nematodes and lungworm in cattle are well documented (Williams *et al.*, 1992, 1997; Jones *et al.*, 1993). However, some studies have shown that cattle treated with MXD pour-on achieved an advantage over the other endectocide compounds in attaining a highly and persistent level of nematode control and weight gains in beef calves (Williams *et al.*, 1999).

Although different management strategies are used to prevent or minimize production losses, the use of anti-parasitic drugs is still the main control measure available against parasitism in lactating dairy animals. The use of strategical anthelmintic treatments during the lactation period has been correlated with a significant enhancement on the volume of milk produced (Ploeger *et al.*, 1989; Gross *et al.*, 1999; Nødtvedt *et al.*, 2002). Topical formulations of MXD are currently approved for use in

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dairy cattle without requiring milk withdrawal time in some countries. The maximum MXD (40 μg/kg) residue limit (MRL) in dairy cattle was determined by the European Union and included in Annex I – Council Regulation No 2377/90 (European Agency for the Evaluation of Medicinal Products, 2001).

The plasma and milk pharmacokinetics of endectocide compounds have been extensively investigated in different animal species. Their disposition kinetics in livestock animals are affected by different factors, such animal species (Alvinerie & Galtier, 1997), animal breed (Sallovitz *et al.*, 2002), dietary management and nutritional condition (Alí & Hennessy, 1996; Lifschitz *et al.*, 1997), type of drug formulation (Lo *et al.*, 1985), route of administration (Imperiale *et al.*, 2004) and natural behaviour (self and allo-licking) (Laffont *et al.*, 2001; Bousquet-Mélou *et al.*, 2004; Sallovitz *et al.*, 2005). Licking is an important part of the natural grooming behaviour of animals in many animal species. In cattle, it serves as an important physiological function in hygiene (skin and hair) and plays a major role in the establishment and maintenance of the herd's social relationship (Sato *et al.*, 1991).

The plasma kinetic behaviour of topical IVM, DRM (Gayrard *et al.*, 1999; Laffont *et al.*, 2001, 2003; Bousquet-Mélou *et al.*, 2004; Sallovitz *et al.*, 2005), EPM (Alvinerie *et al.*, 1999) and MXD (Sallovitz *et al.*, 2002; Bousquet-Mélou *et al.*, 2004) in cattle has been characterized. Altogether, the previously reported work suggests that following topical administration, a fraction of the applied dose of an endectocide compound reaches the systemic circulation by oral rather than by dermal absorption, as a consequence of animal licking. The effect of licking on the pattern of plasma (Laffont *et al.*, 2001, 2003; Bousquet-Mélou *et al.*, 2004) and tissue distribution (Sallovitz *et al.*, 2005) of endectocide compounds after their topical (pour-on) administration to cattle was previously studied. However, the impact of natural licking on the pattern of milk excretion has not been assessed.

Thus, the objective of this study was to estimate the contribution of the oral route (due to licking) to the plasma availability and pattern of milk excretion for MXD after topical treatment, assessing the influence of lingual access to topical treatment of lactating dairy cattle at recommended dosages. The experimental design permitted the evaluation of MXD plasma and milk disposition both in free-licking and licking-restricted lactating dairy cows after topical treatment.

MATERIALS AND METHODS

Experimental animals, treatment and sample collections

Ten lactating Holstein dairy cows with an average body weight of 705 kg in the mid-late stage of lactation (average milk production: 19 L/day/animal) were used. The animals were divided into two experimental groups (n = 5). Natural licking was prevented during 5 days postadministration (group I). Animals in the licking-restricted group were placed in a separate paddock, and each animal was restrained with a stanchion and tied from both sides of the head to restrain from both self- and allo-licking. This system allowed animals to lie down, stand up, eat and drink freely. Animals in the free-licking group (group II) were kept together in a small paddock. Both free-licking and licking-restricted animals were treated with a commercial topical (pour-on) formulation of MXD (Cydectin Pour On, lot 001/05; Fort Dodge, São Paulo, Brazil). MXD (0.5%) was administered on the backline from the withers to the tail-head of each animal at a dosages of 500 μg/kg body weight.

The experimental lactating animals were clinically healthy. After the licking restriction period (5 days posttreatment), animals from both experimental groups were kept under field conditions, grazing on the same pasture and had free access to drinking water during the whole experimental period. The health of the animals was closely monitored prior to and throughout the trial. Dairy cows were milked twice a day with a milking machine, and the milk production was measured prior to and throughout the trial. The average milk production during the trial was 19 L/day/animal.

Blood samples were taken from the jugular vein in heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) prior to treatment, and at 12 h and at 1, 2, 3, 4, 5, 7, 9, 11 and 15 days posttreatment. Milk samples were collected prior to treatment and at 12 h and at 1, 2, 3, 4, 5, 7, 9, 11 and 15 days posttreatment. At each time point, after discarding 20–30 mL and before the complete mechanical milking of each animal, an aliquot of 50 mL of milk sample was collected. 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.

Analytical procedures

The extraction procedures and chromatographical conditions to quantify MXD in fortified and experimental samples (plasma and milk) were performed as describe below.

Drug extraction and derivatization

Pure reference standards of abamectin (ABM) (97.4%, purity) and MXD (91.8%, purity) were used to validate the high-performance liquid chromatography (HPLC) method. Standard solutions of ABM and MXD were prepared by successive dilutions in methanol from the parent stock solution (1 mg/mL) and stored at 4 °C. The fortified and experimental samples (plasma and milk) were added with 100 μL of ABM as internal standard (100 ng/mL).

Plasma and milk samples were extracted using the analytical method previously described for MXD (Imperiale *et al.*, 2004). The samples were then applied to a conditioned Strata C18-T cartridge (Phenomenex, Torrance, CA, USA), and the solid phase extraction was performed manually (milk samples) using a Lichrolut vacuum manifold and an automated solid phase extraction apparatus (plasma samples) (Aspec XL, Gilson, Villiers Le Bel, France), according to the methods described by Alvinerie *et al.* (1995) and modified by Lifschitz *et al.* (1999). The eluent

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was evaporated to dryness under a gentle stream of nitrogen at 60 °C in a water bath and the dry residue of the elution was dissolved with 100 μL of *N*-methylimidazole (Sigma, St Louis, MO, USA) solution in acetonitrile (1:1, v/v) and 150 μL of trifluoracetic anhydride (Sigma) solution in acetonitrile (1:2, v/v) (De Montigny *et al.*, 1990). After the reaction took place, an aliquot (100 μL) of this solution was injected directly into the chromatographical system.

Chromatographical conditions

Concentrations of MXD were determined using a Shimadzu LC-10AT$_{VP}$ 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 acetic acid (0.2% in water, v/v), methanol and acetonitrile (0.5:60:39.5 v/v/v) was pumped at a flow rate of 1.5 mL/min through a Kromasil 100–5C18 (5 μm, 250 × 4.60 mm) reverse phase column (Eka Chemicals, Separation Products, NY, USA) kept in an oven at 30 °C. ABM and MXD were identified by comparison with the retention times of pure reference standards. The areas under the peak were calculated using the integrator software (CLASS LC 10 Software, version 1.2; Shimadzu Corporation) of the HPLC system.

Validation procedures

A complete validation of analytical procedures for extraction and quantification of MXD in plasma and milk was performed before starting the analysis of experimental samples from the specific trial. Calibration lines in the ranges 0.1–20 ng/mL in plasma and 0.1–50 ng/mL in milk were plotted using the peak area ratios between MXD and the internal standard vs. theoretical concentration. The data were analysed for linearity using a linear least-squares regression analysis, and posttest, Run Test and ANOVA, to determine if the data differed from a straight line.

The absolute recovery of MXD was measured by comparison of peak areas from spiked samples with the peak areas resulting from direct injections of standards in methanol. The recoveries of MXD from plasma and milk were determined at different concentrations between 0.1 and 50 ng/mL, using three replicates for each drug concentration. The inter-day precision of the extraction and chromatographical procedures was evaluated by processing four replicate aliquots of samples containing known amounts of MXD (0.5 and 10 ng/mL for plasma; 2 and 20 ng/mL for milk) on different working days. 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. The accuracy was estimated for the matrices under study at MXD concentrations of 0.1, 2 and 20 ng/mL 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, 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 MXD and ABM using the CR10 software, (Shimadzu Corp., Kyoto, Japan), 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, linearity tests and data interpolation.

The milk and plasma concentrations vs. time curves determined after treatment in each individual animal were analysed with the PK SOLUTION 2.0 computer software (Summit Research Services, Ashland, OH, USA). Pharmacokinetic parameters were determined using a noncompartmental method. The peak concentration (C_{max}) and time to peak concentration (T_{max}) were read directly from the plotted concentration-time curves of each animal. The depletion (elimination) half-lives ($T_{1/2\ el}$) were calculated as $\ln 2/\lambda_z$, where λ_z is the terminal rate constant. The areas under the concentration-time curves (*AUC*) were calculated by the trapezoidal rule (Gibaldi & Perrier, 1982) without extrapolation to infinity. The percentage of total dose excreted in milk for each individual animal was estimated using the values of drug concentration at each sampling time interval and the mean daily volume of milk produced during the experiment. Concentration values are presented as mean ± SD. The *t*-test (with Welch correction if appropriate) was used to estimate differences between kinetic parameters determined for plasma and milk in each experimental group. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The linearity of the analytical method to measure MXD was confirmed by the estimated values of the correlation coefficient, where r values ranged between 0.991 and 0.999 for the analysed milk and plasma samples. The inter-assay precision of the analytical method showed a CV between 6.0% and 8.7%. The extraction recovery for concentrations between 0.1 and 50 ng/mL for plasma and milk ranged between 70% and 73%. The LOQ of the method was 0.1 ng/mL for both plasma and milk.

The plasma and milk concentration profiles measured after pour-on administration of MXD in both experimental groups are compared in Fig. 1. MXD was recovered in plasma and milk between 12 h and 15 days posttreatment after its pour-on administration to lactating dairy cows. The impact of the licking restriction (during 5 days after treatment) is clearly evident during this period. Plasma and milk concentrations in the licking-restricted animals were lower compared with the free-licking group during the 5-day licking restriction period posttreatment. Once restricted animals had free access to lick (after day 5), a high proportion of MXD was orally ingested by licking, which is clearly reflected on the pattern of plasma and milk concentrations.

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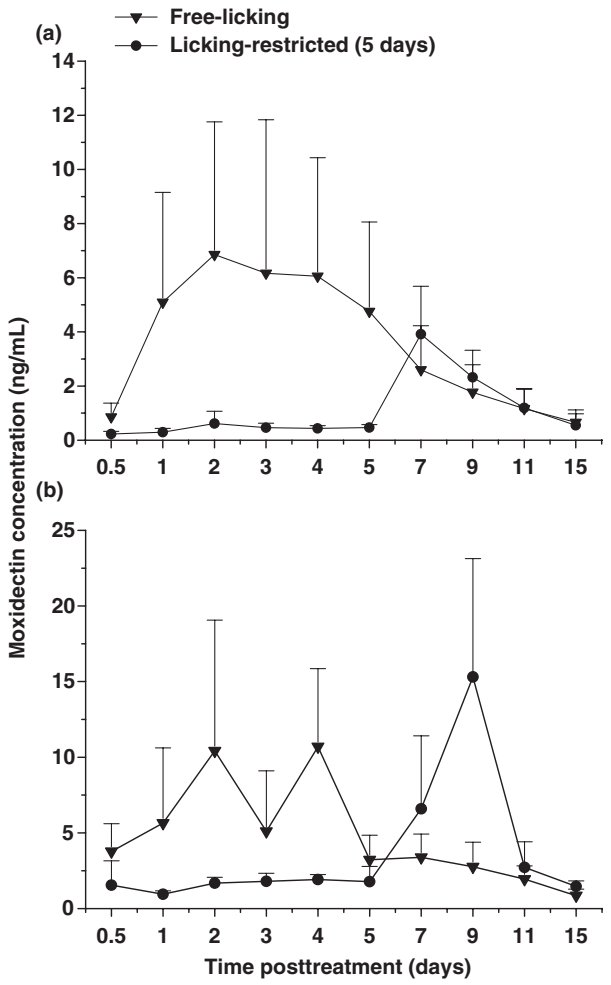

Fig. 1. Comparative mean (±SD) (n = 5) moxidectin (MXD) concentration profiles in plasma (a) and milk (b) after its pour-on administration (500 μg/kg) in licking-restricted (5 days licking-restriction period) and free-licking dairy cows.

The time to peak concentration (T_{max}) in plasma (7.0 ± 0.01 vs. 2.8 ± 1.30 days, $P < 0.01$) and milk (9.0 ± 0.01 vs. 3.2 ± 1.10 days, $P < 0.001$) were significantly different between the experimental groups (Table 1). The plasma and milk C_{max} values in licking-restricted cows were reached once the restriction period was finished, at 7 (plasma) and 9 (milk) days posttreatment. Some kinetics parameters in plasma and milk [C_{max}, *AUC* (0–15 days)] showed no statistical differences between the licking-restricted and free-licking animals. However, the MXD partial *AUC* (0–5 days), absorption and elimination half-lives showed statistical differences between the licking-restricted and free-licking cows. The percentage of MXD recovered in milk was similar in cows from both experimental groups.

The licking restriction period markedly influenced both plasma and milk concentration of MXD. The differences observed in the partial *AUC* up to 5 days posttreatment are shown in Fig. 2, which were statistically significant ($P < 0.05$) for both plasma and milk. The plasma (12.3-fold) and milk (4.4-fold)

Table 1. Mean (±SD) kinetic variables describing the disposition of moxidectin (MXD) from plasma and milk in licking-restricted (5 days licking restriction period) and free-licking lactating dairy cows following pour-on treatment (500 μg/kg) (n = 5)

Kinetic variables	Licking-restricted	Free-licking
Plasma		
T_{max} (days)	7.00 ± 0.01	2.80 ± 1.30**
C_{max} (ng/mL)	3.93 ± 1.80	8.66 ± 5.70
$T_{½\ ab}$ (days)	2.53 ± 0.70	1.14 ± 0.40**
$T_{½\ el}$ (days)	2.87 ± 0.70	3.86 ± 1.40
$AUC_{0–5}$ (ng·day/mL)	2.09 ± 0.80	25.7 ± 17.6*
$AUC_{0–15}$ (ng·day/mL)	19.7 ± 8.30	44.0 ± 28.5
Milk		
T_{max} (days)	9.00 ± 0.01	3.20 ± 1.10***
C_{max} (ng/mL)	15.3 ± 7.80	13.6 ± 7.60
$T_{½\ ab}$ (days)	1.81± 0.40	2.12 ± 1.00
$T_{½\ el}$ (days)	2.20 ± 0.80	4.01 ± 1.20*
$AUC_{0–5}$ (ng·day/mL)	7.79 ± 1.70	34.0 ± 19.9*
$AUC_{0–15}$ (ng·day/mL)	64.5 ± 30.0	57.1 ± 29.0
Dose fraction recovered in milk (%)	0.25 ± 0.09	0.21 ± 0.09

Mean kinetic variables determined for free-licking group are statistically different at *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ from those determined in licking-restricted group after MXD administration.
T_{max}, time to peak concentration; C_{max}, peak milk or plasma concentration; $T_{½\ al}$, absorption half-life; $T_{½\ el}$, elimination half-life; *AUC*, area under the concentration vs. time.

availabilities measured up to 5 days postadministration were higher in free-licking animals compared with licking-restricted animals. After 5 days, the licking-restricted cows were allowed to lick, and the plasma and milk concentration profiles showed an upward trend up to 15 days posttreatment, determining that MXD total availabilities in plasma and milk were similar in both experimental groups (Table 1).

The effect of licking on the plasma disposition of IVM, DRM and MXD in cattle has been reported (Gayrard *et al.*, 1999; Laffont *et al.*, 2001, 2003; Sallovitz *et al.*, 2002; Bousquet-Mélou *et al.*, 2004). The impact of the licking effect on the plasma and milk profiles at the same time was assessed in this work. To achieve this objective, a licking restriction period was imposed to one experimental group. The restriction length was established up to 5 days to maximize, as much as possible, this effect. Sallovitz *et al.* (2003) demonstrated the relevance of licking on drug kinetic behaviour; these authors reported that oral ingestion of topically administrated MXD accounted for higher concentrations in gastrointestinal fluids compared with their respective mucosa tissues. Also, a high individual variability in MXD plasma and tissue concentrations was reported (Sallovitz *et al.*, 2003).

The findings of this work in dairy cattle are in agreement with those earlier reports in beef cattle. Licking restriction markedly changed both plasma and milk concentration profiles. Such changes were observed both during the licking restriction period and after releasing the cows from restriction (Fig. 1). Licking-restricted animals had low MXD plasma and milk concentrations, while restriction was enforced, i.e. 5 days postadministration. Hence, lower partial *AUC* up to 5 days were

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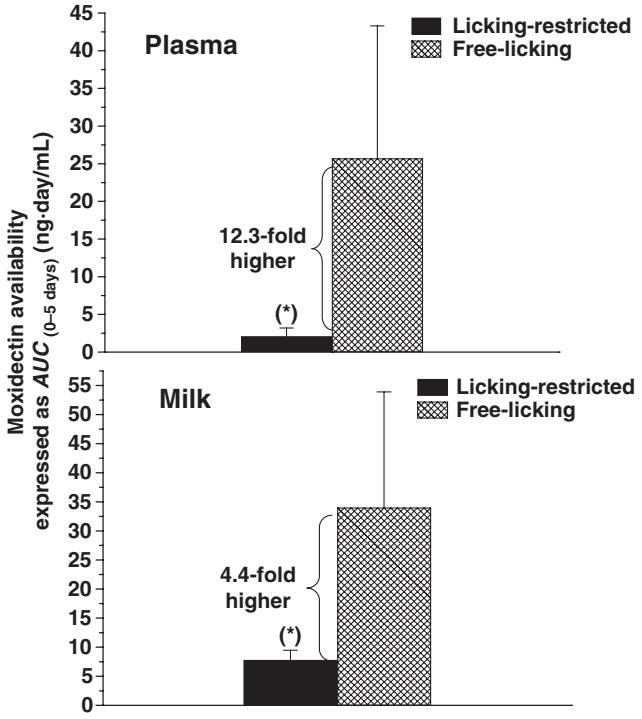

Fig. 2. Comparison of moxidectin (MXD) availabilities in plasma and milk, expressed as partial areas under the plasma and milk concentration vs. time ($AUC_{0-5\ days}$) (±SD) after pour-on treatment (500 μg/kg) in licking-restricted (5 days licking restriction period) and free-licking dairy cows ($n = 5$). Mean partial $AUC_{0-5\ days}$ is statistically different at $^{*}P < 0.05$.

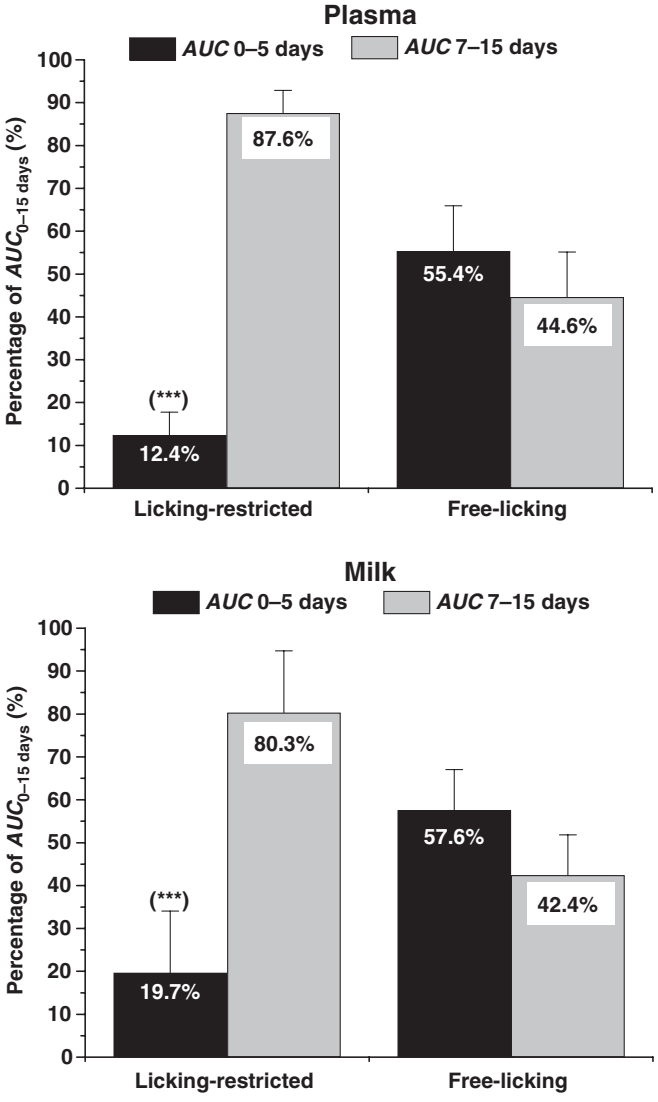

Fig. 3. Effect of licking on the proportion (expressed in percentage ± SD) of the partial areas under the moxidectin (MXD) concentration vs. time curves in plasma and milk after its topical administration to lactating dairy cows. The breakpoint for analysis was the end of the licking restriction period. Mean partial area under the concentration vs. time $(AUC)_{0-5\ days}$ and $AUC_{7-15\ days}$ in licking-restricted group are statistically different at $^{***}P < 0.001$.

observed compared with those of free-licking animals (Table 1). Accordingly, release from restriction determined an uprise of plasma concentrations, as well as a different shape of the curve after day 7 postadministration, due to the subsequent licking taking place (Fig. 1).

The 5-day long licking prevention period after pour-on treatment significantly reduced MXD concentrations in plasma and milk. MXD systemic and milk availabilities, expressed as a partial *AUC* to up 5 days postadministration ($AUC_{0-5\ days}$), were significantly higher ($P < 0.05$) in free-licking compared with licking-restricted cows. The free-licking animals ingested topically administered MXD by self-licking, which may be due to discomfort produced by the formulation and/or allo-licking behaviour due to social activity between individuals in a given herd (Sato *et al.*, 1993). This licking ingestion accounted for the earlier peak concentration observed in plasma (T_{max}, $P < 0.01$) compared with the licking-restricted animals, in which MXD plasma concentrations during 5 days posttreatment were determined solely by the slow and limited transdermal absorption, concentrations being increased when animals were allowed to (self and allo) lick, orally ingesting MXD. Hence, the end of the licking restriction determined a later T_{max}. The oral ingestion of MXD by licking the drug on the skin was able to impose significant differences in both plasma and milk availabilities during the first 5 days posttreatment. However, over the length of the study (up to 15 days posttreatment) MXD plasma and milk availabilities ($AUC_{0-15\ days}$) showed no statistically significant differences between restricted and free-licking cows. This was due to the increase on MXD concentrations observed in the licking-restricted dairy cows after day 5 posttreatment, as a consequence of licking. Therefore, the oral (self- and allo-licking) ingestion that took place once the restriction period was over was sufficient to compensate the MXD plasma and milk concentrations measured in the free-licking cows. Hence, the curve shows a characteristic shape that is determined by the end of the licking restriction, with later T_{max}. A further kinetics analysis of the concentration vs. area plot showed interesting findings. Comparison of partial *AUC* determined that even though the total *AUC* from 0 to

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15 days postadministration were similar, the temporal distribution of the administered dose was different in licking-restricted animals, considering the end of the time of restriction as the breakpoint for this analysis.

Partial *AUC* in the free-licking group were almost equal (evenly distributed) on both sides of the previously determined breakpoint (Fig. 3; $P > 0.05$). Conversely, in the licking-restricted group, *AUC* on each side of the breaking point were markedly different to each other ($P < 0.0001$). When comparing the partial *AUC* up to day 5 postadministration, the free-licking group's curves presented rapidly rising concentrations, while the licking-restricted group's curves were characterized by steady concentrations and low peak concentrations. In the former situation (free-licking), both plasma and milk concentrations were due to two drug inputs: percutaneous and intestinal absorption, happening almost simultaneously. Meanwhile, in the licking-restricted animals, MXD concentrations in both fluids were due to percutaneous absorption only, which accounted for a lower rate and smaller amount of drug absorbed than the combined absorption (percutaneous and intestinal) taking place in the free-licking group. These situations also determined a great individual variability in both experimental groups. While in the licking-restricted group, an important individual variability due to differences in the amount of absorbed drug was observed (expressed as *AUC* up to 5 days, 38% and 22% in plasma and milk respectively), and a greater variability was present in the free-licking group (68% and 58% respectively). MXD is being simultaneously absorbed by two absorption routes (percutaneous and intestinal) in free-licking animals, which could account for the observed large variability (as the summation of variation of each absorptive surface).

It is also interesting to note that at 5 days postadministration (restricted cows), enough MXD was available to be licked and intestinally absorbed to uprise plasma and milk concentrations. This finding may agree with slow and constant percutaneous absorption in cattle.

Although the licking activity drastically enhanced the milk residues of MXD topically administered, the residual concentrations assessed under the current experimental conditions did not overpass the permitted residual concentrations at any time. This includes the individual C_{max} values in milk (range between 5.0 and 25.0 ng/mL), which were lower than the MRL (40 ng/mL) approved for bovine milk. However, the influence of licking on the pattern of milk residues should be carefully monitored, particularly for those macrocyclic lactone compounds, whose approved MRL values in milk are much lower than the MXD MRL. For instance, the MRL for IVM (10 ng/mL) (JECFA, 2000) in bovine milk is much lower than the established for MXD, which could represent a greater risk situation if topically treated dairy cows are ingesting the drug by licking. Some preliminary data available in our laboratory indicate that IVM milk residual concentrations over the established MRL value are often found after topical treatment. This work provides information that should be considered to assure the avoidance of unwanted milk residues for different drug compounds therapeutically used by topical administration in dairy animals.

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