*Article*

# Determination of Moxidectin in Serum by Liquid Chromatography-Tandem Mass Spectrometry and Its Application in Pharmacokinetic Study in Lambs

*Rafaela C. Baptista,[a] Maria A. M. Fernandes,[b] Susana Gilaverte,[b] Sonia C. N. Queiroz,[c] Marcia R. Assalin,\*,[c] Vera L. Ferracini,[c] Alda L. G. Monteiro[b] and Felix G. R. Reyes[a]*

[a]*Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Rua Monteiro Lobato 80, 13083-862 Campinas-SP, Brazil*

[b]*Laboratório de Produção e Pesquisa em Ovinos e Caprinos (LAPOC), Fazenda Experimental da Universidade Federal do Paraná, 80035-050 Curitiba-PR, Brazil*

[c]*Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), CP 69, 13820-000 Jaguariúna-SP, Brazil*

The widespread use of moxidectin (MOX), a parasiticide used in the sheep breeding, has induced the parasite resistance in Brazilian farms. As a consequence, the farmers often increase the dose and frequency of drug utilization, and disregards safety of meat or milk. In order to establish adequate therapeutic treatment it is necessary to know the pharmacokinetics of the drug in the animal's body. Thus, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) method was developed for the determination of MOX in serum lamb. Serum samples were treated with acetonitrile to precipitate proteins. A clean up by dispersive extraction in solid phase (SPE-d), using primary/secondary amine (PSA) and C18 sorbents, followed by freezing was performed. Method validation presented precision (coefficient of variation) and accuracy (recovery%) between 1.7-6.7 and 80.0-107.3%, respectively. The limit of quantification (LOQ) of the method was 2.0 ng mL$^{-1}$ and a linear response was obtained over a range of 2.0 to 100 ng mL$^{-1}$. This method was successfully applied to the determination of MOX in serum from suffolk lamb to evaluate the pharmacokinetic profile.

**Keywords:** serum lamb, moxidectin, veterinary drug, pharmacokinetic, LC-MS/MS

## Introduction

The infections with gastrointestinal nematodes have constituted major obstacle to the expansion of the sheep industry in Brazil. The gastrointestinal parasitism is associated with physiological changes in animals, such as bowel dysfunction and nutritional stress that result in decreased body condition, lower weight gain and death of animals. Among the most important parasites that affect sheep flocks we can highlight the *Haemonchus contortus*.[1] The parasiticide agents allowed for controlling endo and ecto-parasites in sheep, the macrocyclic lactones, have been widely employed over the world because of its high efficiency and broad-spectrum activity.[2] The moxidectin (MOX) (Figure 1), semisynthetic derivative of nemadectin, is a macrocyclic lactone obtained by the fermentation of *Streptomyces cyanogriseus*.[3] In the last decade MOX has emerged in sheep flocks in Brazil due to its efficacy against a wide variety of nematodes and arthropod parasites, even at extremely low doses.[4] However, similarly as occurred with other drugs, indiscriminate use of MOX could induce parasite resistance, affecting the efficiency of the drug.[5] Actually, similarly to ivermectin and the benzimidazole, parasite resistance against MOX has been reported in Brazil.[6] As an action of parasite resistance control, alternatives have been studied in order to minimize the spread of animal diseases and the damage to the breeding and may be referred: correct management and grazing, proper sheep nutrition, selection of resistant animals, biological control, use of vaccines, herbal medicine and/or the use of techniques for assessing the degree of infection.[7]

Chromatographic methods for the determination of MOX in plasma from different species (alpacas, cattle, horses, pig, rabbit and sheep) have been reported applying liquid chromatography coupled with fluorescence detection (LC-FLD) after a derivatization
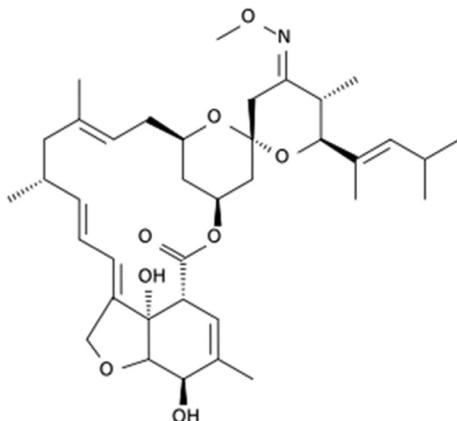
*\*e-mail: marcia.assalin@embrapa.br*

**Figure 1.** Moxidectin chemical structure.

step.[8-13] Rarely are described analytical methods by using mass spectrometry as a detector for MOX.[14] However, it is important to emphasize that analytical methods based on liquid chromatography coupled to mass spectrometry (HPLC-MS) are sufficiently sensitive and selective to quantify the compounds in very low amounts, not being necessary a derivatization step.

Regarding the sample preparation, most procedures described for MOX residue quantitation in plasma involve clean up steps using solid phase microextraction cartridges (SPME)[8,11-13] and derivatization step. According to literature, the pharmacokinetic (PK) parameters of a veterinary drug vary according to the applied dose, animal species, type of animal and the application site, among others.[15] In this regard, there is a lack of data available in the literature related to PK studies for MOX in lambs. Thus, based on the systems of sheep breeding in Brazil, the aim of this study was to evaluate the disposal of MOX in the serum of lambs. For that purpose, a simple and throughput liquid chromatography with mass spectrometry detection analytical method was developed and validated.

## Experimental

### Chemicals

Moxidectin (purity of 94.8%) was obtained from European Pharmacopoeia Reference Standard (Strabourg, France). The solvents used were high performance liquid cromatography (HPLC) grade. Acetonitrile (ACN) was obtained from J. T. Baker (USA). Methanol (MeOH) was obtained from J. T. Baker (Mexico). Triethylamine (TEA) (purity of 99%, grade PA) was obtained from Merck (Germany). Octadecyl (C18) and primary/secondary amine (PSA) was obtained from UCT, Inc. (USA). Water was purified with a Milli-Q system (Millipore, USA).

### Standards solutions

Standard stock solution was prepared in ACN at a nominal concentration of 1.0 mg mL$^{-1}$. Standard intermediate solution was prepared daily by appropriate dilution of stock solution in ACN, at a concentration of 10 µg mL$^{-1}$ and stored under refrigeration at 4 °C. The working solutions were prepared daily by dilution of intermediate solutions of MOX in the mobile phase (MP) used in the chromatographic separation.

### Animals and study design

The study protocol was approved by the Ethics Committee in the Use of Animals of the Animal Welfare Policy of the Federal University of Paraná (UFPR), Curitiba city, PR, Brazil (Protocol No. 055/2011). Serum samples were provided by the Laboratory of Production and Research in Sheep and Goats (LAPOC/UFPR), Curitiba city, PR, Brazil. Weaned Suffolk breed lambs (n = 6), aged 69-83 days and body weight ranging from 32.2 to 39.0 kg, who had never received treatment with anthelmintic, were randomly selected. The animals were housed in individual pens with floor slatted suspended, equipped with individual feeders and drinkers, and fed a diet containing ryegrass hay. They received a single MOX subcutaneous injection at a dose of 0.2 mg kg$^{-1}$ bw. All animals underwent a fasting solid of 12 h and were weighed before dose application. Blood samples (1.5 mL) were collected from each animal at 6, 12, 18, 24, 30, 36 h, and 2, 3, 5, 9, 14, 21, 28, 35, 42 days after MOX administration. The samples were kept under refrigeration (4 °C) for a maximum of two hours, the serum was separated from whole blood by centrifugation at 2500 g, for 10 min, and stored in Eppendorf tubes at −20 °C prior to analysis.

### Sample preparation

Blood serum samples (0.5 mL) were extracted with 2 mL of ACN, by vortexing (Quimis) for 30 s and centrifugation (Heraeus Multifuge 3L-R centrifuge, Thermo Scientific) at 13800 × g, for 5 min at 5 ºC. Using a Pasteur pipette the supernatant was transferred to another polypropylene tube of 50 mL containing dispersive-SPE, with 100 mg of octadecyl (C18) and 75 mg of primary and secondary amine exchange material (PSA). At the end, the Pasteur pipette was washed with 1 mL of ACN. The extracts were again shaken in vortex for 30 s, centrifuged (13800 × g, 5 min, 5 °C) and refrigerated for 1 h at 4 °C. After that, an aliquot of the supernatant (2.7 mL) was transferred to round-bottom flasks of 50 mL, the solvent was evaporated

to dryness in a rota-evaporator at 40 °C (HB4 Basic rotating evaporator, IKA) and the residue was resuspended in 0.5 mL of MP. In order to facilitate analyte resuspension, samples were sonicated in an ultrasonic bath for 30 s. The obtained solutions were filtered through a 0.45 μm nylon syringe filter directly into the vial for analysis in the high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) system.

## HPLC-ESI-MS/MS system conditions

The chromatographic separation was performed using a reverse phase analytical column C18 (50 × 2.1 mm, 2.7 μm) Poroshel 120 EC-18 (Agilent Technologies), connected to a pre-column with the same stationary phase (30 × 2.1 mm, 2.7 μm). The chromatographic separation was performed at room temperature (25 °C), on a HPLC system (Varian) consisting of a binary pump (Varian ProStar 210), and an automatic injector (Varian ProStar 410, Auto Sampler model). The HPLC was coupled to a Varian mass spectrometer triple quadrupole analyzer (QqQ) (Varian model 1200L), equipped with electrospray ionization source (ESI). The software Varian MS Workstation, version 6, was used for data acquisition.

The MP was composed of (A) ACN and (B) MeOH:$H_2O$ (50:50 v/v) and TEA added in sufficient amount to obtain a pH equal 8. The ratio of the phases A:B was 75:25, v/v. The injection volume was 5 μL and the MP was pumped isocratically at a flow rate of 0.2 mL min$^{-1}$.

Optimized ionization conditions for MOX in the ESI source were established in order to obtain the higher MOX detectability in the mass spectrometer (MS/MS). For this purpose, 1.0 μg mL$^{-1}$ MOX standard solution was directly infused in the MS/MS, using a flow rate of 20 μL min$^{-1}$. A fine adjustment of each parameter was performed, obtaining the following optimum conditions: source temperature 50 °C, capillary voltage −85 kV, pressure and temperature of the desolvation gas 29 psi and 280 °C, respectively. The voltage of the detector was configured in 2000 V. The selected reaction monitoring method was the multiple reaction monitoring (MRM), conducted in the negative electrospray ionization mode. The selected transition ions (*m/z*) were 638 > 108 (quantification); 638 > 235 (confirmation).

## Method validation

The developed analytical method was in-house validated based on the guidelines provided by the Brazilian Ministry of Agriculture, Livestock and Supply and the European Community,[16,17] which establish performance criteria for analytical methods for the determination of residues and contaminants in food of animal origin. The limit of quantification (LOQ) parameter was determined according to Shah *et al.*[18] The validation parameters were: linear range and linearity, sensitivity, selectivity, intra and inter-day precision, accuracy and limit of quantification.

The selectivity was evaluated by checking the lack of sign from interfering compounds at the MOX retention time, by comparison of the chromatogram obtained for blank samples (n = 10) with those originated from the blank matrix fortified with MOX (n = 10). The linearity and sensitivity were established through the matrix-matched analytical curve in eight different concentrations levels (2.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 ng mL$^{-1}$). The results were analyzed by the least squares method and the linearity expressed as the linear correlation coefficient (r).

The precision of the method was determined in two steps: (*i*) repeatability (intra-day) and (*ii*) the intermediate precision (inter-day). The precision was evaluated in three fortification levels (5, 10.0 and 20.0 ng mL$^{-1}$) and 5 replicates for each level. The repeatability was expressed as the coefficient of variation (CV%) of the results of each level of fortification analyzed on the same day with the same sample, using the same instrument. The intermediate precision was expressed by the CV (%) of the results of the analysis performed on three different days (n = 3), by the same analyst using the same instrument.

The accuracy was determined as percent recovery at three fortification levels (5, 10.0 and 20.0 ng mL$^{-1}$). Each fortification level was assessed using 5 replicates. LOQ was determined according to Shah *et al.*[18] using eight samples independent of standards and determining the coefficient of variation (CV < 20%).

The matrix effect was evaluated by comparing the chromatogram area from the analyte in the fortified extract, with the analyte in solvent. For the calculation, results obtained from the fortified extract, at concentration levels corresponding to 20, 40, 60 ng mL$^{-1}$, were compared with the results obtained for the same concentration levels of the analyte in the solvent. The analyte signal obtained from the extract at each concentration level was divided by the signal from the analyte in the solvent. There were conducted the F and *t*-Student tests and the results expressed as percentages.

## Data analysis

The curves of serum concentrations *versus* time obtained in the MOX disposition study, from each individual animal, were fitted with the Curve Expert Pro software, version 2.0.3.[19] PK analysis of data was performed using a one

compartment model and all estimated PK parameters were determined according to Gibaldi and Perrier[20] and are reported as mean ± standard deviation (SD).

## Results and Discussion

### Determination of moxidectin by HPLC-MS/MS

The determination of macrocyclic lactones by using MS/MS QqQ as the detection system can be accomplished by positive and negative electrospray ionization mode. Both of them were evaluated. Nonetheless, in the positive electrospray ionization mode sodium $[M + Na]^+$ and potassium $[M + K]^+$ adducts were predominantly observed instead the MOX protonated ion $[M + H]^+$, which showed a low signal intensity, impairing quantitation limit of the analytical method. Sodium and potassium adduct formation occurs due to the facts of these ions are inherent in the matrix, and its monitoring is not recommended because they result in non-linear analytical curves.[21] In the negative electrospray ionization mode, the mobile phase used favored the MOX deprotonation resulting in a significant increase in signal. Thus, as previously mentioned, optimized ionization conditions for MOX in the ESI source were established in order to obtain the higher MOX detectability in the mass spectrometer.

The Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) and the European Community (EC) specify that the methods employing mass spectrometric detection are particularly suitable to the confirmation of the presence of residues in food and biological samples by providing measurements of molecular masses and information on the chemical structure.[16,17] The use of high and low resolution mass spectrometric techniques meets the criteria for the residue identity confirmation based on the system of identification points (IP), which is related to the amount of fragment ions. In the case of low-resolution mass analyzers, such as the triple quadrupole analyzers, four IPs are needed for identity confirmation.[17] Thus, the proposed method achieves the 4 IP required to confirm the identity of MOX, by monitoring of the molecular ion (equivalent to 1 IP) and two fragment ions (1.5 IP for each fragment ion).

A typical chromatogram obtained for MOX by HPLC-ESI-MS/MS QqQ from lamb serum sample is presented in Figure 2.

### Optimization of extraction procedure

The optimization of the sample preparation is an important process to achieve greater efficiency in the extraction of MOX from serum samples in order to obtain
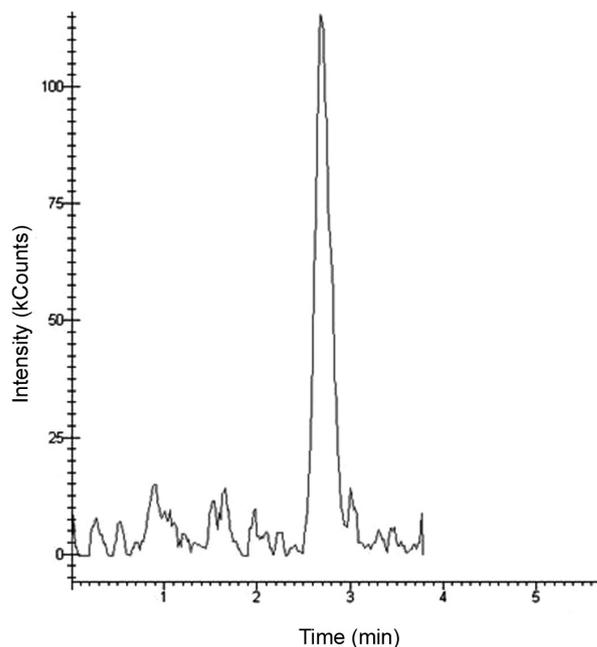


**Figure 2**. Typical total ion chromatogram (TIC) obtained for moxidectin by HPLC-ESI-MS/MS QqQ from a lamb serum sample.

better recovery. The amount of co-extractants obtained after the extraction process established for veterinary drugs determination is a relevant parameter in laboratory routine not only because it can affect the method performance, but also for the maintenance of the analytical equipment. Thus, the clean-up step by SPE-d, using PSA (75 mg) and C18 (amounts tested 75, 100 and 150 mg) as sorbents, was introduced in the sample preparation step. As reported by Kinsella *et al.*[22] the concomitant use of PSA and C18 is more effective in the clean-up extract step, if compared to their use in a separate manner. The amount of PSA used (75 mg) was based in a study carried out by Hashimoto *et al.*[23] PSA sorbent has a high chelating effect, helping samples to get free from fatty acids present in the matrix.[24] Our research has showed that the use of C18 amounts greater than 100 mg are not able to improve the MOX recovery values. The C18 sorbent has the function to removing others nonpolar compounds present in the matrix. Freezing step (−4 °C for 1 h) was necessary to eliminate phospholipids that have been kept in the sample. The compounds are prone to produce unwanted matrix effects.

### Method validation

In terms of selectivity we verified the absence of interferences co-eluting with the analyte (MOX) or near to its retention time. The MOX showed linearity in the concentration range of 2.0 to 100 ng mL$^{-1}$ with linear correlation coefficient higher than 0.99. The calculated

values for intraday and interday precision, expressed as coefficient of variation (CV%), were in accordance with the values preconized by the EC and MAPA which recommend that CV values must be lower than 20% at concentration levels below 100 ng mL$^{-1}$.[16,17] The average values of accuracy, obtained through of recovery assays, also meets the requirements described in the validation guides that establish values between 70 to 110%. The LOQ was established as the lowest level concentration from the calibration curve (2 ng mL$^{-1}$) corresponding to CV value of 16%.[18] No significant difference ($p < 0.05$) was verified with respect to a decrease or increase of analytical signal of MOX in the extract in relation to MOX in solvent showing absence of matrix effect (Table 1).

**Table 1.** Method validation parameters for quantification of moxidectin in lamb serum by HPLC-ESI-MS/MS QqQ

| Validation parameter | |
| --- | --- |
| Linear range / (ng mL$^{-1}$) | 2.0-100.0 |
| Linearity (r) | 0.99 |
| Sensitivity / (au mL ng$^{-1}$) | 274066 |
| Matrix effect / % | |
| 20 ng mL$^{-1}$ | 1.8 |
| 40 ng mL$^{-1}$ | 8.0 |
| 60 ng mL$^{-1}$ | 1.9 |
| Limit of quantification (LOQ) / (ng mL$^{-1}$) | 2.0 |
| Intraday precision (CV) / % | |
| 5.0 ng mL$^{-1}$ | 6.7 |
| 10.0 ng mL$^{-1}$ | 2.0 |
| 20.0 ng mL$^{-1}$ | 1.7 |
| Interday precision (CV) / % | |
| 5.0 ng mL$^{-1}$ | 6.0 |
| 10.0 ng mL$^{-1}$ | 2.1 |
| 20.0 ng mL$^{-1}$ | 2.9 |
| Accuracy (recovery) / % | |
| 5.0 ng mL$^{-1}$ | 82.4-89.8 |
| 10.0 ng mL$^{-1}$ | 80.0-98.3 |
| 20.0 ng mL$^{-1}$ | 83.7-107.3 |

CV: coefficient of variation.

The method validation parameters presented in Table 1 indicate that the HPLC-ESI-MS/MS QqQ method showed to be adequate for the determination of MOX residues in the lamb serum for the pharmacokinetic study.

## Pharmacokinetic study of moxidectin

In the PK study, the first serum sample analyzed was related to that collected 6 h after MOX subcutaneous administration. The parameters that describe the kinetic layout of the MOX are shown in Table 2. It was verified that MOX concentration increased progressively until reaching a maximum average concentration ($C_{max}$) of 7.4 ± 3.1 ng mL$^{-1}$ after 0.8 ± 0.2 day ($T_{max}$). The results obtained (Figure 3) showed large intra specie variability. For instance, regarding the $C_{max}$ values the variability among lambs was between 4.72 to 12.25 ng mL$^{-1}$. A large variability on $C_{max}$ values within specie was reported by Death *et al.*,[14] which evaluated the MOX profile in marsupials obtaining $C_{max}$ values between 55.7 and 142 ng mL$^{-1}$ after subcutaneous administration of 0.2 mg kg$^{-1}$ bw, and by Hunter *et al.*,[25] who evaluated the profile of MOX in llamas and alpacas after topical administration of 0.5 mg kg$^{-1}$ bw reporting $C_{max}$ values between 0.286 and 1.27 ng mL$^{-1}$ for llamas and from 0.213 to 0.879 ng mL$^{-1}$ for alpacas. In relation to other macrocyclic lactones, in sheep after subcutaneous administration of 0.2 mg kg$^{-1}$ bw it was reported mean $C_{max}$ values for ivermectin and doramectin of 13 ± 1 and 38 ± 20 µg L$^{-1}$, respectively,[2] and for ivermectin (0.2 mg kg$^{-1}$ bw) administered by oral route a mean $C_{max}$ values of 5.4 ± 2.3 ng mL$^{-1}$.[26] Therefore, it can be concluded that reported data in this study and in the literature indicate that there is a large variability inter and intra-species related to MOX.

Dupuy *et al.*[27] studied the PK of MOX in the plasma of Lacaune breed lambs with body weight between 20 to 37 kg, six months of age, after subcutaneous administration of 0.2 mg kg$^{-1}$ bw. The $T_{max}$ reported by those authors was of 0.29 days, which is significantly lower than that observed in this study indicating, in our case, a slower passage to blood circulation. This fact may be related to body composition of the animals, since the Lacaune breed has greater ability to produce milk and Suffolk for meat, they exhibit different body composition, which may interfere in the PK of the drug.

The area under the concentration-time curve is generally considered as the most relevant for the assessment of the drug exposure extent. The verified value was 78.5 ± 12.7 ng day mL$^{-1}$, which is similar to that reported by Dupuy *et al.*[27] in lambs (73.26 ± 25.60 ng day mL$^{-1}$) and smaller than the reported by Escudero *et al.*[28] in goats of the Murciano-Granadina breed (136 ng day mL$^{-1}$), with body weight between 31 and 45.8 kg, and 1.5 to 3 years old. The lower area under the curve values obtained in the present study, when compared with Escuredo *et al.*,[28] may be related to the minor body fat storage in lambs when compared to adult animals.[29] Previous studies have also shown that the PK of endectocides presents variability according to the specie.[29,30]

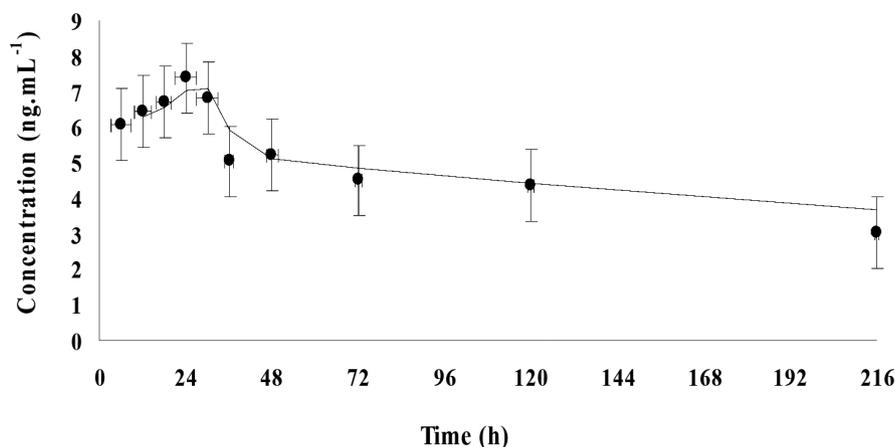The results showed that MOX levels in serum decreased progressively after 24 hours post dose administration.

**Figure 3**. Serum concentration profile (mean ± SD) of moxidectin in lambs following subcutaneous administration of 0.2 mg kg$^{-1}$ body weight (n = 6).

**Table 2.** Pharmacokinetic parameters of moxidectin in lamb serum, after subcutaneous administration of 0.2 mg kg$^{-1}$ body weight

| Parameter | Moxidectin (MOX) (mean ± standard deviation) (n = 6) |
|---|---|
| $C_{max}$[a] / (ng mL$^{-1}$) | 7.4 ± 3.1 |
| $T_{max}$[b] / day | 0.8 ± 0.2 |
| $AUC_{0-Cmax}$[c] / (ng day mL$^{-1}$) | 4.5 ± 2.6 |
| $AUC_{0-9}$[d] / (ng day mL$^{-1}$) | 38.9 ± 9.5 |
| $AUC_{0-\infty}$[e] / (ng day mL$^{-1}$) | 78.5 ± 12.7 |
| $t_{\frac{1}{2}}$[f] / day | 9.0 ± 3.8 |
| $Ke$[g] / day | 0.004 ± 0.002 |

[a]$C_{max}$ = maximum concentration observed; [b]$T_{max}$ = time to reach $C_{max}$; [c]$AUC_{0-Cmax}$ area under the concentration-time curve truncated at $C_{max}$; [d]$AUC_{0-9}$ area under the concentration-time curve truncated at 9 days; [e]$AUC_{0-\infty}$ = area under the concentration-time curve from time zero to infinity; [f]$t_{\frac{1}{2}}$ = elimination half-life; [g]$Ke$ = rate constant elimination in the terminal phase.

The constant disposal rate was 0.004 ± 0.002 days. MOX elimination half-life time value was 9.0 ± 3.8 days. Thus, the elimination half-life of MOX in lambs was similar than the values reported for male cattle, body weight between 180 and 210 kg, 10 months old (14.5 ± 1.2 days) and adult goats (9.9 ± 1.1 days ),[28,30] that received the same dose by the same route of exposure. The deposit of MOX in adipose tissue functions as a reservoir for the drug, releasing it gradually into the bloodstream and contributing for its long half-life elimination and, consequently, to the persistence of the molecule activity on target parasites. Young animals, by presenting less body fat content, tend to store less amount of the compound, influencing in the storage and other PK parameters of the drug. Thus, our results corroborate the long residence time of the drug in the organism and indicate the need to conduct a residue depletion study to establish the withdrawal period in the edible tissue for MOX in lambs.

## Conclusions

The results of the validation parameters showed that the developed and validated analytical method was suitable for MOX residue quantitation in lamb serum.

The PK parameters of MOX in lambs, after subcutaneous administration of 2 mg kg$^{-1}$ bw, corroborate the high intra specie variability, slow subcutaneous absorption, slow release of the drug in the organism and, thus, higher systemic exposure, and indicate the need to conduct a residue depletion study to establish the withdrawal period in the edible tissue for MOX in lambs.

## Acknowledgments

## References

1. Vieira, L. S.; *Rev. Tecnol. Ciên. Agropec*. **2008**, *2*, 28.

2. Antonić, J.; Grabnarb, I.; Cinski, L. M.; Skibina, A.; Süssingera, A.; Cnika, M. P.; Cerkvenik-Flajsa, V.; *Vet. Parasitol*. **2011**, *179*, 159.

3. Sumano, L. H.; Ocampo, C. L. In *Farmacología Veterinaria*, 3$^{rd}$ ed.; MacGraw-Hill Interamericana: México D. F., México, 2006, p. 481.

4. Putter, I.; MacConnell, J. G.; Preiser, F. A.; Haidri, A. A.; Ristich, S. S.; Dybas, R. A.; *Experientia* **1981**, *37*, 963.

5. Chagas, A. C.; Oliveira, M. C.; Esteves, S. N.; de Oliveira, H. N.; Giglioti, R.; Giglioti, C.; Carvalho, C. O.; Ferrezini, J.;

Schiavone, D. C.; *Rev. Bras. Parasitol. Vet.* **2008**, *17*, 126.

6. Cezar, A. S.; Toscana, G.; Camilloa, G.; Sangionia, L. A.; Ribasb, H. O.; Vogela, F. S. F.; *Vet. Parasitol.* **2010**, *173*, 157.

7. Besier, R. B.; Kahn, L. P.; Sargison, N. D.; Van Wyck, J. A.; *Adv. Parasitol.* **2016**, *93*, 181.

8. Craven, J.; Bjørn, H.; Hennessy, D. R.; Friis, C.; *J. Vet. Pharmacol. Ther.* **2002**, *25*, 227.

9. Gokbulut, C.; Nolan, A. M.; McKellar, Q. A.; *Equine Vet. J.* **2001**, *33*, 494.

10. Gokbulut, C.; Biligili, A.; Kart, A.; Turgut, C.; *Lab Anim.* **2010**, *44*, 138.

11. Pérez, R.; Palma, C.; Núñez, M. J.; Navas, M.; Olmos, G.; Cox, J.; *J. Vet. Pharmacol. Ther.* **2009**, *32*, 596.

12. Lifschitz, A.; Suarez, V. H.; Sallovitz, J.; Cristel, S. L.; Imperiale, F.; Ahoussou, S.; Schiavi, C.; Lanusse, C.; *Exp. Parasitol.* **2010**, *125*, 172.

13. Cocquyt, C. M.; Amstel, S. V.; Cox, S.; Rohrbach, B.; Martín-Jiménez, T.; *Res. Vet. Sci.* **2016**, *105*, 160.

14. Death, C. E.; Taggart, D. A.; Williams, D.; Milne, R.; Schultz, D. J.; Holyoake, C.; Warren, K. S.; *J. Wildl. Dis.* **2011**, *47*, 643.

15. Canga, A. G.; Prieto, A. M. S.; Liébana, M. J. D.; Martínez, N. F.; Vega, M. S.; Vieitez, J. J. G.; *Vet. J.* **2009**, *179*, 25.

16. Ministério da Agricultura, Pecuária e Abastecimento (MAPA); *Guia de Validação e Controle de Qualidade Analítica: Fármacos em Produtos para Alimentação e Medicamentos Veterinários*; 2011, http://www.agricultura.gov.br/arq_editor/file/Laboratorio/Guia-de-validacao-controle-de-qualidade-analitica.pdf, accessed in May 2016.

17. European Comission (EC); Official Journal of the European Communities, 17.8.2002, L221/8, available at http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002D0657&from=EN, accessed in May 2016.

18. Shah, V. P.; Midha, K. K.; Digh, S.; Mcgilveray, I. J.; Skelly, J. P.; Yacobi, A.; Layloff, T.; Viswanathan, K. T.; Cook, C. E.; McDowall, R. R.; Pittman, K. A.; Spector, S. J.; *Pharm. Res.* **1997**, *9*, 588.

19. http://www.curveexpert.net/curveexpert-pro-2-0-3-released/ accessed in May 2016.

20. Gibaldi, M.; Perrier, D.; *Pharmacokinetics*, 2nd ed.; Marcel Dekker: New York, USA, 1982.

21. Durden, D. A.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2006**, *850*, 134.

22. Kinsella, B.; Lehotay, S. J.; Mastovskab, K.; Lightfieldb, A. R.; Fureyc, A.; Danahera, M.; *Anal. Chim. Acta* **2009**, *637*, 196.

23. Hashimoto, J. C.; Paschoal, J. A. R.; Queiroz, S. C. N.; Ferracini, V. L.; Assalin, M. R.; Reyes, F. G. R.; *J. AOAC Int.* **2012**, *95*, 913.

24. Prestes, O. D.; Friggi, C. A.; Adaime, M. B.; Zanella, R.; *Quim. Nova* **2009**, *32*, 1620.

25. Hunter, R. P.; Isaza, R.; Koch, D. E.; Dodd, C. C.; Goately, M.; *Small Ruminant Res.* **2004**, *52*, 275.

26. Alvinerie, M.; Dupuy, J.; Kiki-Mvouaka, S.; Sutra, J.; Lespine, A.; *Vet. Parasitol.* **2008**, *157*, 117.

27. Dupuy, J.; Larrieu, G.; Sutra, J. F.; Lespine, A.; Alvinerie, M.; *Vet. Parasitol.* **2003**, *112*, 337.

28. Escudero, E.; Carceles, C. M.; Diaz, M. S.; Sutra, J. F.; Galtier, P.; Alvinerie, M.; *Res. Vet. Sci.* **1999**, *67*, 175.

29. Dupuy, J.; Chartier, C.; Sutra, J. F.; Alvinerie, M.; *Parasitol. Res.* **2001**, *87*, 294.

30. Lanusse, C.; Lifschitz, A.; Virkel, G.; Alvarez, L.; Sánchez, S.; Sutra, J. F.; Galtier, P.; Alvinerie, M.; *J. Vet. Pharmacol. Ther.* **1997**, *20*, 91.