Determination and Withdrawal Time of Fosfomycin in Chicken Muscle, Liver and Kidney

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Abstract: A HPLC-MS/MS method was developed for the determination of fosfomycin in chicken muscle, liver and kidney. Organ samples were dispersed in silica gel with a subsequent clean up and elusion in a vacuum chamber followed by filtration with activated carbon to eliminate matrix components which are prone to produce unwanted matrix effects. Response was linear and recovery was determined to be between 81-106% for muscle, 92-102% for liver and 99-107% for kidney. The method developed is suitable to be used in withdrawal time studies. The withdrawal time of fosfomycin in broiler chickens, considering a MRL of 0.5 µg/g was studied. Twenty-four broiler chickens were assigned to two groups; in group one, fosfomycin was orally administered daily with 40 mg/kg bw and to the other group a dose of 10 mg/kg bw of the antibiotic was intramuscularly administered. Broilers were slaughtered 24, 48, 72 and 96 h later of oral treatment withdrawn and post intramuscular treatment. Although the longer WDT was of 2.55 days, a WDT of 3 days could be assigned as a precautionary principle for public health, without a significant economic impact for the broiler producer.

Key words: HPLC MS-MS, fosfomycin, chicken tissues, maximum residues limit, withdrawal time

INTRODUCTION

Fosfomycin (cis-1, 2-epoxyphosphonic acid) is a broad-spectrum bactericidal antibiotic, not structurally related to other classes of antimicrobial agents. Fosfomycin acts inside the bacterial cytoplasm (Popovic et al., 2010). Its mechanism of action is based in the inhibition of cell wall and early murine/peptidoglycan synthesis in proliferating bacteria (Kahan et al., 1974). It inhibits an initial peptidoglycan synthesis step, triggered by uridine diphosphate N-acetyl-glucosamine-enol-pyruvyltransferase and its co-enzyme phosphonole-pyruvate (Kahan et al., 1974; Lin, 1976; Popovic et al., 2010), causing bactericidal activity against Gram positive and Gram negative bacteria (Gobernado, 2003). Therefore, when compared with other antibiotics, the in vitro fosfomycin activity has a broader spectrum of action than penicillins and semi-synthetic cephalosporins (Mata et al., 1977). On the other hand, no cross-resistance with other antibiotics has been reported (Gobernado, 2003). The use of fosfomycin in animals and humans has been suggested due to its low toxicity and potential efficacy (Gallego et al., 1974). Fosfomycin forms salts easily due to its acidic nature. Orally, it is used in its calcium salt form and intravenously and intramuscularly, as the more water-soluble disodium salt. Fosfomycin-tromethamine salt is highly hydro-soluble and has a good oral bioavailability in humans (Patel et al., 1997; Borsa et al., 1988; Popovic et al., 2010). Fosfomycin is also widely used in animal production due to its rapid effect, good tolerance and absence of side effects (Aramayona et al., 1997; Carraminana et al., 2004). Particularly in broilers, this antibiotic is used for the treatment of infectious diseases caused by mycoplasmas (Mycoplasma gallisepticum and sinoviae) and Gram negative and Gram positive bacteria (Salmonella, E. coli, Haemophilus paragallinarum, Pasteurella multocida, Staphylococcus and Listeria) (Fernandez et al., 2001; 2002).

Fosfomycin pharmacokinetic has been extensively studied in humans, (Damaso et al., 1990; Falagas et al., 2008; Gallego et al., 1971; Grassi, 1990; Kirby, 1977; Mensa et al., 1994; Segre et al., 1987; Simon et al., 1987; Vargas et al., 1987), broiler chickens (Aramayona et al., 1997, Soraci et al., 2011a,b), rabbits (Fernandez Lastra et al., 1986; 1987), cows (Sumano et al., 2007), horses (Zozaya et al., 2008), dogs (Gutierrez, 2008) and pigs (Soraci, 2011a). Likewise, different analytical methods for determination of fosfomycin in biological matrices have been described in the literature (Pianetti, 1997; Dios-Vieitez et al., 1996; Yu-Ling Hu et al., 1999; Hernandez et al., 2001; Loste et al., 2002; Tzanavaras and Themelis, 2002; Petsch et al., 2005), including microbiological assays (Gutierrez, 2008; Zozaya et al., 2008). Most of them are time consuming and include a
derivatization step for the analysis. Currently, HPLC MS/MS is the method of choice for xenobiotics determination. For fosfomycin, this method has the advantage that derivatization is not needed, becoming a much easier, less time consuming and highly specific methodology at the same time (Li et al., 2007; Dieguez et al., 2011; Soraci, 2011a). However, when working in HPLC-MS/MS with biological matrices, care must be taken to avoid interferences which are prone to alter signal by ion suppression/enhancement mechanisms.

The administration of veterinary drugs to food-producing animals without an adequate Withdrawal Time (WDT) may lead to violative concentrations of residues in foods intended for human consumption. These residues represent a risk to public health, including stimulation of bacterial resistance, alterations on intestinal microflora and hypersensitivity reactions. Therefore, to ensure the delivery of safe animal products to consumers, the Withdrawal Time (WDT) of drugs must be respected. In general terms, the WDT is the period of time required after completion of treatment needed for tissue concentrations of the drug and/or its metabolites to deplete to less than the established Maximum Residue Limits (MRLs) (Riviere et al., 1998; KuKanich et al., 2005).

With the aim of minimizing the risk to human health represented by residues in food products, MRLs for many drugs have been established by regulatory agencies of different countries. However, the European Agency for the Evaluation of Medicinal Products (EMEA) and the European Commission have not established the MRLs for fosfomycin. The Japan Food Chemical Research Foundation has established MRLs of 0.5 µg/g for cattle muscle, liver, kidney and fat and a MRL of 0.05 µg/mL for milk. No MRLs has been established for chicken tissues. This study was also aimed to determine WDTs of calcium fosfomycin in poultry after an oral treatment and of disodium fosfomycin after intramuscular administration. For this purpose, the cattle tissues MRL (0.5 µg/g) established by Japan, was considered.

MATERIALS AND METHODS
This work was performed at the Laboratory of Toxicochemistry and the experimental farm of the Faculty of Veterinary Sciences, UNICEN, Tandil, Buenos Aires, Argentina.

Animals: For residues analysis and withdrawal time calculation, forty-eight healthy male broiler chickens (Ross genetic) of 14 days-old, were used. Animals were placed on the floor and maintained under conditions of controlled temperature (25±5°C), light cycle (12/12 h) and relative humidity (45-65%). One week before experimentation (acclimation period), no clinical signs of disease were apparent. Food (the ration was formulated ad libitum). Experimental animal’s conditions were in agreement with the Animal Welfare Guidelines approved by the Bioethics Committee of the Faculty of Veterinary Sciences, UNICEN, Tandil, Argentina and the recommendations of the European Council Directive 2007/43. Birds were sacrificed under the Animal Welfare Rules of the European Council Directive 93/119/CE 1993. On day 21, chickens were weighed and divided randomly into two experimental groups of twenty four animals (A and B groups). The A group (24 broiler chickens) was treated individually with 40 mg/ kg bw of calcium fosfomycin (98.9% of purity, Bedson Laboratory, Pilar, Buenos Aires, Argentina) orally administrated once daily for five consecutive days (every morning, between 7 and 8 am). The drug was weighted, diluted in water and administrated using a plastic gastric catheter to assure the complete ingestion of the dose. The total volume of administration was 2.5 mL for animal. For intramuscular assay, (B group, 24 animals), disodium fosfomycin (98.9% of purity, Bedson Laboratory, Pilar, Argentina) was diluted in sterile water and administered in the pectoral muscle, at 10 mg/kg. bw. The total volume of administration was 1.2 mL for animal.

Method development: Fosfomycin calcium salt (analytical standard, STD), purchased from Sigma (St. Louis, USA), was used. HPLC grade acetonitrile, isopropanol and methanol were from JT Baker (Deventer, Holland). Ultra purified de-ionized water was obtained using a water purification devise (Pure Lab UHQ from ELGA [Lane End, UK]). Silica Gel 60 (0.040-0.063 mm) was from Merck (New Jersey, USA).
**Instruments:** The HPLC-MS/MS system was from Thermo Electron Corporation (San Jose, CA, USA), consisting of a Finnigan Surveyor auto sampler, a Finnigan Surveyor MS quaternary pump. The detector was a Thermo Quantum Discovery Max triple quadrupole mass spectrometer, equipped with an ESI source. Nitrogen used as nebulizer and sheath gas was obtained through a nitrogen generator from Peak Scientific Ltd. (Inchinnan, Scotland). Data processing was done using Xcalibur software, also from Thermo.

**Mass spectrometer conditions:** The mass spectrometer was operated in negative ionization mode. The tuning parameters were optimized with 10 µg/mL individual aqueous solutions of fosfomycin directly infused in the ion source by means of a syringe pump at 10 µl/min, with influence of mobile phase delivered from the LC pump through a T connection to give the corresponding chromatographic flow rate. The spray voltage was set to -3800 eV, the capillary temperature was 350°C and argon 99.999% purity was used for Collision Induced Dissociation (CID) at 1.6 mTorr in the collision cell. Source CID energy was set to -8eV. Fosfomycin detection and quantification were achieved by single reaction monitoring of transitions m/z 137→79 with an optimized collision energy of 25.

**Chromatographic conditions:** Separation was achieved on a Phenomenex CN (cyano) stationary phase, 75 mm x 4.6 i.d., 5 µm column. The mobile phase consisted on acetonitrile:water 20:80, working in isocratic mode, at a flow rate of 100 µl/min. The column was maintained at 30°C, while the samples in the auto sampler were at 10°C. Sample injection volume was 20 µl and chromatographic run time was 10 min.

**Sample collection:** For method development and validation, five healthy, 30 days-old chickens were used. Animals received an antibiotic-free diet and water ad libitum. After sacrifice, tissue samples were collected. Organs were processed by a hand blender and stored at -18°C until analysis.

**Standard solutions preparation:** Stock solution of fosfomycin was prepared by dissolving 10.0 mg of drug in 25 mL of purified water.

**Quality control samples (QC):** Fosfomycin solutions to fortify drug free tissues before extraction were prepared daily, by diluting adequate volumes of stock solution in water. Fifty µl of each of these solutions were thoroughly mixed in 1 g of tissue, to obtain effective concentrations of 2, 5, 10, 20, 40, 60 and 80 µg/mL.

**Calibration solutions:** Drug free extracts were spiked with 100 µl fosfomycin working solution at different levels just before injection into HPLC-MS/MS system, to obtain concentrations corresponding to 100% extraction over the range of 0.1-4 µg/mL.

**Sample extraction:** One g of tissue was placed in a glass mortar containing four g of silica gel and mortared until the sample was completely dispersed into the silica. Columns were assembled in 20-mL syringes, placing a Whatman GF/B filter on the bottom, followed by a mixture of silica gel with the tissues. Another Whatman GF/B filter was placed above. Columns were pressed with a piston. Each column was placed in a vacuum chamber, where different volumes of solvents were applied to wash and elute the sample. Clean up began with 6 mL of acetonitrile. Pressure was kept constant and leakage was uniform in all columns. Then 5 mL of isopropanol were applied. The next step was the addition of 6 mL of methanol. All the solvents were collected in glass tubes and discarded. Properly labeled clean glass tubes were placed in the vacuum chamber. 9 mL of methanol-water (2:1) were added and eluted. The nine mL eluate was filtered in syringes containing activated carbon, 1 mL aliquots were filtered with 0.22 µm nylon filters, placed in vials and analyzed by HPLC MS-MS.

**Method validation:** Validation parameters, as well as their acceptance range, were in accordance with international guidelines (U. S. Department of Health and Human Services, 2001; European Commission Decision 2002/657/EC, 2002). Quantification was achieved by calculating fosfomycin area as the assay response. Calibration curves were prepared in quintuplicates, and assayed within one week, in order to assess Linearity by Hartley’s Test. Least square linear regression was used for curve fitting.

QC samples fortified at 3 levels were processed in triplicates on 4 separate days, in order to assess Accuracy and Precision of the method. The accuracy was expressed as Relative Error (RE) and it was required to be ±15% (except for the limit of detection that can reach up to 20%). Within day precision (repeatability) was calculated by the mean Coefficient of Variation (CV) which was required to be less than 15% for all concentrations (except for the limit of detection where it can reach up to 20%).

Lower limit of quantification was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20% and it was obtained by analyzing fortified tissues at the lower level of the calibration curve, in 5 replicates on three different days. Recovery of fosfomycin following extraction was calculated by comparing the fosfomycin mean peak area of QC samples with the values obtained for post-extraction spiked samples, which represented 100% recovery.
Selectivity was determined by analyzing tissues from 6 healthy chickens, which had never received antimicrobial treatment, each coming from different poultry farms.

**Decision limit (CC**) and Detection capability (CC$)$:**
The decision limit (CC”) is defined as the limit above which it can be concluded with an error of probability of ”, that a sample contains the analyte. The detection capability (CC$)$ is defined as the lowest concentration of analyte at which the method is able to detect and quantify contaminated samples with a statistical certainty of 1-$(European Commission Decision, 2002).

**Matrix effects evaluation:** For matrix effects evaluation, two types of studies were conducted, as described by Matuszewski et al. (1998). On one hand, peak area ratios obtained with fosfomycin aqueous solutions, at three concentration levels within the linear range, were compared with those obtained with extracted blank tissues, spiked at the same concentration, just before injection. On the other hand, each blank tissue extract was injected at the same time a fosfomycin aqueous solution was being directly infused into the ion source. Matrix effects were observed by enhancement or reduction of the signal at certain regions of the chromatogram.

**Residues analysis and withdrawal time calculation**

**Residues analysis**

**Oral assay**

**Testing and sampling:** On the fifth day of trial fosfomycin dosage was discontinued. Twenty-four h later, the first group of animals (six broilers) was sacrificed. The next day, the second group of animals was euthanized (six broilers). After 72 h of drug dosage suspension, other six broilers were sacrificed. The last group of six chickens was euthanized 24 h later. All groups were killed by puncture of the foramen magnum after 12 h of fasting.

**Samples:** Samples were collected in plastic bags (Ziploc ® type), quickly cooled and stored at -80°C until assayed.

**Muscle:** A total of forty-eight samples were obtained (twenty-four samples of thigh muscle and twenty-four samples of pectoral muscle).

**Liver:** A total of twenty-four livers were obtained (six livers each day of sacrifice).

**Kidneys:** Twenty-four kidneys were obtained, six each slaughtering day.

Fosfomycin determination in chicken tissues was based on the validated method previously mentioned. Data processing was performed by Xcalibur software (Thermo Corporation). Quantification was achieved by calculating fosfomycin area as the assay response. Samples analyzed were:

**Muscle:** One hundred and twenty samples (Forty eight oral samples and seventy-two intramuscular samples, all analyzed by duplicate).

**Liver:** Forty-eight samples (twenty-four oral samples and twenty-four intramuscular samples, all analyzed by duplicate).

**Kidney:** Forty-eight samples (twenty-four oral samples and twenty-four intramuscular samples, all analyzed by duplicate).

**Determination of withdrawal times:** The fosfomycin area at each sampling time was considered for the determination of WDTs in muscle, liver and kidney, adopting the European Agency for the Evaluation of Medicinal Products recommendations (EMEA, 1995). The WDTs were estimated from the linear regression analysis of log-transformed tissue concentration and was determined at the time when the 95% upper one-sided tolerance limit was below the MRL with 95% confidence. For this purpose, the statistical program WTM 1.4 was used.

**RESULTS**

**Method development**

**Optimization of mass spectrometer conditions:** High spectrometric response was observed when working in...
ESI negative ion mode for fosfomycin STD. The predominant ion obtained was de-protonated fosfomycin, which m/z value was 137, in the Q1 (or Q3) full scan spectra.

The direct infusion from syringe pump of independent solutions of fosfomycin STD, allowed the observation of changes in response as well as in MS parameters. Thus, the most suitable conditions of the ion source in order to obtain the highest signal from parent ions were found to be the following: Spray voltage: -3800eV; Spray temperature: 350°C; Source CID energy: -8eV and Sheath gas pressure: 1.5.

Using the same technique, collision pressure and collision energy in Q2 were evaluated. For fosfomycin, a predominant fragment ion of m/z 79 was formed when collision pressure was 1.6 and collision energy -25 eV. The intensity of parent ions showed a more than 80% reduction.

Optimization of chromatographic system: The use of a Cyano (CN) stationary phase greatly improved the retention, when compared to a C18, which agrees with the results by Li Li’s et al. (2007) and Dieguez’s et al. (2011). In addition, good performance was achieved with water:acetonitrile 80:20 mobile phase, as described by Dieguez et al. (2011) and Soraci et al. (2011a).

Optimization of extraction and clean up procedure: For method development, different sample preparation techniques were evaluated, using chicken muscle as initial study tissue. These techniques included: a.) Deproteinization and lipids extraction, b.) Double de-proteinization, c.) Dispersion plus C-18 reverse phase cartridge, d.) Dispersion plus normal-phase silica cartridge, e.) Different solvents elusion.

a.) De-proteinization and lipids extraction: Method development was initially evaluated by liquid-liquid extraction done by crushing a piece of pectoral muscle (fat free) with a mixer. Four samples of 2.5 g were taken (two as blank tissue and two for STD spiking with 10 µg/mL and 30 µg/mL of fosfomycin). Methanol was added for de-proteinization. Samples were stirred 10 min and then centrifuged at 3200 rpm for 15 min. Each sample supernatant was evaporated at 60°C and the residue was re-suspended in 200 µl of water and 1 mL of hexane-methanol 1:0.2 (Folch reactive) was added for sample clean up. To discard the hexane phase, samples were stirred and centrifuged again. Forty µl from each sample were taken and carried to 800 µl with HPLC water, filtered and injected into the chromatograph. It was observed that methanol was suitable for the deproteinization of the sample and the extraction of lipids with Folch reactive was also a good option to samples clean up. However, these two steps were not effective enough, due to interferences in blank samples at analyte same retention time due to co-elution with some component of the biological matrix.

b.) Double de-proteinization: Due to the possibility of remnants of polar nature proteins that could precipitate with a second extraction, a double protein precipitation with methanol was tested. Two samples of 1 g and 2.5 g of muscle were used to post-extraction spiking (4 and 10 µg/mL). Five mL of methanol were added to 1 g samples and 6 mL to those of 2.5 g. Samples were homogenized, stirred for 20 min. in a shaker and centrifuged for 15 min. at 3200 rpm. Supernatant was evaporated to dry. The waste underwent a second precipitation with 5 mL of methanol and samples were stirred and centrifuged again. The supernatant of each sample was re-evaporated to dry, followed by a re-suspension with 200 µl of HPLC water and 1 mL of Folch reactive. Then, they were stirred and centrifuged, the hexane phase was discarded and 40 µl of each sample were taken to 800 µl with HPLC water, filtered and injected into the chromatograph. Interferences continued to appear in blank tissues. Therefore, the clean up procedure had to be improved.

c.) Dispersion plus C-18-reverse phase cartridge: The intention was to retain some non-polar compounds that could have remained in the sample in order to obtain a peak with better resolution. Three, 1 g-samples (one blank, one spiked with 1 µg/mL and one spiked with 4 µg/mL of fosfomycin) were mixed with 4 g of silica gel and homogenized in a mortar; columns were assembled in syringes and cleaned up in a vacuum chamber with 6 mL of acetonitrile and 6 mL of methanol. Samples were eluted with nine mL of methanol-water (2:1). This portion was filtered and 3 mL aliquots were taken. Previously activated and conditioned C-18 cartridges were used with the intention of retaining any non-polar compound that could have remained in the sample. This passage permeate was injected into the chromatograph. The result was not encouraging and it was concluded that it was an unnecessary step.

d.) Dispersion plus normal-phase silica cartridge: In this case, it was attempted to adhere the analyte to the solid phase (silica gel), exploiting the polarity of the molecule and then, to elute it with different solvents according to their polarity. Two samples of 0.5 g (a blank and a sample spiked with 150 µl of a 100 µg/mL solution) were used. Two g of silica were added in a mortar and the mixture was homogenized. Columns were assembled in syringes by first placing a GF/B filter, followed by the mixture and finally, by another GF/B filter. Samples were eluted in manifold with 6 mL of methanol and centrifuged at 4°C and 10000 rpm for 15 min; the supernatant was diluted with HPLC water, 1:50, 1:10 and 1:5 and then injected. Fosfomycin began to diverge even if the resolution was not optimal. On the other hand, it is noteworthy that the peak previously described to appear on blank tissues was not observed with this method.
Different solvents elutions: Elutions with acetonitrile (Fig. 1), acetonitrile-methanol (1:1) (Fig. 2) and methanol: water 2:1 (Fig. 3) were attempted. There was an increase in the instrumental signal and improved resolution with the use of methanol:water (2:1). As there were interferences at the front of the chromatogram, a preliminary clean up step with different solvents was performed.

Acetonitrile was used with the intention of precipitating the proteins; isopropanol was added because of its powerful activity for lipids elimination and methanol because it has an effective potential for de-proteinization of the sample. The clean up procedure was followed by the elution with methanol-water (2:1). A well-defined peak and no interferences on the blank tissues was detected (Fig. 4). Therefore, the definitive method was defined.

Method validation
Validation parameters
Selectivity: No signal above the base line at fosfomycin retention time was observed in tissues from chickens to which antimicrobials had not been administered. Figures 5a, 6a and 7a show a comparison of typical blank chromatograms of muscle, liver and kidney with spiked tissue samples (Fig. 5b, 6b and 7b).

Linearity: A typical linear regression curve was constructed over the range 0.01-0.4 µg/mL (representing 0.1-4 µg/mL of chicken muscle, liver or kidney). Good linearity was obtained within the concentration range, being $r^2$ coefficient above 0.995 for all matrix replicates. As a $r^2$ coefficient above 0.995 is not enough to determine the method linearity, a Hartley’s Test was
Fig. 5a: Blank muscle

Fig. 5b: 0.1 µg/mL spiked muscle

Fig. 6a: Blank liver

performed to demonstrate homoscedasticity, so the variance around each regression line might be the same for all values of the predictor variable. Fmax was always less than Tabulated F.

**Accuracy and precision:** Accuracy and precision were evaluated for spiked samples at different levels. Accuracy, expressed as RE, was 0.49%-2.20% for muscle, 0.97%-3.76% for liver and 0.30%-1.21% for kidney. Repeatability (within day precision) was less than 6% for all concentrations studied, except for the 0.1 µg/mL spiked samples of kidney, which was 15.16%, a value that was accepted because it was our detection limit (CV<20%).

**Extraction recovery:** Drug recovery was tested for chicken tissue samples spiked at different levels. Mean extraction recoveries were between 81-106% for muscle, 92-102% for liver and 99-107% for kidney.

**Decision limit (CC") and detection capability (CC$):** CC" and CC$ values were calculated using the intercept (value of the signal, y, where the concentration, x, is equal to zero) and the Standard Error of the Intercept.
Fig. 6b: 0.1 µg/mL spiked liver

Fig. 7a: Blank kidney

Fig 7b: 0.1 µg/mL spiked kidney

(SEI). CC$^*$ is the concentration corresponding to the intercept + 2.33 x the SEI. CC$^+$ is the concentration corresponding to the signal at CC$^*$. CC$^*$ ranges from 0.03 µg/mL (liver and kidney) to 0.06 µg/mL (muscle). CC$^+$ was of 0.05 µg/mL (liver), 0.06 µg/mL (kidney) and 0.10 µg/mL (muscle).

Table 1 summarizes the validation parameters for each matrix.

Residues analysis: The matrix-matched calibration curves of muscle, liver and kidney were linear ($r > 0.99$) and were used for drug quantification in experimental
Table 1: Validation parameters summary

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acceptance criteria</th>
<th>Tissue</th>
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<tr>
<td></td>
<td></td>
<td>Muscle</td>
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<td></td>
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<td>Liver</td>
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<td></td>
<td></td>
<td>Kidney</td>
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<tr>
<td>Lineality-Hartley’s Test</td>
<td>FMAX&lt;FTABULADO</td>
<td>26.38&lt;50.70</td>
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<td></td>
<td></td>
<td>11.16&lt;50.70</td>
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<td></td>
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<td>24.33&lt;50.50</td>
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<td>Precision (CV%)</td>
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<td>0.83-3.33</td>
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<td></td>
<td></td>
<td>0.23-15.16</td>
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<tr>
<td>Accuracy (ER%)</td>
<td>&lt;15</td>
<td>0.49-2.20</td>
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<td></td>
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<td>0.97-3.76</td>
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<td>0.30-1.21</td>
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<td>%R</td>
<td>80-120</td>
<td>81.34-105.95</td>
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<td>91.91-101.80</td>
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<td></td>
<td>99.91-106.75</td>
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<td>Limit of quantification</td>
<td>0.10 µg/mL</td>
<td>0.10 µg/mL</td>
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<tr>
<td></td>
<td>CV% = &lt;20</td>
<td>CV% = 12.60</td>
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<td>ER% = &lt;20</td>
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<td>ER% = 15.16</td>
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<td>ER% = 3.33</td>
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<td>CC (µg/mL)</td>
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<td>CC$ (µg/mL)</td>
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Table 2: Fosfomycin average concentration for chicken thigh, pectoral and site of injection muscles, liver and kidney at different time-points after drug administration

<table>
<thead>
<tr>
<th>Average concentration (µg/g)</th>
<th>Muscle</th>
<th>Liver</th>
<th>Kidney</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Oral assay</td>
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<td>24 h</td>
<td>&gt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<td>48 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<tr>
<td>72 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<tr>
<td>96 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<tr>
<td>IM Assay</td>
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<tr>
<td>24 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<tr>
<td>48 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<td>72 h</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<tr>
<td>96 h</td>
<td>&lt;0.10</td>
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Table 3: WDTs for each matrix and assay

<table>
<thead>
<tr>
<th>Withdrawal time (days) - MRL 0.5 µg/g</th>
<th>Muscle</th>
<th>Liver</th>
<th>Kidney</th>
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</thead>
<tbody>
<tr>
<td>Oral assay</td>
<td>1.12</td>
<td>1.27</td>
<td>2.55</td>
</tr>
<tr>
<td>IM assay</td>
<td>1.72</td>
<td>0.42</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Withdrawal time calculation

Estimation of Withdrawal Time (WDT): In Table 3, the duration of the WDTs for each assay considering a 0.5 MRL µg/g are shown.

DISCUSSION

Selection and validation of suitable analytical methods must be previously accomplished in drug tissue depletion studies for determination of WDTs. The HPLC-MS-MS method was selected because of its high specificity and accuracy, which were considered critical factors for this study. HPLC-MS-MS has been used for similar purpose by other authors (Clemente et al., 2006; Hermo et al., 2006; Tang et al., 2006; San Martin et al., 2007; Lopez et al., 2008; Dieguez et al., 2011; Soraci et al., 2011a).

Solid phase dispersion with silica gel was previously used by some authors to determine residual concentrations in biological tissues as Gutierrez Valencia and Garcia Camacho (2010) and Lei Zhang et al. (2005), among others. The results of the analytical method validation indicate that the proposed method is suitable for the evaluation of fosfomycin tissue depletion in chickens.

In this study, fosfomycin tissue concentration and WDTs were determined in broilers chickens after the administration of a daily oral dose (40 mg/ kg bw for five consecutive day) and after an intramuscular dose (10 mg/kg bw) considering the only established MRL for fosfomycin, 0.5 µg/g (Japan Food Chemical Research Foundation).

Aramayona et al. (1997) found fosfomycin mean concentrations ranging from 0.63 µg/g in fat to 13.48 µg/g in kidney. The values for Fosfomycin in kidney are higher than those found in this study. The administration of oral calcium fosfomycin for five consecutive days at 40 mg/kg in broilers, showed low residual levels in chicken muscle, liver and kidney. Such concentrations are associated with poor oral bioavailability of calcium formulation (F: 39%) and an absence of fosfomycin tissue protein binding. The administration of disodium fosfomycin at 10 mg/kg in broilers has also showed low residual levels in chicken tissues. In both assays, fosfomycin distribution was higher in thigh muscle over pectoral muscle. These differences found between thigh and pectoral muscles, are probably related to a greater blood flow to the first muscle. However, in contrast to our findings, Aramayona et al. (1997) detected fosfomycin in all tissues, except muscle. The low concentrations of fosfomycin in kidney may be associated with a short plasma half-life (1.81 h) and rapid clearance (Cl(area) ml/h/kg = 139.9) (Soraci et al., 2011a,b). Fosfomycin distribution in kidney was higher compared to other tissues (liver or muscle, after oral administration for five samples. Table 2 shows the average concentration (µg/g) for each tissue, corresponding to each sampling time of oral and intramuscular assay.
consecutive days), probably due to the fact that this organ represents the main route of elimination for the systemically available fosfomycin dose. Otherwise, the FDA has established that the edible tissue from which residues deplete most slowly should be considered as the target tissue to evaluate the WDT (Ellis, 2004). According to the EMEA (2002), target tissues for poultry are muscle, liver and kidney. For the calculations of WDTs, the EMEA (1995) recommends to perform a linear regressions analysis of the logarithmic transformed concentrations during the tissue depletion phase of the drugs and/or their metabolites. Using the model of the EMEA, the WDT of fosfomycin was determined as the point at which the upper 95% tolerance limit for the residue is less than MRLs with 95% confidence. In agreement with the Codex Alimentarius Commission (1995), the lengths of the WDTs are defined by MRLs of each veterinarian drug. In this study, the WDTs of fosfomycin were determined considering the only MRL defined by Japan. It is worth pointing out that this MRL was established for muscle, liver, kidney and fat from cattle, not for broiler chickens. On this regard, based on the 0.5 µg/g MRL of Japan, it is important to note that in both oral and intramuscular assays, concentrations of fosfomycin in muscle, liver and kidneys were always below the MRL. Also, after 72 h of fosfomycin food withdrawn and of fosfomycin intramuscularly administration, the values of the residual concentrations of drug in tissues were below the 0.1 µg/g detection limit of our validated method. Fosfomycin WDT in muscle was among 1-2 days, being of 1.12 days for calcium fosfomycin (oral assay) and 1.72 days for disodium salt (IM assay). Differences between fosfomycin muscle WDTs may be due to the distinct formulations and routes of administration. The same applies to periods of withdrawal in liver and kidney, which are also longer after oral calcium fosfomycin consumption in food (1.27 vs. 0.42 days and 2.55 vs. 0.92 days, respectively). In conclusion, according to our results, it is possible to suggest that a WDT of 3 days could be assigned as a precautionary principle for public health, without a significant economic impact for broiler producers.

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REFERENCES


