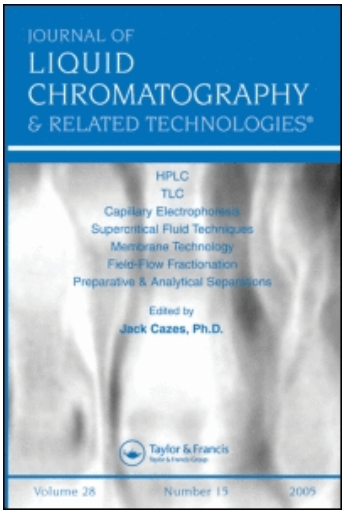

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DETERMINATION OF ANTIBIOTIC FOSFOMYCIN IN CHICKEN SERUM BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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□ *A high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method was developed for the determination of fosfomycin in chicken serum using fudosteine as the internal standard. Serum samples were treated with methanol to precipitate proteins. A subsequent clean up using liquid-liquid extraction followed by dilution was performed to eliminate phospholipids which are prone to produce unwanted matrix effects.*

The HPLC-MS/MS system involved the use of an isocratic mobile phase on a cyano stationary phase column and electrospray ion source operating in negative ion mode. Single reaction monitoring of transitions m/z 137 → 79 and 178 → 91 was performed on a triple quadrupole mass spectrometer to quantify fosfomycin and fudosteine, respectively.

Response was linear over the range of 0.1 to 50 $\mu g\,mL^{-1}$. Recovery ranged from 95 to 108%. Accuracy determined for spiked samples at 5, 10, and 20 $\mu g\,mL^{-1}$ was −7.8, 1 and −0.7%, respectively, expressed as relative error. Within day and between days precision, in terms of coefficient of variation, were less than 10% for all concentrations. The developed method was successfully used in a pharmacokinetic study after oral administration of calcium fosfomycin to broiler chickens.

Keywords chicken, fosfomycin, high performance liquid chromatography-tandem mass spectrometry, matrix effect, validation

INTRODUCTION

Fosfomycin [(−)-(1 R,2S)-(1,2epoxyproyl) phosphonic acid], is a broad spectrum antibiotic that inhibits cell wall synthesis as it interferes with peptidoglycan production causing bactericidal activity against Gram positive and Gram negative bacteria.[1] Fosfomycin is widely used in animal

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production due to its rapid effect; it is well tolerated, gives no side effects, and creates little cross-resistance with other antimicrobials.[2–3] Fosfomycin is used for the treatment of infections caused by multidrug resistant non-fermenting Gram negative bacilli.[4] This antibiotic is used in broilers for the treatment of infectious diseases caused by micoplasmas, Gram negative, and Gram positive bacteria (*Salmonella, E. Coli, Haemophilus paragallinarum, Pasteurella multocida, Estafilococus* and *Listeria, Micoplasma gallisepticum* and *sinoviae*).[5,6]

Different analytical methods for determination of fosfomycin in biological matrices have been described in the literature.[7–15] Most of them are time consuming and include a derivatization step. High performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) has been the recent method of choice for xenobiotics determination. This method is particularly advantageous for the analysis of fosfomycin as derivatization is not required, becoming easier, less time consuming, and highly specific at the same time.[16] However, in the analysis of biological matrices, care must be taken to avoid interferences that are prone to alter signal by ion suppression/enhancement mechanisms. This phenomenon is specially observed in plasma or serum, where high concentrations of phospholipids are present.[17–20]

In the present study, a simple, rapid, and highly selective HPLC-MS/MS method was developed and validated to determine fosfomycin in broiler chickens serum. The method developed has been used to study serum concentration profiles after oral administration of calcium fosfomycin.

EXPERIMENTAL

Materials

Fosfomycin calcium salt (analytical standard, STD) was purchased from Sigma (St. Louis USA), Fudostein (Internal Standard, ISTD) was purchased from EH Field Co., Ltd. (Nanjing, China). HPLC grade methanol, acetonitrile, hexane, and ethanol were from J.T. Baker (Deventer, Holland). Ultra purified de-ionized water was obtained using a water purification devise Pure Lab UHQ from ELGA (Lane End, UK).

Instruments

The HPLC-MS/MS system was a Thermo Electron Corporation (San Jose, CA, USA), consisting of a Finnigan Surveyor auto sampler, a Finnigan Surveyor MS quaternary pump, and a detector Thermo Quantum Discovery Max triple quadrupole mass spectrometer, equipped with electrospray

(ESI) ion 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 (Thermo).

A Turbo Vap workstation from Caliper (Massachusetts, USA) with bath temperature and air flow control was used for solvent evaporation.

Mass Spectrometer Conditions

The mass spectrometer was operated in negative ionization mode. The tuning parameters were optimized with 10 μg mL^{-1} individual aqueous fosfomycin and fudosteine solutions. A syringe pump directly infused the solutions into the ion source at 10 μL min^{-1}, while mobile phase was delivered from the LC pump through a T connection to give the corresponding chromatographic flow rate. Spray voltage was set to −3800 eV, capillary temperature was 350°C. 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 −8 eV.

Fosfomycin and fudosteine detection and quantification were achieved by single reaction monitoring of transitions m/z 137 → 79 with optimized collision energy of 25, and 178 → 91 with optimized collision energy of 14, respectively.

Chromatographic Conditions

Separation was achieved on a Phenomenex CN (cyano) stationay phase, 150 mm × 4.6 i.d., 5 μM column. The mobile phase consisted of acetonitrile:water 20:80 working in isocratic mode, at a flow rate of 250 μL min^{-1}. The column was maintained at 30°C. Samples in the auto sampler were kept at 10°C. Sample injection volume was 20 μL and chromatographic run time was 6 min.

Sample Collection for Method Development

Blood samples were collected from five healthy, four-week old chickens. Animals received an antiobiotic-free diet and water *ad libidum*. Serum was obtained after centrifugation at 3500 rpm for 5 min. Serum from all tubes was pooled, homogenized, and stored fractioned in 1 mL vials at −18°C until analysis.

Standard Solutions Preparation

Stock fosfomycin and fudosteine solutions were prepared by dissolving 10.0 mg of each drug in 25 mL purified water.

Quality Control Samples (QC)

Fosfomycin solutions used to fortify drug free serum before extraction were prepared daily by diluting adequate volumes of stock solution in water. 20 μL of each of these solutions were thoroughly mixed in 100 μL serum to obtain effective concentrations of 0.1; 5, 10, and 20 $\mu g\,mL^{-1}$. Fudosteine working solution (150 $\mu g\,mL^{-1}$) was prepared by diluting an adequate volume of stock solution in water.

Calibration Solutions

Drug free extracts were spiked with 20 μL fudosteine working solution, and 20 μL fosfomycin solutions at different levels just before injection into HPLC MS/MS system to obtain concentrations corresponding to 100% extraction over the range of 0.1 to 50 $\mu g\,mL^{-1}$.

Sample Extraction

20 μL of a 150 $\mu g\,mL^{-1}$ fudosteine solution were added to 100 μL serum (30 $\mu g\,mL^{-1}$). Protein precipitation was carried out by addition of 1 mL methanol and vortex mixing for 1 min. The precipitate was removed by centrifugation for 10 min at 3500 rpm.

The supernatant was evaporated to dryness at 50°C under air flow.

The dry extract was reconstituted in 200 μL water. Other impurities were removed by liquid-liquid partition by addition of 1 mL hexane:ethanol 83:17. The organic (superior) layer was discarded. Total volume of remaining aqueous layer was thoroughly measured with a precision Hamilton syringe (this volume was 400 μL ± 1.8 μL).

20 μL were taken from the aqueous layer and further diluted to 400 μL with purified water. After micro filtration, 20 μL of the extracts were injected into HPLC-MS/MS system.

Method Validation

Quantification was carried out using the ratio between fosfomycin and its IS fudosteine as the assay response.

Validation parameters, as well as their acceptance range, were in accordance with international guidelines.[21]

Calibration curves were prepared in triplicates, and assayed within one week, in order to assess linearity. 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 where it could reach up to 20%). Within day precision (repeatability) was calculated in terms of mean coefficient of variation (CV) that was required to be less than 15% for all concentrations (except for the limit of detection where it could reach up to 20%). Between day's precision (intermediate precision) was expressed as between day's coefficient of variation, which was calculated using the following equation

$$CV_{bd} = \frac{SD_{bd}}{\mu}$$

Being:

μ: average media
SD_{bd} = between day standard deviation (calculated as the square root of between days variance)

Between day's variance was obtained after substracting the contribution of within day variability, using the following equation

$$SD_{bd}^2 = SD^2(\mu) + \frac{n-1}{n} SD_{wd}^2$$

Being:

$SD^2(\mu)$: variance of every day mean
n: number of observations per day
SD_{wd}^2: average within day variance

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 serum at the lower level of the calibration curve, in 5 replicates on three different days.

Fosfomycin recovery was calculated by comparing the STD/ISTD mean peak area ratio of QC samples with the values obtained for post-extraction spiked samples which represented 100% recovery.

Selectivity was studied by the analysis of serum from six healthy chickens to which no antimicrobials had been administered during all their lives, each coming from different poultry farms.

Matrix Effects Evaluation

Two types of studies were conducted in order to evaluate matrix effects as described by Matuszwski, 1998.[22] On one hand, peak area ratios

obtained with fosfomycin aqueous solutions, at three concentration levels within the linear range, were compared to those obtained with extracted blank serum, spiked at the same concentration just before injection.

On the other hand, a blank serum 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 decrease of the signal at certain regions of the chromatogram (Figure 1).

Drug Stability

Stock solutions kept at 4°C were tested regularly in order to assure a constant concentration throughout the study. To evaluate bench top stability during in-day manipulation, fosfomycin standard solutions (within the range of calibration curve) were kept at room light and temperature for 6 hr. Aliquots were taken every hour and injected into the HPLC-MS/MS system. Mean peak area ratios were compared with those obtained for a freshly prepared solution.

Stability of fosfomycin in serum extracts was also evaluated. Samples obtained from chicken treated with fosfomycin were extracted and quantified. These samples were left inside the autosampler (at 10°C) and requantified every 5 days for a period of 2 weeks.

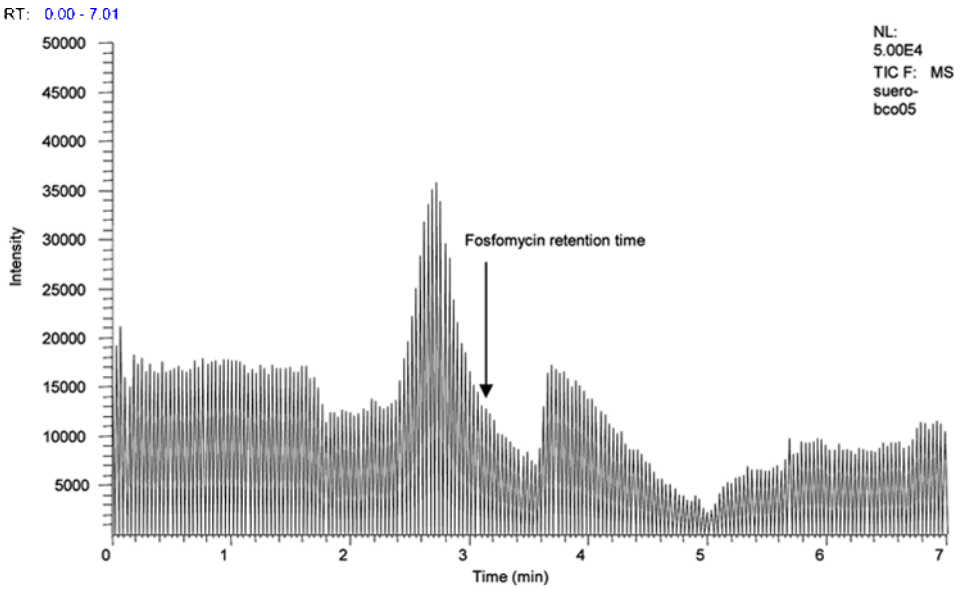

FIGURE 1 Evaluation of matrix effect by injection of blank serum extract into HPLC-MS/MS system, while directly infusing a fosfomycin solution. (Figure available in color online.)

Animals and Treatments

36 broiler chickens (21 days old) were maintained under controlled temperature (25°C), light cycle (12/12 hr) and humidity (45–60%) conditions. Food and water were supplied *ad libidum*.

Fosfomycin was administered in food at a dose of 40 mg Kg^{-1} body weight. Sampling was carried out at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 8, and 12 hr after oral administration of the antibiotic. At each sampling time three chickens were sacrificed and blood samples were collected.

After centrifugation, serum was recovered and kept at −22°C until analyzed. Mean (±SD) serum concentration-time curves were constructed.

RESULTS AND DISCUSSION

Optimization of Mass Spectrometer Conditions

For both STD and ISTD, high spectrometric response was observed in ESI negative ion mode. The predominant ions obtained were deprotonated fosfomycin and fudosteine, which m/z values were 137 and 178, respectively, in the Q1 (or Q3) full scan spectra.

By direct infusion from the syringe pump of independent solutions of each STD and ISTD, it was possible to observe changes in response as changes in MS parameters were performed. The most suitable conditions of the ion source that yielded the greatest signal from parent ions were found to be the following:

- Spray voltage: −3800 eV
- Spray temperature: 350°C
- Source CID energy: −8 eV
- Sheath gas pressure: 1.5

Collision pressure and collision energy in Q2 were evaluated using the same technique. For fosfomycin, a predominant fragment ion of m/z 79 was formed when collision pressure was 1.6 mTorr and collision energy −25 eV. For fudosteine, a predominant fragment ion of m/z 91 at collision pressure of 1.6 mTorr, when collision energy of −14 eV was applied. In both cases, the intensity of parent ions showed more than 80% reduction.

Optimization of Chromatographic System

Different chromatographic columns were evaluated. The use of cyano (CN) stationary phase greatly improved retention when compared to

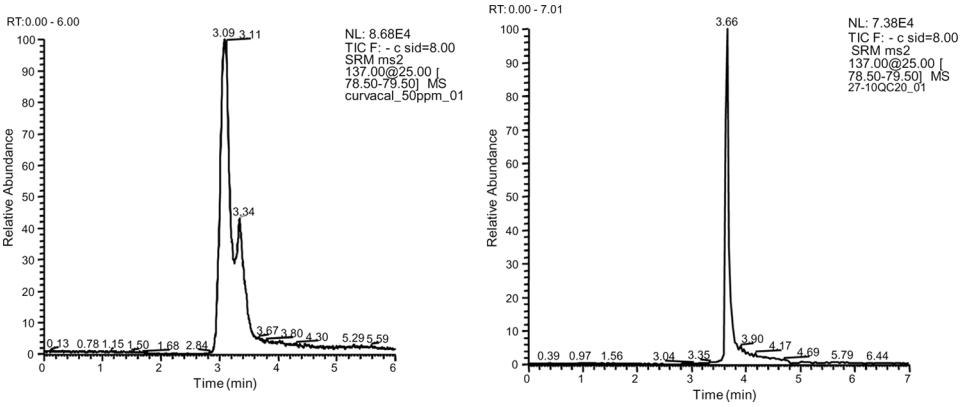

FIGURE 2 (A) Fortified chicken serum extracted and analyzed using a CN, 75 mm column; (B) Fortified chicken serum extracted and analyzed using a CN 150 mm column.

C18, which agrees with Li Li's results.[16] To evaluate the effect of column length, 75 and 150 mm long columns (CN stationary phase) were compared. Baseline resolution was achieved using the longer column, since a compound eluting very near the end of fosfomycin peak in the 75 mm one made integration less accurate (Figure 2).

In addition, a number of mobile phases were tested using water and different proportions of methanol and acetonitrile as eluting solvent. The highest signal to noise ratio and minimum interferences were obtained with water:acetonitrile 80:20. Good performance was not achieved with 90:10 water:methanol mobile phase as used by Li Li.[16]

Optimization of Extraction and Clean up Procedure

Sample clean up by protein precipitation followed by dilution is described by Li Li for fosfomycin determination in human plasma by HPLC-MS/MS.[16] In the present study, fosfomycin could not be determined in extracts prepared from chicken serum following this technique, even spiking blank extracts before injection.

Although protein precipitation is a common preparation method for polar compounds, in chicken serum it obviously leads to ion suppression mechanisms between analyte of interest and other co-eluting compounds. Thus, it was imperative to further work on the extraction procedure.

As endogenous phospholipids have been identified as a major source of matrix effects by many researchers,[17–20] a strategy to eliminate these compounds from serum samples was followed. After protein precipitation with methanol, different methods for phospholipids clean up were tested using normal phase solid phase extraction (NP-SPE) with silica columns, reverse

phase solid phase extraction (RP-SPE) with C18 columns, and liquid-liquid extraction with different solvents such as hexane, chloroform, chloroform: methanol 3:1, methylene chloride, and methylene chloride:methanol 3:1 (data not shown). Best results were obtained by a double liquid-liquid extraction using hexane:ethanol 83:16 as cleaning solvent, as stated in the Sample Extraction section. A further step of RP-SPE to the aqueous layer was tested in order to obtain a cleaner extract, but as no improvement was observed, this step was omitted (data not shown).

Validation Parameters

Selectivity

No signal above base line at fosfomycin retention time was observed in serum from chickens to which no antimicrobials had been administered. Figure 3 shows a comparison of typical chromatograms obtained when fosfomycin is (A) and is not present (B) in chicken serum.

Linearity

A typical linear regression curve was constructed preparing calibration solutions over the range of 0.025 to 0.625 $\mu g\,mL^{-1}$ (representing 0.1 to 50 $\mu g\,mL^{-1}$ of chicken serum). Good linearity was obtained within the concentration range, being r^2 coefficient above 0.995 for all replicates.

Accuracy and Precision

Accuracy and precision were evaluated for spiked samples at 5, 10, and 20 $\mu g\,mL^{-1}$. Accuracy, expressed as relative error was −7.8%, 1.0%,

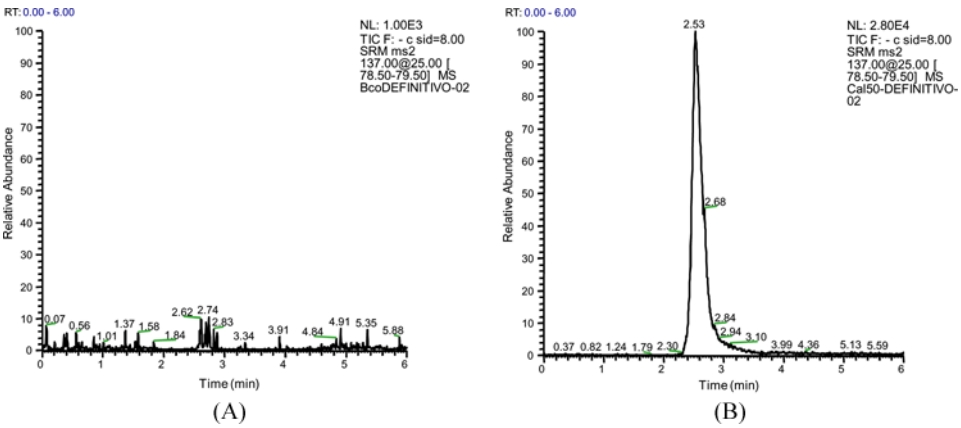

FIGURE 3 Chicken serum after extraction and clean up procedure: (A) blank sample; (B) sample taken from a chicken which has received fosfomycin treatment. (Figure available in color online.)

TABLE 1 Accuracy Expressed as Relative Error by Analyzing Spiked Serum Samples in Four Different Days

Spiked Concentration [$\mu g\,mL^{-1}$]	Measured Concentration [$\mu g\,mL^{-1}$]					
	Day 1	Day 2	Day 3	Day 4	Mean	RE (%)
5	0.062	0.079	0.066	0.063	0.067	−7.8%
10	0.122	0.129	0.119	0.125	0.124	1.0%
20	0.247	0.260	0.243	0.257	0.252	−0.7%

and −0.7%, respectively (Table 1). Repeatability (within day precision) and intermediate precision (between days precision) were less than 10% for all concentrations studied. Table 2 summarizes data analyzed for serum spiked at $10\,\mu g\,mL^{-1}$ as an example.

Lower limit of quantification was $0.1\,\mu g\,mL^{-1}$, which improves $0.5\,\mu g\,mL^{-1}$ obtained by Aramayona[2] using a microbiological method for the determination of fosfomycin from chicken serum. This is an important benefit when working at low doses of antibiotic for the treatment of microorganisms with low minimal inhibitory concentration values (MIC), as stated by Sumano for S*treptococcus* sp. for which the MIC_{90} is $0.25\,\mu g\,mL^{-1}$.[23]

Extraction Recovery

Drug recovery was tested for chicken serum samples spiked at 5, 10, and $20\,\mu g\,mL^{-1}$. Mean extraction recoveries were between 95 and 108%. Mean recovery of IS was 91%. High recovery extraction agrees with the fact that plasma protein binding of fosfomycin is negligible,[14] hence, such values are expected.

Matrix Effects Evaluation

Table 3 shows the decrease in peak areas due to the presence of matrix.

TABLE 2 Estimation of Repeatability (Within Day Precision) and Intermediate Precision (Between Day Precision) for Blank Serum Samples Spiked at 10 μg/mL (Obtaining a Final Concentration of 0.125 μg/mL to be Injected into HPLC System)

	Day 1	Day 2	Day 3	Day 4	MEAN (μ)	SD^2 (μ)
Mean	0.122	0.129	0.118	0.125	0.123	2.17×10^{-6}
SD	0.001	0.006	0.001	0.003		
CV%	0.82	4.6	0.84	2.4		

Within day precision: 2.2%.
Between day precision: 4.2%.

TABLE 3 Area Ratio Obtained by Fosfomycin Aqueous Solution Compared with that Obtained by Spiking Blank Extract after Extraction (Just Before Injection into HPLC System)

	Area Ratio		
Concentration [μg/mL]	Fosfomycin Aqueous Solution	Spiked Blank Extract	D (%)
0.063	0.103	0.056	45.631
0.125	0.200	0.112	44.000
0.250	0.432	0.213	50.694
0.375	0.691	0.338	51.085
0.500	0.942	0.448	52.442
0.625	1.208	0.594	50.828

%D: percentage difference between the two determinations.

Blank serum extracts spiked at different fosfomycin concentrations yielded between 44 and 52.4% less area ratios than those obtained with STD aqueous solution of the same concentration. This effect is explained by the fact that when fosfomycin solution is directly infused into the ion source constantly while a blank serum extract is simultaneously injected through the chromatographic column, some degree of signal drop off is observed around fosfomycin retention time (Figure 1). Ion suppression/enhancement is observed in different regions of the chromatogram (Figure 1), even though matrix has been cleaned up from most probable interfering molecules. These unwanted effects are caused by substances that still remain after extraction procedure. In addition, the eluent itself may have a detrimental effect in the ionization process.[24,25]

Drug Stability

No significant differences in concentrations ($\alpha < 0.05$) were observed neither between stock fosfomycin solution kept at 4°C for 4 months, nor for a 10 μg mL^{-1} fosfomycin solution left on bench top for 6 hr, compared to freshly prepared ones. Evaluation of drug stability in serum samples, showed no significant differences ($\alpha < 0.05$) between freshly prepared samples and those kept in the autosampler for 2 weeks. The stability of fosfomycin in analytical conditions and in the biological matrix allowed us to simplify analytical procedures. These findings agree with Li Li's[16] results for human plasma.

Mean Serum Concentration Profiles of Fosfomycin

Figure 4 shows the mean (±SD) serum concentration profiles after oral administration of calcium fosfomycin in broiler chickens. Maximum serum concentrations were 24.00 ± 1.21 μg mL^{-1}, which were observed at 3.0 hr.

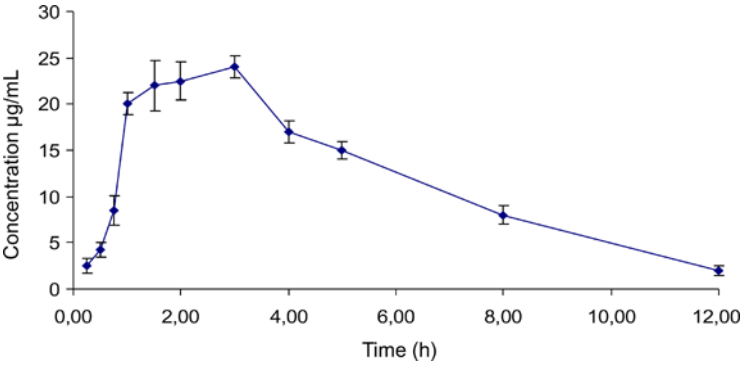

FIGURE 4 Mean (±SD) serum concentration profiles after oral administration of 40 µg/Kg calcium fosfomycin in broiler chickens. (For each sampling point N = 3 animals). (Figure available in color online.)

CONCLUSIONS

A highly selective HPLC-MS/MS method for the determination of antibiotic fosfomycin in chicken serum was developed and validated. This method proved to be in conformity with international accepted validation parameters. The clean up technique is rapid and simple. Derivatization is not required, which lowers the costs and analytical time compared to most methods found in the literature to determine fosfomycin in different biological matrices.[10–13]

This method has been successfully applied to conduct a pharmacokinetic study in broiler chicken serum after oral administration of calcium fosfomycin.

It would be interesting to further evaluate its implementation in tissues and serum from different species.

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