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New HPLC Method for Determination of Cefquinome in Cattle Plasma and its Application for Pharmacokinetic Study

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IA, HH and LH designed the study. PS, IA and HH performed analysis and interpretation of the work. IA, ZI, FA, MI and ZS drafted the manuscript and revised it. IA, HH and SA performed experimental procedure. IA wrote the manuscript.

Key words
Cefquinome, HPLC-UV, Plasma, Cattle, Pharmacokinetics

ABSTRACT

The cefquinome samples from plasma were extracted and separated by HPLC on a reversed phase C-18 column with mobile phase of acetonitrile and formic acid (0.1%) solution- (90:10, v/v). The calibration curves were linear ranged from 0.01-5µg/mL. The limit of quantification (LOQ) and limit of detection (LOD) were 0.04 and 0.01 µg/mL. The recovery of cefquinome from plasma was 73.4%, 78.33%, and 77% for low, medium and high concentration samples. The intraday and inter day relative standard deviation were less than 15%. The Cefquinome were found to be stable for more than 3 month at -70°C (degree symbol) and more than 24 h at 4°C. The present method has been applied successfully for the pharmacokinetics study in cattle. After intramuscular administration, the mean peak serum Concentrations (C_{max}) was found 2.95µg/mL and achieved after (T_{max}) 1.50h. The elimination half-life ($t_{1/2(el)}$) was 2.03±0.06h. The Mean Residence Time (MRT) was 2.58±0.05h and the area under curve from zero time to infinity ($AUC_{0-\infty}$) were 9.67±0.72µg/mL/h. The newly proposed method was shown to be simple, rapid, economical and sensitive compared to the previously reported method, and thereafter applicable for the pharmacokinetics study in animals.

INTRODUCTION

Cephalosporins, a group of ß-lactam antibiotics, used for the treatment of several types of infections since 1954 (Zaffiri *et al*., 2012). They are considered as low toxic, broad spectrum antibiotics and inhibit synthesis of bacterial cell wall. Cefquinome sulphate ($C_{23}H_{24}N_6O_5S_2$) an aminothiazolyl cephalosporin, a member of the family cephalosporins fourth generation is recommended especially for use in animals (Aarestrup and Skov, 2010; Agnieszka *et al*., 2014; Léon *et al*., 2020; Xiao *et al*., 2015). World widely cefquinome is permitted for use in numerous animal species (Smiet *et al*., 2012). This drug has ß-lactamases stability, produced by some clinically important bacteria (Dumka *et al*., 2013). Cefquinome is also used for the respiratory tract diseases treatment, calf septicemia, acute mastitis and foot rot in cattle. Its pharmacokinetic study has been examined in horse, mice,

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ducks, rabbits and cattle (Ehinger *et al*., 2006; Hwang *et al*., 2011; Shan *et al*., 2013; Winther *et al*., 2010; Yuan *et al*., 2011). It also has zwitterionic property so it has high bioavailability and can easily penetrate into the biological membrane (Shymaa *et al*., 2019). This drug is an organic acid having no lipid solubility and low oral bioavailability; after intramuscular administration it has rapid absorption and short elimination half-life in cattle, pig, horse and dogs. Cefquinome effects kidney and liver due to less binding with plasma protein (Zhao *et al*., 2013).

The cefquinome is time dependent drug, the PK/PD indices responsible for the efficacy of this drug is the times the plasma drug concentration exceeds the MIC and mostly used in severe animal diseases (Maleck *et al*., 2014; Wang *et al*., 2014). Therefore, it is important to conduct the pharmacokinetic (Pk) study for the efficacy of cefquinome. It is essential to establish the reliable method for the determination of this drug in biofluid due to widely use in veterinary medicine. There are numerous methods established for the observation of cefquinome in animal tissue, milk and plasma. In several method HPLC-MS were used for the examination of cefquinome in tissue and serum (Zhang *et al*., 2014). The HPLC-UV method is also used by some of the investigators (Dumka *et al*., 2013) and (Shan *et al*., 2013). It is an economic and easily accessible for the examination of drug in plasma. Some researchers developed HPLC-UV method for the observation of cefquinome in plasma, as they used complex gradient mode and or used hazardous mobile phase like TFA, which is considered to be no longer safe for use in column. According to our knowledge, there are no published methods available on establishment of HPLC method for the cefquinome determination in cattle plasma.

A new modified HPLC based method is developed in a compliance to the FDA guidelines for industrial application (Food and Drug administration, 2001). In this method simple isocratic mode, small sample volume, using low-cost chemical, less solvent absorption, solvents suitable for column were used. The new developed method for quantification of plasma cefquinome is sensitive, rapid, and highly applicable for pharmacokinetic study in cattle.

MATERIALS AND METHODS

HPLC analysis

Chemical and reagents

The reference standard of cefquinome (95% purity) was kindly supplied by Dr. Ehenstorfer (Augsburg, Germany). Commercial injectable cefquinome sulphate used for pharmacokinetics study was taken from local distributor (Shanghai Tongren Pharmaceutical Company Ltd.). Acetonitrile (ACN), formic acid and methanol (MeOH) were purchased from TEDIA (USA). Solid phase extraction (SPE) cartridges (Waters Oasis^{TM} HLB, waters) were used in sample preparation. Thoughout the study de-ionized water (Milli-Q Millipore Corp.) was used.

Apparatus and chomatographic condition

HPLC system contains Waters separation module (2695) with UV detector (2487) (USA). The column used for reverse phase chomatography was analytical ZORBAX SB-C_{18} (250mm×4.6mm i.d., 5µm) (Agilent Technology, USA). High speed centrifuge (HIMAC CR 21 G III, Japan) and vortex shaker (XW-80A, China) were used though the sample preparation. The optimized methods used were of isocratic mode with mobile phase A (0.1% formic acid) and mobile phase B ACN. Prior to use the mobile phase solvents were ultra sonicated and degassed. The column temperature was set at 30±5°C. The UV detector was set at a wavelength of 268 nm. The injection volume and flow rate were 50 µl and 0.9 mL/min.

Standard stock solution

A stock solution of 1000µg.mL^{-1} was prepared and stored in dark at -70°C, protected from light by using aluminum foil. Further dilutions were prepared by using 15 % ACN to prepare for working standard solutions.

Sample preparation procedure

Blood samples were collected from different cattles. Aliquots of cattle plasma (250 µl)were collected in 1.5mL tube, methanol (500 µl) was added before shaking for 20 sec, and then centrifuged at 8000 rpm/10min at 4 °C. The supernatant was collected, added into 10mL water and then cleaned up by HLB SPE cartridges (3 mL, Waters Corp., Milford, MA, USA), which were preconditioned with 3 mL water and 3 mL methanol. The cartridge was washed with 3 mL water and 3mL methanol (10%) after filtering the sample and theanalytes were eluted with 3 mL acetonitrile. The collected analytes were dried with a stream of nitrogen at 50 °C. The dry residues were then dissolved with 500 µl (15% ACN) and vortexed for 20 seconds. The resulting solution was then filtered through 0.22 μm nylon Millipore chomatographic filter, an aliquot of 50 μL was analyzed by HPLC (Ahmad *et al*., 2015).

Method validation

Selectivity

The selectivity was evaluated by analyzing blank cattle plasma to evaluate possible endogenous interference in samples. Plasma spiked with cefquinome and chomatographic conditions were optimized to guarantee that no interference incurred at the retention time of the tested compound.

Limit of quantification and detection

The limit of detection **(**LOD) and quantification (LOQ) were determined by signal to noise ratio, evaluation of sample spiked from 0.01 to 0.1µg/mL of the drug concentration. The LOD parameter can be detected from background noise but not quantities, which provides the lowest concentration in a sample. The least signal to noise ratio of LOD is 3. LOQ is defined as the lowest concentration of analytic with a signal to noise ration of at least 10 with adequate precision and accuracy.

Calibration curve

The calibration curve linearity was determined by using different concentration, both in standard solution and plasma. Calibration curve were run before and after the sample. Blank sample were included with each set. Calibration curve were checked on different days, to assess the linearity of each calibration curve. For every standard curve c the intercept, slope, and correlation coefficient were recorded. Unknown concentrations were calculated using the equation drawn from calibration curve.

Precision and accuracy

The cefquinome at high, medium and low concentration were used. Spiked quality control sample were prepared at the concentrations of LOQ (0.04µg/mL), 2 LOQ (0.08µg/mL) and 4 LOQ (0.16µg/mL). Thee time for five days we run that concentration. For the intraday we ran three sets of each concentration. Six replicates of the three concentrations were analyzed in five different days for inter-day experiment. The precision was defined by relative standard deviation (RSD). To explain the term accuracy, the mean complete recovery was analyzed by comparing the peak area of extracted sample with those of the standard working solution. For examining the absolute recoveries of cefquinome, spiked QC samples were prepared at LOQ, 2LOQ and 4LOQ. The recoveries were analyzed by comparing the observed with that standard area.

Stability

Stability of stock solution was determined using two different conditions at 4°C for 24 h and at -70°C for three month. The stability was examined by comparison between the peak areas of the standard stock solution with that of freshly prepared drug solution. For each storage condition, four replicated were analyzed. The LOQ and 4 LOQ were used for stability of standard solution. For incurred sample we used the plasma obtained 2 month before from the animals and then spiked by using the 2 concentration. For the drug administered to the animals we used the sample at 1 and 1.5 h for stability.

Pharmacokinetic application

The study was performed in accordance with the procedures of the Committee for Care and Use of Laboratory Animals of China. The study was approved by the Animal Care Center, Hubei Science and Technology Agency in China (SYXK 2013-0044). All efforts were made for minimization of animals suffering. The study was conducted in 4 healthy female cattle, Body weights were 195±10 kg, they were housed in 8×10 m cattle pen, and pen was cleaned daily. The animal house was maintained at a room temperature of 25±2°C and a relative humidity of 45%~65%. All animals were allowed a seven-day acclimation period before the studies. The water and feed for animal were available *ad libitum*. In animals cefquinome is commonly used intramuscularly. In our study we administered cefquinome at a dose rate of 1.25mg/kg b.w. by intramuscular route. The blood sample (1mL) were collected before and then after 5, 10, 15, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 6, and 8th h following cefquinome injection. Blood samples collected in EDTA added tubes and, plasma were separated immediately by centrifugation @3000 rpm/20min at 4°C and then saved at -20°C. The plasma concentration versus time data of cefquinome for each cattle were used to examine the PK data. The obtained plasma was used for HPLC and analyzed by Winonlin software (Pharsight Corporation, CA, USA) to get the suitable pharmacokinetic parameters for each animal. Drug concentration versus time were plotted on a log linear graph to find the best fit model. Pharmacokinetics parameters included maximum concentration (C_{max}), Time to reach C_{max} (T_{max}), half-life ($T_{1/2}$) of drug, area under the plasma drug concentration time curve from zero to infinity ($AUC_{0-\infty}$), mean residence time (MRT), clearance of drug and Area under first moment curve (AUMC) were measured. All the data were expressed as the mean±SD. A non-compartmental method was used for the analysis.

RESULTS

Selectivity and calibration curves

For selectivity we examined the plasma from six different cattle corresponding with spiked plasma. Figure 1 shows the blank standard solution, 1µg/mL of cefquinome in standard solution, blank plasma, and plasma spiked with 1µg/mL of drug. No interference was found at the retention time of cefquinome. For the linearity we used eight concentrations ranged from 0.01-5µg/mL for both standard and drug added plasma. The correlation coefficients were 1 and 0.9998 for standard and plasma over the concentration range used as shown in Figure 2.

Table I. Recovery, interday and intraday RSD for three concentrations in plasma.

Sample/Day	Concentration (0.04 µg/mL)		Concentration (0.08 µg/mL)		Concentration (0.16 µg/mL)	
	Rec.(%)	Intraday (%)	Rec.(%)	Intraday (%)	Rec.(%)	Intraday (%)
1	82±3	3.65	88±4.58	5.20	87.33±6.42	7.36
2	84.33±1.52	1.81	87.33±5.85	6.70	85±8.18	9.62
3	67±2.64	3.94	68.66±4.16	6.06	64.66±6.42	9.94
4	63.33±9.86	1.55	72±1	1.38	74±3.46	4.68
5	71.66±5.13	7.16	75.66±0.57	0.7	74±2	2.70
Interday (%)	12.48		11.33		11.99	

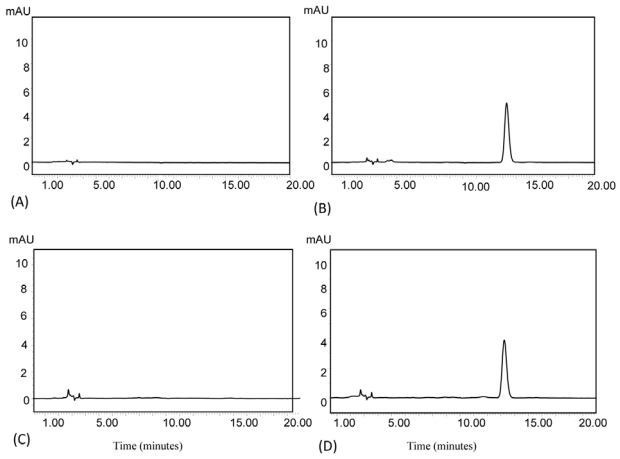

Fig. 1. Chromatograms of blank standard (A), 1µg/ml of cefquinome standard solution (B), blank plasma (C) and 1µg/ml of cefquinome in plasma (D).

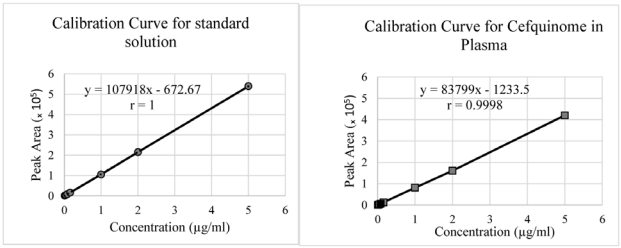

Fig. 2. Calibration curve for cefquinome is standard (A) and for plasma (B).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were determined on the basis of signal to noise ratio 3:1 and 10:1. The LOD and LOQ were 0.01 and 0.04µg/mL.

Recovery, precision and accuracy

The recovery of cefquinome in plasma are shown in Table I. Recovery of cefquinome from plasma was more than 73% for low, medium and high concentration. Extraction recovery and repeatability (intraday and interday) were used to evaluate the precision and accuracy respectively. The intraday RSD% was less than 10, while in interday RSD% was less than 15% as shown in Table I.

Stability

The stability of the cefquinome was checked with two different temperature 4C and -70°C for 24h and 3 month. No significant changes were observed as shown in Table II.

Table II. Stability of cefquinome stored at -70 °C, 4 °C for 24 h and 3 months duration.

Samples	Concentration at 0 time	Concentration after	
		24 h (4°C)	3 months (-70°C)
Standard sample	0.04	0.039	0.038
	0.16	0.16	0.16
Drug added in plasma	0.035	0.035	0.032
	0.13	0.13	0.12
Blood sample post drug administration			
1 h	2.50	2.51	2.50
1.5 h	2.87	2.86	2.84

Pharmacokinetics

Following a single intra-muscular administration of cefquinome, mean plasma concentration of cefquinome and time curve were plotted as shown in Figure 4. Figure 3 indicates chomatogram after 1.5h of cefquinome administration. Pharmacokinetic parameters of cefquinome were determined by non-compartmental analysis. There was gradual decrease in plasma drug concentration after 1.5 h of post administration. The C_{max} of 2.95±0.13µg/mL was achieved at 1.5 h. After drug administration no adverse reactions were observed. The absorption of drug was rapid even after 5 min post drug administration we obtained in good concentration. The elimination half-life was 2.03h, showing rapid elimination after intramuscular

administration. The area under the concentration time curve AUC0-Last, AUC0-∞ was 9.67±0.72. The mean residence time was 2.58±0.05h. In Table III also shows the other pharmacokinetic parameters.

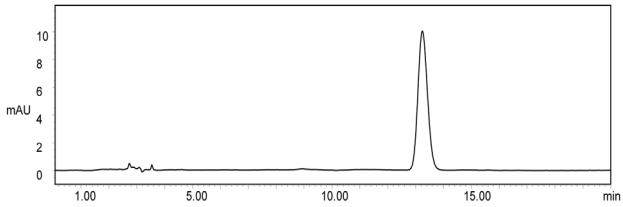

Fig. 3. Chromatogram of cefquinome in cattle plasma after 1.5 h of intramuscular drug administration of 1.25mg/kg.

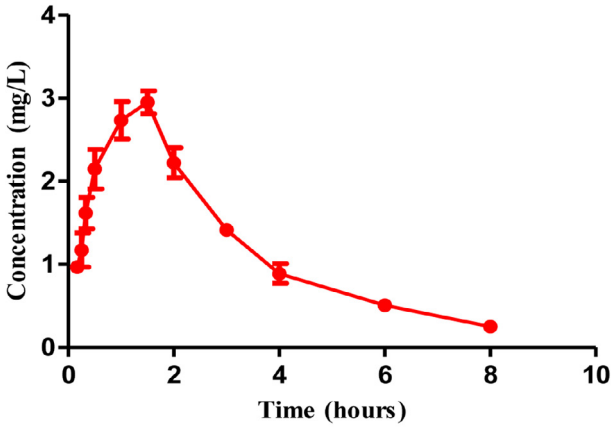

Fig. 4. Plasma concentration of cefquinome (Mean±SD) versus time curve after I/M administration (1.25mg/kg) in cattle (n=4).

Table III. Pharmacokinetics parameters after I/M administration of cefquinome in 4 cattle.

Parameters	Unit	Values (Mean±SD)
C_{max}	µg/ml	2.95±0.130
T_{max}	h	1.50±0.000
$T_{1/2}$	h	2.03±0.060
$AUC_{0\text{-}inf}$	µg.h/ml	9.67±0.720
$AUC_{0\text{-}last}$	µg.h/ml	9.67±0.720
MRT	h	2.58±0.050
Cl/F	l/kg/h	0.11±0.008
$AUMC_{last}$	µg.h^2/ml	25.05

C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; $T_{1/2}$, elimination half-life; $AUC_{0\text{-}inf}$, area under the plasma concentration-time curve from 0 to infinity; $AUC_{0\text{-}last}$, area under the plasma concentration-time curve from 0 to last time; MRT, minimum residence time; Cl/F, Clearance of drug; $AUMC_{last}$, Area under first moment curve.

DISCUSSION

Chomatographic conditions and sample preparation

Sample preparation and chomatographic conditions for the planned method was improved for the preclinical pharmacokinetics studies. Before start the proposed method, chomatogram of cefquinome standard and quality control sample were analyzed. Sample preparation is the most important factor for cefquinome separation from endogenous substances. Some initial test was performed for separating cefquinome from plasma protein. And then we selected the above described method for sample preparation. The wavelength and mobile phase composition is also important factor for analysis. Different wavelength were set and checked the sensitivity of the drug, result showed that good sensitivity were observed at 268 nm. At the start some test were performed by using gradient mode by making some changes in the previous established method (Wang *et al*., 2014). In these conditions the cefquinome was not separated properly from endogenous substances. Efficient separation of cefquinome was obtained by using the isocratic mode (A water 90% + 0.1% formic acid, B ACN 10%). The total run time for each of the samples was 20 min, at the retention time of cefquinome at 13 min no significant interfering peaks were observed.

Protein precipitation is important step to get desired result. In the previous studies, some researchers have used simple method for extraction of cefquinome from plasma of different animals (Uney *et al*., 2011; Dinakaran *et al*., 2013). In the preliminary studies some trials were conducted with different organic solvent methanol, ACN and ammonium acetate buffer but the methanol has given good result. I have used direct extraction method but the result quality was not good. In the present study, we were able to get good recovery without the use of SPE for extraction of cefquinome from plasma.

The outcome of eluent and its volume effectiveness on extraction was investigated. The methanol and ACN were tested in this experiment for eluting of cefquinome. Comparatively ACN showed higher efficiency as an eluting agent than methanol. To make sure that the target analytes were completely eluted from the SPE, optimum volume of eluent was examined though altering the volume of acetonitrile from 2 to 3 mL. From the results it was observed that the cefquinome recovery increased while increasing the volumes of ACN from 2 to 3mL. Therefore, 3 mL ACN was chosen as the optimum eluent.

Sample volume affects the effectiveness of the extraction process. In these experiments, blood samples were obtained from the jugular vein, 1mL each time, before and after dosing. Limitation by the practical condition, 250 µL of plasma was considered reasonable for sample

preparation. The loaded sample volume was comparatively small in this investigation; sample flow rate (0.9 mL/min) was suitable to improve the analytes recoveries.

Connection with other preparation techniques

In this study, bio-samples of cefquinome in the plasma were frequently pre-treated using SPE process. In the reviewed literature, cefquinome was extracted from 500µL plasma (Uney *et al*., 2011). In our developed method the SPE columns were used for extraction of cefquinome from plasma, the volume used was 250µL. some researcher used SPE for the pretreatment of rabbit plasma sample (Zhao *et al*., 2013). In our method, 250 µL cattle plasma samples were prepared with 500 µL methanol used as a protein precipitation agent, the supernatant was diluted in (10mL) water. The mixture was then transferred slowly into SPE cartridges (Waters Oasis™ HLB, waters) conditioned already in advance. After washing, drug adsorbed on SPE column, then eluted with 3mL ACN, and the elute was allowed to evaporate and dry in nitrogen stream at 50°C. The residue was suspended in 500µL mobile phase, and then 50µL of the supernatant was used for chomatographic method. The injection volume was varied between 10 and 50µL and was finally set at 50µL in order to increase the sensitivity of the method.

In this study, SPE method was developed for the sample preparation from plasma of cattle. As the results shown in Table I, the current method showed suitable analytical performance with excellent specificity, low level of LODs, high recoveries and good accuracy and precision. Furthermore, target analytes in 250µL plasma obtained from cattle could be entirely extracted. The sample volume was less and pretreatment was relatively time-saving and easy. In relation with the reviewed literature, the technique for preparation of sample was proposed in this study has its own obvious advantages.

Method performance characteristics

Several important parameters evaluated were linearity, specificity, repeatability, recovery, LOD and LOQ. The specificity was confirmed by the analysis of six different blank cattle plasma with subsequent spiked plasma. Figure 1 shows blank plasma and plasma spiked with drug concentration. The method was free of interference in the existence of recipient, no significant changes observed at the retention time of analytes, which showed that the procedure is specific for cefquinome.

The LOD as the lowest concentration that can be reliable detected, while the LOQ is the lowest drug concentration that can be measured with a desired level of precision and accuracy. For determination of LOD and LOQ, we used four different concentrations from 0.01-0.04. On the basis of signal to noise ratio of 3:1 and 10:1 the LOD and LOQ for cefquinome in cattle plasma were 0.01 and 0.04.

The proposed method linearity was observed for both standard and drug in plasma in the concentration range 0.01-5 µg/mL showing its suitability. For obtaining the calibration data, the experiment at all concentration was repeated on six different days. The result showed that the correlation coefficient value (r values) were linear in the particular range. These results clearly showed that the detection circumstances were practical and balanced.

The accuracy and precision were the important criterion for evaluating the performance of a diagnostic method. For the proposed method the accuracy is predicted as the percentage difference among the mean values obtained by the method and the true or known concentrations. The precision defines the closeness of analytes measures when the method is applied frequently to numerous aliquot of a single homogenous amount of biological medium. The results of recovery and reproducibility of the method over the concentration range on 5 separate days were calculated. The intraday RSD% was less than 10, while in interdays RSD% was less than 15%. These results indicate that the method is consistent and reproducible. The recovery was determined by comparing the peak areas of prepared sample with those of the standard solutions. The recovery of cefquinome from plasma was 73.4%, 78.33, and 77% for low, medium and high concentration samples as shown in Table I. The result indicated the described method was efficient and consistent.

There were no significant changes observed in 24 h storage at 4 C and for 7 months at -70°C. The results indicated that cefquinome were stable for three month in incurred samples during storage at −70 °C and for 24 h at 4°C. This result suggests that cefquinome can be used for the above duration without any significant loss.

PK characteristics

Cefquinome is used for the treatment of severe infection disease where the susceptibility test indicates that other drugs are not useful (Corum *et al*., 2019). Pharmacokinetics study of this drug is important; therefore, the method described here was effectively applied for the cefquinome pharmacokinetics study in cattle, no adverse effect were observed in all the cattle. After i.m. administration of cefquinome the plasma drug concentration-time curve were best fit a non-compartmental model in all cattle. The result showed the drug absorption is good from the site of injection as compared to goat and camel (Al-Taher, 2010; Tohamy *et al*., 2006). This drug has good pharmacokinetic characteristics due to their rapid absorption after intramuscular administration. In the

present study the peak concentration C_{max} was achieved at 1.5h post drug administration which are longer than 0.28-0.83h in pig (Zhang *et al*., 2014), and shorter than pharmacokinetic studies in camels (Al-Taher, 2010), after intramuscular administration. The elimination half-life was 2.03 h presenting rapid elimination after i.m. administration which was shorter than 2.76h in pig, 4.36 h in piglet (Li *et al*., 2008) and (Yang *et al*., 2009) and 10.24 h in camels (Al-Taher, 2010). The clearance after intramuscular administration was (0.11±0.008l/kg/h), similar data found in horses (0.11±0.03l/kg/h) (Smiet *et al*., 2012). The area under concentration time curve $AUC_{0\text{-}Last}$, $AUC_{0\text{-}\infty}$ was 9.67±0.72 after intramuscular administration of cefquinome (1.25mg/kg), which are similar with the previous study in horses and cattle after intramuscular administration at the dose of 1mg/kg (Shan *et al*., 2013; Uney *et al*., 2017). The mean residence time was 2.58±0.05h. Pharmacokinetic data are shown in Table III.

The proposed HPLC-UV method was precise, perfect and specific for simultaneous determination of cefquinome in cattle plasma. In addition, this method has more advantages than previously reported methods, such as use of less plasma, less time consuming and simplicity. The main difference of this method compare with other methods is its ability to determine cefquinome simultaneously.

CONCLUSIONS

The described HPLC-UV method is effective, sensitive, fast and economical. Good linearity was demonstrated over the concentration range 0.01-10µg/mL. This study describes the HPLC method developed and validated to compute cefquinome in cattle plasma. The technique was validated in a broad concentration range (0.04-10 µg/mL, therefore it can be applied to routine therapeutic drug monitoring and also to other pharmacokinetics based studies planned to investigate, for example the bioequivalence and bioavailability of drug formulation of cefquinome.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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