Agricultural Journal 6 (6): 374-377, 2011
ISSN: 1816-9155


# Comparison of Microbiological Assay and High-Performance Liquid Chromatography/Mass Spectrometry for the Pharmacokinetics of Cefquinome in Pigs

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**Abstract:** A simple and sensitive Agar well diffusion bioassay with *Providencia alcalifaciens* ATCC 9886 and Liquid Chromatography/Mass Spectrometric (LC/MS) method for cefquinome in pig plasma was evaluated and validated. The proposed microbiological and LC/MS methods for the determination of cefquinome in plasma showed good inter-assay and intra-assay precision, accuracy and linearity. A microbiological assay and LC/MS assay for the determination of cefquinome yielded statistically identical results ($r^2$ = 0.83). The developed microbiological method in pig plasma has been successfully utilized for pharmacokinetic study of cefquinome in pigs. Following intramuscular injection of cefquinome at 1 mg $kg^{-1}$ in pigs, it was rapidly absorbed with mean $C_{max}$ of 0.40 μg $mL^{-1}$ at 2.33 h. Thereafter, the plasma concentration of cefquinome was declined with 2.36 h of elimination half-life in pigs.

**Key words:** Cefquinome, microbiological assay, *Providencia alcalifaciens*, pharmacokinetics, LC/MS, pigs

## INTRODUCTION

Cefquinome, an aminothiazolyl cephalosporin is a member of the 4th generation of cephalosporins which have been developed solely for veterinary use (Limbert *et al*., 1991; Murphy *et al*., 1994). It has a broad-spectrum and is susceptible to clinically important bacteria such as *Streptococcus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Moraxella* sp., *Haemophilus* sp., corynebacteriae, enterococci, *Escherichia coli* and gram-positive anaerobes tested *in vitro* (Guerin-Faublee *et al*., 2003; Limbert *et al*., 1991; Murphy *et al*., 1994; Shpigel *et al*., 1997). Moreover, it is highly stable to β-lactamases produced by most pathogenic bacteria. It is approved for the treatment of respiratory tract diseases and mastitis for livestock in worldwide. Several methods have been reported for the determination of cefquinome in biological fluids using microbiological assay, High-performance Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry (LC/MS) in the literature (Daeseleire *et al*., 2000; Maes *et al*., 2007; Sorensen and Snor, 2000; Uney *et al*., 2011). Despite the speed, precision and accuracy of LC techniques most of them suffer interference from the biomatrix and are not simple for routine analysis as they need sophisticated equipment and expensive reagents that are not yet available in many laboratories (Souza *et al*., 2006). The development of alternative analytical methodologies such as an Agar diffusion microbiological assay for antibiotics which is simple and operationally inexpensive represents a great advantage for laboratories that do not have specialized and sophisticated instruments (Lourenco *et al*., 2007; Perea *et al*., 2000; Souza *et al*., 2006). The aim of this study was to develop and validate a microbiological assay for the determination of cefquinome in swine plasma comparing with LC/MS. Moreover, these assay methods were successfully applied to pharmacokinetic studies of cefquinome after single intramuscular injection of 1 mg $kg^{-1}$ to pigs.

## MATERIALS AND METHODS

**Chemicals:** Cefquinome, ceftiofur (Internal Standard, IS) and other analytical grade chemicals was obtained from Sigma (Missouri, USA) and HPLC grade methanol and acetonitrile were purchased from Mallinckrodt Baker (New Jersey, USA). Cefquinome 2.5% injectable suspension (Cobactan® 2.5% suspension, Intervet/Schering-plough Animal Health, The Netherlands) was supplied by a local distributor and used in the pharmacokinetic study.

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**Animal experiment:** Six healthy male growing pigs (Landrace x Yorkshire x Duroc, 4 months old, 39.7±4.68 kg) were used in this study. A single dose of 1 mg $kg^{-1}$ of cefquinome solution (Cobactan® 2.5% suspension, Intervet/Schering-Plough Animal Health, the Netherlands) was intramuscularly injected to pigs. Blood samples were obtained from the jugular vein at different time points (0, 0.08, 0.16, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h) after the oral administration of cefquinome. The samples were centrifuged at 150 g for 10 min to obtain plasma and stored at -70°C until analysis. The pharmacokinetic parameters of cefquinome were calculated by non-compartmental analysis using BA-Calc 2007 (Korea food and drug administration, Seoul, Korea). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Chungnam National University.

**Sample preparation and chromatographic conditions for LC/MS determination:** To 100 μL of plasma samples were added to 10 μL of IS (1 μg $mL^{-1}$) and 200 $\mu L^{-1}$ acetonitrile. The mixture was vortexed for 10 min and then centrifugated at 1,200 g for 10 min. After centrifugation, the supernatant was transferred to another tube and then 600 $\mu L^{-1}$ of methylene chloride were added. After vortexing for 15 sec, the sample was centrifuged at 1,000 g for 10 min and 20 $\mu L^{-1}$ of the top layer was injected into LC/MS. Samples were analyzed on Agilent 1100 series LC/MS system. Separation was achieved on $C_{18}$ reverse phase column (Eclipse plus®, 3.5 μm, 4.6×150 mm, Agilent, USA) with a guard column filled with the same material. The mobile phase consisted of 0.01% trifluoroacetic acid in 10 mM ammonium acetate (A) and acetonitrile (B) using a gradient elution of 10-33% (v/v) B at 2-6 min, 33-100% B at 6-9 min and 100% B at 9-11 min. The flow rate was 0.6 mL $min^{-1}$. The Electrospray Ionization (ESI)-MS analysis was performed on an Agilent 5989 mass spectrometer with an ESI interface fitted with a hexapole ion guide. The optimal condition for the analysis of cefquinome and IS employed pneumatic nebulization with nitrogen (45 p.s.i.) and a counterflow of nitrogen (9 L $min^{-1}$) heated to 350°C for the nebulization and desolvation of the introduced liquid. Mass spectrometer was performed using the positive ion mode and the Selected Ion Monitoring (SIM), detecting at 529.2 m/z for cefquinome and 524.2 m/z for IS with a dwell time of 300 m sec.

**Microbiological assay:** Parallel to the LC/MS determinations, the concentrations of cefquinome in plasma were measured by an Agar well diffusion method using *Providencia alcalifaciens* ATCC 9886 as the test microorganism. Bacterial suspension was grown overnight in trypticase soy broth and adjusted to an optical density of 0.5 at 550 nm. The bacterial suspension of *Providencia alcalifaciens* ATCC 9886 was added to tempered trypticase soy agar at $10^6$ cfu $mL^{-1}$ agar and immediately poured as a layer of 2.2 mm to assay plates. The plates were allowed to solidify for 45 min and 0.5 cm of wells were punched to fill with 50 μL of plasma samples or cefquinome standards ranging in concentrations from 0.075-5.0 μg $mL^{-1}$. The Agar plates were incubated for 24 h at 30°C. Zones of bacterial inhibition were measured using a digital vernier caliper. Each sample or standard was assayed in triplicate and mean values for three measurements of the zone diameters were determined.

**Statistical analysis:** Pharmacokinetic parameters were presented as mean±Standard Deviation (SD). Statistically significant differences of pharmacokinetic parameters estimated on the basis of concentrations measured by the two analytical methods were determined with Student's t-test or Wilcoxon test. Level of significance was set at $p<0.05$.

## RESULTS AND DISCUSSION

The strain of *Providencia alcalifaciens* ATCC 9886 was found to be an appropriate test microorganism because of its sensitivity to cefquinome and its capacity to form sharply defined inhibition growth zones allowing these measurements with precision. The lower limit of quantification of the assay in swine plasma for cefquinome was 3 ng using 40 μL of plasma. Negative-control samples did not cause bacterial inhibition. Plots of zone diameters vs. standard cefquinome concentrations were linear between 0.075 and 5.0 μg $mL^{-1}$ with a mean correlation coefficient of 0.992.

The precision and accuracy values were <20% for QC samples indicating the assay method is consistent and reliable. There were no significant interferences at the retention times of the analyte and IS. The peak of cefquinome and IS were shown at 7.7 and 8.4 min, respectively (Fig. 1). Peak area ratio of cefquinome to IS was used for the construction of the calibration curves ranged from 0.02-5 μg $mL^{-1}$ and the standard curves were fitted to linear regression. The calibration curve represented the good linearity ($r^2>0.99$) over the range of 0.02-5 μg $mL^{-1}$. Inter-assay precision values were <13.36% and the accuracy values ranged from 93.09-116.2% for four replicates QC samples at the concentration levels of 0.02, 1 and 5 μg $mL^{-1}$. The intra-assay precision values were <12.5% and the accuracy values ranged from 96.4-118.4% for the QC samples. There were no side effects such as nausea, swelling and hardness at the injection site and severe pains, associated with the intramuscular injection of cefquinome in pigs. There were no adverse effects following the intramuscular injection of cefquinome in pigs. The pharmacokinetic parameters for cefquinome based on the plasma

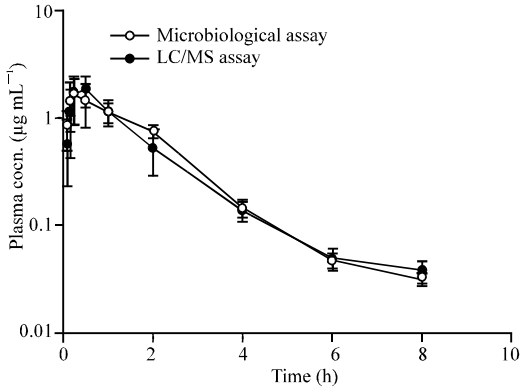


Fig. 1: Mean plasma concentrations of cefquinome after single oral administration at 1 mg $kg^{-1}$ of body weight in pigs determining by microbiological and LC/MS assay. Each point represents the mean±SD

Table 1: Pharmacokinetic parameters of cefquinome after an oral administration at 1 mg $kg^{-1}$ in pigs

| Pharmacokinetic parameters* (unit) | Methods | |
|---|---|---|
| | LC/MS | Microbiological assay |
| $C_{max}$ (μg $mL^{-1}$) | 0.40±0.15 | 0.30±0.16 |
| $t_{max}$ (h) | 2.33±0.63 | 1.99±0.82 |
| $t_{1/2\lambda z}$ (h) | 2.36±0.34 | 1.88±0.24** |
| $AUC_{0-\infty}$ (μg h $mL^{-1}$) | 3.26±0.62 | 3.42±0.54 |
| $V_z/F$ | 1.08±0.27 | 0.82±0.21 |
| Cl/F | 0.32±0.06 | 0.30±0.05 |
| MRT (h) | 1.89±0.14 | 1.83±0.14 |

*$C_{max}$: Maximum observed concentration; $t_{max}$: Time of maximum observed concentration; $t_{1/2\lambda}$: Terminal half-life; $AUC_{0-\infty}$: Area Under Curve from 0 h to infinity; $V_z/F$: Apparent Volume of distribution; Cl/F: Apparent total body Clearance; MRT: Mean Residence Time; **$p<0.05$: A significant difference as compared to the result of LC/MS

concentration vs. time curve derived from microbiological and LC/MS assays were calculated (Fig. 1 and Table 1). The scatter plot of concentrations of cefquinome in plasma samples obtained by the microbiological assay versus those observed from the LC/MS assay demonstrated the agreement between these two methods with a good correlation ($r^2 = 0.83$). As a consequence, the corresponding values of calculated pharmacokinetic parameters exhibited consistency between a microbiological assay and LC/MS ($p>0.05$), except for terminal half-life (Table 1).

Cefquinome was rapid and well absorbed with mean $C_{max}$ values of 0.40 μg $mL^{-1}$ at 2.33 h following a single i.m. injection of cefquinome at 1 mg $kg^{-1}$ in pigs. Thereafter, the plasma concentration of cefquinome was declined with 2.36 h of elimination half-life (Table 1).

Although, microbiological assay is less accurate, precise and specific in comparison with LC assays, microbiological assay requires no specialized equipment, additional extraction procedures or toxic solvents (Perea *et al*., 2000). The microbiological assay has been proposed as an alternative method for LC assay (Lourenco *et al*., 2007; Perea *et al*., 2000; Souza *et al*., 2006). However, analytical methods used for the quantitative measurement of medicines are the key determinants in generating reproducible and reliable data (Shah *et al*., 2000). The absence of metabolites could be improved the sensitivity and precision of microbiological assay (Spoo *et al*., 1995). There was no evidence for the presence of metabolites in the LC/MS analysis of plasma samples following intramuscular injection of cefquinome in pigs. Therefore, the microbiological assay and LC/MS assay for the determination of cefquinome could yielded statistically identical results ($p>0.05$, $r^2 = 0.83$).

However, the plasma concentration of cefquinome in elimination phase assessed using a microbiologic assay considerably underestimated its plasma concentrations measured by LC/MS. Because the underestimated plasma concentration attributed on the estimation of elimination constant, the elimination half-lives were significantly shortened in the microbiologic assay ($p<0.05$). This is likely caused by the fact that the LOQ in microbiological method was higher than that of the LC/MS method, leading to the underestimation of terminal half-life (Atef *et al*., 2001). In additions, non-compartmental analysis is model-independent and relies on the time points of the plasma samples, typically yielding different values than a compartment modeling approach (Deleu *et al*., 1994; DiStefano 3rd, 1982; Hamidi, 2010).

Following i.m. administration at 1 mg $kg^{-1}$, cefquinome was rapidly and well absorbed in pigs. The mean $C_{max}$ of 2.33 μg $mL^{-1}$ was shown at 0.40 h. The $C_{max}$ and $T_{max}$ values in pigs were relatively higher and earlier than those in calves (4.5 μg $mL^{-1}$, 2 h after 10 mg $kg^{-1}$ of i.m. doses; Limbert *et al*., 1991), dogs (7.5 μg $mL^{-1}$, 0.38 h after 10 mg $kg^{-1}$ of s.c. doses; Limbert *et al*., 1991), camel (1.23 μg $mL^{-1}$, 4.25 h after 1 mg $kg^{-1}$ of i.m. dose; Al-Taher, 2010). It was similar to $C_{max}$ of 3.01 μg $mL^{-1}$ at 1 h in sow and $C_{max}$ of 4.01 μg $mL^{-1}$ at 0.28 h in piglets after i.m. administration at 2 mg $kg^{-1}$ of cefquinome (Block *et al*., 2005; Li *et al*., 2008).

A relatively longer terminal half-life was observed in growing pigs ($t_{1/2\lambda z}$ = 2.36 h) than that in calves ($t_{1/2\lambda z}$ = 1.33 h) and dogs ($t_{1/2\lambda z}$<0.98 h; Limbert *et al*., 1991) which is consistent in the terminal half-life of 1.85 h in piglets and 1.5-2.3 h in sows (Block *et al*., 2005). However, Tohamy *et al*. (2006) reported that prolonged elimination half-life of cefquinome were shown in buffalo calves (12.86 h), cattle calves (13.46 h), cows (7.10 h) and goats (8.68 h). The reason for the difference in half-life and absorption may be attributable to differences in species and pharmaceutical formulation.

## CONCLUSION

In this study, the proposed microbiological and LC/MS methods for the determination of cefquinome in plasma showed good inter-assay and intra-assay precision, accuracy and linearity. The microbiological assay, although less sensitivity in comparison with LC/MS method is relatively simple and has sufficient precision and accuracy to be used to monitor drug level in plasma when an LC or LC/MS system is not available.

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