Development and Validation of Analytical Method for the Determination of Cefovecin Sodium in African lions (*Panthera leo*) Plasma by HPLC: FDA Bioanalytical Assay Guidance

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Summary: The aim of this study was to develop and validate a simple high performance liquid chromatography (HPLC) method for the determination of cefovecin sodium in small volume of lion's plasma. Analytical separation was obtained in less than 5 minutes (4.29-4.42 min) using a C-18 column with UV detection at λ_{max} 254nm. The extraction of cefovecin sodium was carried out in methanol and acetonitrile. The mobile phase was composed of water: acetonitrile: methanol in a ratio of 60:20:20 at pH 3.1. The method was validated according to FDA Bioanalytical assay guidance. The limit of detection of cefovecin sodium was 0.05 ug/ml, and the limit of quantification was 0.1ug/ml. The range of linearity consisted of 5 points with 3 runs for each point that result in excellent linearity with $R^2 = 0.9998$. The recoveries were 98-99.25 ± %RSD (NMT 2%). The intra-day and inter-day precision (%CV) of this method was 2.3-10.26% ± SD and 2.44-7.22% ± SD respectively.
A straightforward, easy and successful HPLC method was developed that determined and quantified cefovecin sodium concentrations in African Lion's plasma that can be used in the future work in a pharmacokinetic study of cefovecin sodium.

Keywords: Antimicrobial, Cefovecin sodium, HPLC, Total quality control.

Introduction

Cefovecin sodium (Convenia®) is a third generation cephalosporin antimicrobial. Cefovecin sodium monosodium is manufactured by Zoetis as a powder to be reconstituted for parenteral use. It is a beta-lactam bactericidal agent that inhibits bacterial cell wall synthesis by the formation of a covalent bond cross-linking within the microorganisms cell wall's peptidoglycan [1]. It is approved to treat bacterial skin infections that are caused by broad spectrum Gram-positive and Gram-negative bacteria in cats and dogs such as Staphylococcus intermedius, Staph. Canis, and Pasteurella multocida [2] and also shows efficacy in urinary tract infections caused by E. coli in cats [3].

Cefovecin sodium shows a unique pharmacokinetic profile in different animal species unlike other beta-lactam inhibitors [4-7]. It has a long half-life (up to 5-7 days in dogs and cats respectively), very high protein binding and high bioavailability after subcutaneous administration [6, 7]. Furthermore, cefovecin sodium has time-dependent antibacterial properties, and it could be effective against bacteria for extended periods of time that could reach up to two weeks after subcutaneous injection [2, 8, 9].

Optimal efficacious drug treatment of veterinary animals requires patient compliance especially when concerns of veterinarian safety and/or animal extinction play a role. In addition, a drug given in a dosing regimen as a single subcutaneous injection in such a dosage that persists over an extended period of time due to a long elimination half-life could be crucial when treating or withdrawing blood samples of wild animals such as African lions (*Panthera leo*). In a case report, a female African lion suffering from septic peritonitis was treated successfully with cefovecin sodium. In this report, abdominal culture media showed growth of *E. coli* and the lion recovered from the infection within few days after the start of cefovecin sodium therapy [10].

There has not been a cefovecin sodium pharmacokinetic study performed in African lions. Therefore, the aim of this paper as the first part of a cefovecin sodium pharmacokinetic study is to develop and validate a simple yet efficient high performance liquid chromatography method to determine cefovecin sodium concentrations in the African lion (*Panthera leo*).

Experimental

Animals

African lions (n=6) that were housed at the Oregon Zoo were included in the study. Before they

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were included in the study, all lions were visually examined and hematologically evaluated by a panel of specialists. Diagnostic test results were within reference limits, and all lions were deemed healthy and at ideal body condition. The lions had access to the exhibit and holding area, and their diet was strictly managed to ensure body condition remained stable throughout the study. To minimize stress, all lions were trained to allow blood collection from both lateral coccygeal veins and the right lateral saphenous vein by use of static chute with removable training windows. During the study, injection and phlebotomy sites as well as general health of the lions were monitored by the zookeeper and veterinary staff.

Ethical approval

The study was approved by the Oregon Zoo Animal Welfare Committee in accordance with the Association of Zoos and Aquariums standards for research and technology (PRN 2015-2609).

Reagents and Chemicals

All reagents and solvents were HPLC grade. Lyophilized Cefovecin sodium (Convenia®) powder was purchased from Zoetis (NJ, USA). Deionized purified water was used in this study. Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (NJ, USA). Acetic acid, which was used to adjust the pH of the mobile phase and to denature lion plasma samples and increase cefovecin sodium recovery, was obtained from VWR International (PA, USA).

Instrumentation

Chromatographic analysis was conducted using a Shimadzu Prominence High Performance Liquid Chromatographic system (Kyoto, Japan) model LC-2010A HT and equipped with UV Detector. Centrifugation was performed on Eppendorf centrifuge 5415C (Brinkmann Instruments, NY, USA). Plasma samples were filtered via 0.22 um nylon filters (VWR International, USA) before injection onto the HPLC.

Method development and Chromatographic conditions

An HPLC chromatographic method for cefovecin sodium analysis was performed with a 4.6×150 mm column (Kinetex 5-um, C-18, Phenomenex, CA, USA) connected to pre-column (Security guard 2.1 – 4.6 mm, Kinetex, Phenomenex, CA, USA) and samples flow through in-line filter (KrudKatcher ULTRA HPLC, Kinetex, Phenomenex, CA, USA). The mobile phase consisted of acetonitrile, water, and methanol in a ratio of 60:20:20 with a pH 3.1 (pre-adjusted with acetic acid). The mobile phase was pumped at a 0.7ml/min flow rate. The UV-Detector wavelength was set at 254, and 290 nm and the column temperature was set at room temperature. The sample injection volume was15 ul while the run time was 10 minutes.

Standard solutions and calibration curves preparation

A standard solution of the cefovecin sodium (1 mg/ml) was prepared in methanol. Standard curve solutions of cefovecin sodium in Lion plasma were prepared by adding appropriate volumes of the cefovecin sodium (1 mg/ml) stock solution to drug-free African lions plasma to produce concentrations of 0.1, 0.5, 5, 10, 50, 100 ug/ml. Each standard cefovecin sodium Lion plasma concentration was injected in triplicate using the HPLC method conditions. Three quality control levels were also tested at low, middle and high levels (0.5, 10, and 100 ug/ml) by injecting three replicates for each level to check the method's inter- and intra-day Accuracy (Recovery ± SD%). All six Lion plasma cefovecin sodium standard concentrations were analyzed in triplicate for within-day and for three consecutive days to check method's inter- and intra-day precision (%CV).

The average of peak area under the absorbance curves (AUC) of cefovecin sodium in lions' plasma was calculated and plotted against the corresponding concentrations in ug/ml. The slope, intercept, and correlation coefficient (R^2) were determined by linear regression analysis. Linearity was checked with F-test lack-of-fit analysis. The goodness of fit of the linear regression analysis was evaluated by the coefficient of determination (R^2) and by observation of residuals vs. concentration, and the outliers were observed using studentized residuals vs. concentrations over six runs for each level. Excel software was used to provide analysis curves and results.

Sample preparation

For calibration curves and quality control analyses samples, an appropriate amount of the lion's plasma was spiked with the appropriate volume of the cefovecin sodium stock solution in order to achieve the calibration curve range (0.1-100 ug/ml). For all unknown lion's plasma samples, standard

curve samples, and quality control samples (calibrators, quality control levels, and test samples), 0.5 ml of plasma was pipetted into a 2 ml Eppendorf centrifuge tube, an equal amount of methanol, and 50 ul of glacial acetic acid were added. Then, the tube contents were mixed for 1 minute on a vortex mixer, and then allowed to denature for 15 min. After that, the mixture was centrifuged at 4500 rpm×25 min. The supernatant was filtered through 0.22 filtered and injected onto the HPLC-UV for quantitation.

Result and Discussion

Sample preparation and plasma analysis

A simple, effortless separation method to extract cefovecin sodium from lion's plasma using methanol to separate drug from plasma (added at a 1:1 ratio) gave much greater drug recovery than acetonitrile. Even though cefovecin sodium has high protein binding (96-99%), acidification of plasma sample with a minimal volume of strong acetic acid provided a very high percentage of drug recovery [6, 7].

The developed chromatographic method provides good separation between lion's plasma and cefovecin sodium. There were no interfering peaks with the cefovecin sodium peak at 4.29-4.42 min (retention time) when compared to blank peaks from lion's plasma (Figs. 1 A-D). The addition of methanol during sample preparation and to the mobile phase (with Water: Methanol: Acetonitrile 60:20:20 ratio) produced better efficiency to increase the analysis resolution and eliminate (dilute) any possible interference from excipients. The mobile phase flow rate of 0.7 ml/min and the injection volume of 15 uL were optimal to give separate and sharp peaks.

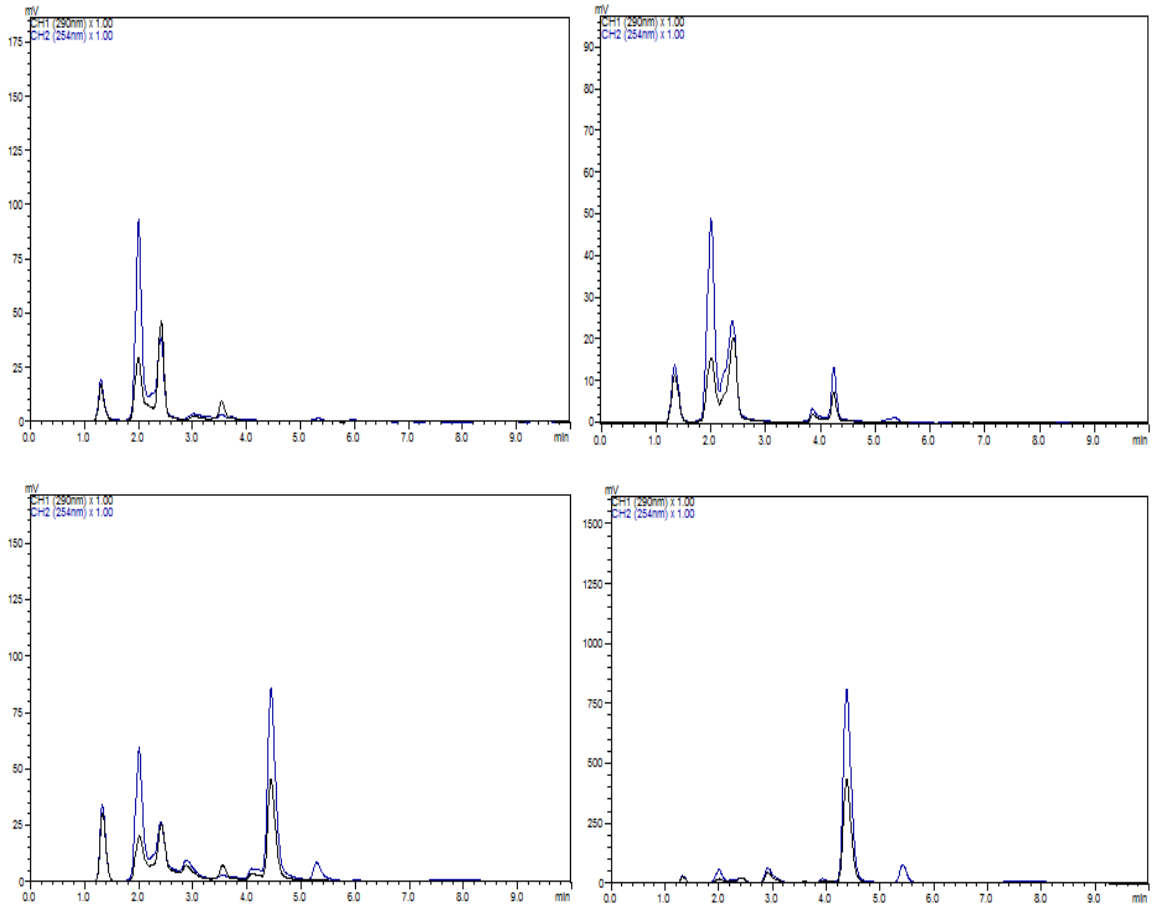

Fig. 1: (A) Blank lion plasma chromatogram. 1B: Cefovecin Chromatogram at 1 ug/ml concentration in lion plasma. 1C: Cefovecin Chromatogram at 10ug/ml concentration in lion plasma. 1D: Cefovecin Chromatogram at 100 ug/ml concentration in lion plasma.

Cefovecin sodium UV absorbance at 254nm provided higher and larger peak areas than 290 nm wavelengths. HPLC oven was on off-mode and its temperature was measured at 27-30° C (room temperature), and the mobile phase adjusted to pH= 3.1 were optimal for analysis.

Method validation Criteria

Selectivity

Cefovecin sodium 100 ug/ml solution was evaluated against the blank lion's plasma and spiked plasma with 100 ug/ml samples. There were no interfering peaks with cefovecin sodium peak at its retention time of 4.29-4.42 minutes (Figs. 2 A-B).

Linearity

From the previously described method condition, an excellent linear relationship was found when the plotting average cefovecin sodium chromatogram peaks versus concentration range (25 – 125 ug/ml). The cefovcin concentration range used contains 5 different concentration with triplicate injections. Linear regression model and one-way ANOVA test were used to calculate the slope and intercept as y=8.3667x+7.5549 with R^2 = 0.9998 (Fig. 3).

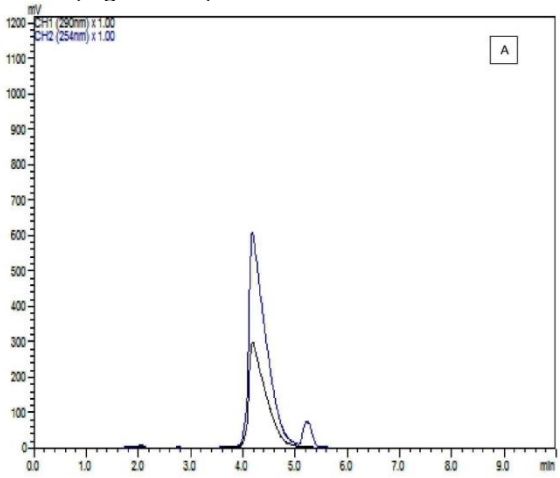

Fig 2: (A) Cefovecin Chromatogram at 100 ug/ml concentration in methanol (selectivity).

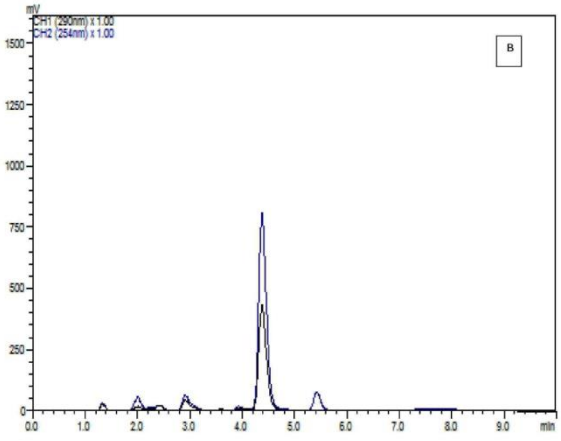

Fig 2: (B) Cefovecin Chromatogram at 100 ug/ml concentration in lion plasma (selectivity).

Parameters	25%	50%	75%	100%	125%
Conc. (µg/ml)	25	50	75	100	125
Injection # 1	197.000	393.200	576.000	763.000	959.500
Injection # 2	193.200	395.800	567.300	757.500	951.500
Injection # 3	189.600	398.700	565.000	764.000	953.500
Average Area	193.26667	395.90000	569.43333	761.50000	954.83333
Slope	7.5549				
Intercept	8.3667				
Correlation	0.9998				

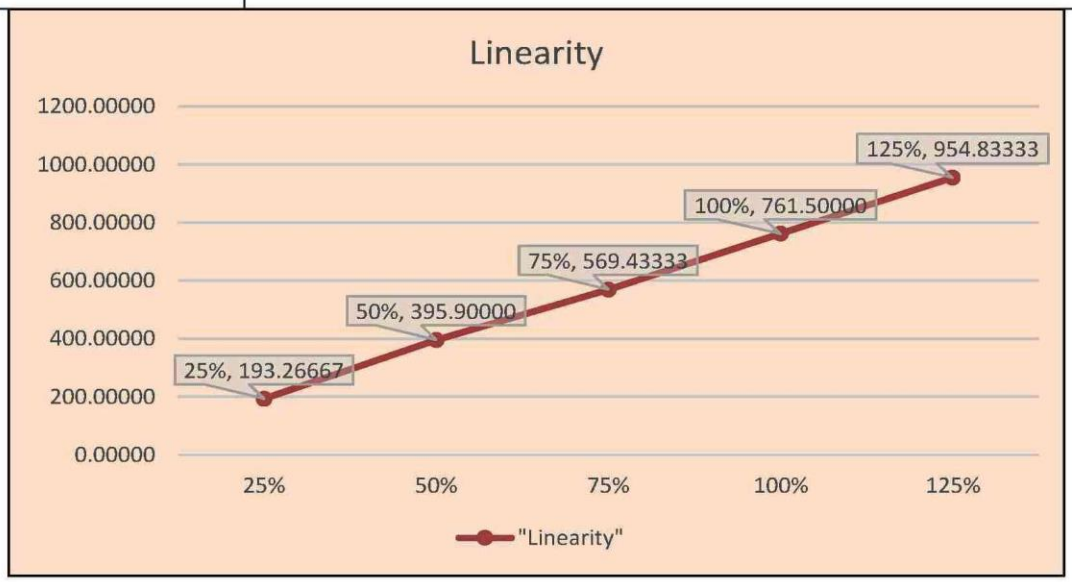

Fig. 3: Concentration (ug/ml) versus HPLC Peak average calibration curve of cefovecin levels (Range 25 – 125 ug /ml).

The goodness-of-fit was also determined by plotting scatter plots of residuals versus concentration and studentized (standardized) residuals against concentration for all concentration levels (5 points) for 3 runs for each level. The standardized residuals versus concentration curve show no level has a larger value than 2. (Figs 4 and 5).

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

The lower limit that can be reliably detected with the HPLC method was 0.05 ug/mL (LOD). The limit of quantification that the cefovecin sodium concentrations cannot be accurately measured and the calibration curve shows a non-linear pattern at concentration below this level was 0.1 ug/mL (LOQ).

Accuracy

Three replicate analyses for three quality control concentration levels 0.5, 10, 100 ug/ml (low, middle and high levels) were performed to assess recovery of cefovecin sodium. The HPLC method showed a sufficiently high recovery rate of cefovecin sodium from lion's plasma. The cefovecin sodium recovery percentage ranged between 98-99.25% ± 3% RSD (Table-1).

Table-1: HPLC-UV method Accuracy.

Recovery		
Concentration (ug/ml)	**Acceptance Criteria**	**%Recovery (RSD)**
0.5		**98.0 ± 1.6**
10	**100±3%**	**99.0 ±0.84**
100		**99.25 ± 0.78**

Note: %RSD for recovery sample NMT 2%

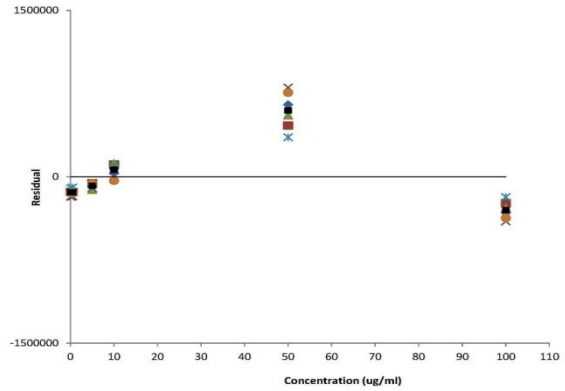

Fig. 4: Residual curve that shows cefovecin concentration (ug/ml) versus UV-absorbance AUC regression line model residuals over six runs for four days.

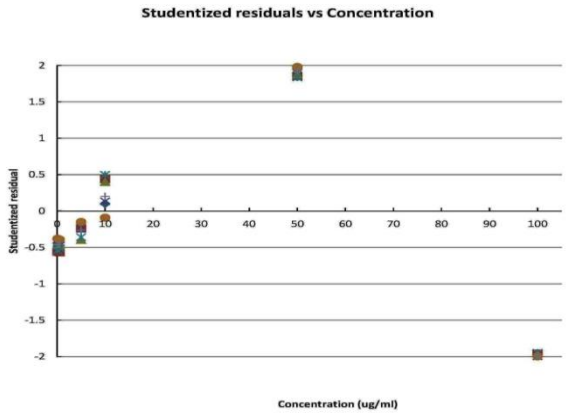

Fig. 5: Studentized residual curve that shows cefovecin concentration (ug/ml) versus UV absorbance AUC regression line model residuals over six runs for four days.

Precision

The coefficient of variation (%CV) was calculated for six averages of cefovecin sodium peak areas corresponding to the six standard curve concentrations 0.1, 0.5, 5, 10, 50, 100ug/mL. The intra-day %CV was between 2.3 – 10.26%. The inter-day %CV was between 2.44 – 7.22% (Table-2). The highest CV% value was with the lowest concentration which is limit of quantification, which is acceptable according to the United States Food and Drug Administration (FDA) bioanalytical criteria.

Table-2: Inter-day and Intraday HPLC-UV method precision.

Concentration (ug/ml)	Intraday Precision (CV%)	Interday precision (CV%)
0.1	**10.26**	**7.06**
0.5	**4.4**	**7.22**
5	**4.27**	**6.52**
10	**4.97**	**3**
50	**1.79**	**6.05**
100	**2.3**	**2.44**

Several researchers tried to develop validated analytical method for determination of cefovecin sodium while using different lab animals and/or different lab conditions. E.g. Sherry developed simple HPLC method for determination of cefovecin sodium while using sea animals (white bamboo sharks and horseshoe crabs). This study showed a recovery of 95% which is almost in agreement to our results (91.92%). This slight difference may be attributed to the animals used [11]. Similarly James et al carried out a research on cefovecin sodium however their intension was to determine the pharmacokinetics of cefovecin sodium while using white bamboo sharks and horseshoe crabs [12]. This is the first HPLC method that evaluates any kind of analysis of drug concentrations in the African lions (*Panthera leo*) plasma. Presented is a straightforward and efficient HPLC-UV method for cefovecin sodium determination in lion plasma. Many previous studies evaluated cefovecin sodium in different animals (dogs, cats, bamboo sharks, patagonian sea lions) using HPLC techniques that utilized either mass-spectroscopy or ultraviolet detection [6, 7, 13].

Unlike other studies, reported here is a simple recovery method using equivalent volumes of methanol with Lion's plasma that provides sufficient extraction of cefovecin sodium for analysis. Evaporation of organic solvent after sample dilution is either a tedious process or requires extra resources (*i.e*., nitrogen evaporation). The use of methanol in the separation process and the mobile phase gave a higher resolution than acetonitrile and may also help in further dilution of excipients and diminish them from appearing in the chromatogram [10].

Extraction of the sample provided superior analytical results where the use of strong acid to denature protein in the sample would not be appropriate as injection directly onto HPLC could cause possible plugging of the pump or tubing and may lead to the formation of microorganism contamination in the system and affect the results [13].

Cefovecin sodium UV detection at 254 nm as found by Encinas T. *et al*., 2000, and unlike Steeil *et al*., 2008 (280nm) provided peaks with greater areas allowing superior quantification [6, 7, 13]. Acidification of the sample and mobile phase provided a higher

recovery percentage of high protein binding drug (cefovecin sodium) from plasma (dogs, cats, crab, sea lions). The mobile phase flow rate used (0.7 ml/min) was optimal and decreases the load on the column by a decrease in the pressure [6, 7].

An internal standard was not used because, unlike other studies, dilution and evaporation of plasma or extract from plasma were not performed and plasma spiked with cefovecin sodium showed similar peak areas at the same time as cefovecin sodium in methanol. The HPLC method used provided acceptable accuracy and precision similar to dilution and evaporation techniques [7, 10].

This HPLC analytical method was successfully applied when cefovecin sodium was administered to an African lions and pharmacokinetic parameters were calculated using noncompartmental analysis (Fig. 6 and Table-3). Since Lions present a unique challenge to study pharmacokinetics in them, a simple HPLC assay should help to develop therapeutic profiles and effective dosing of antibacterial agents like cefovecin sodium and may also improve treatment compliance with such wild and dangerous animals.

Table-3: Pharmacokinetic parameters for an African lion using the proposed HPLC-UV method after single subcutaneous administration of cefovecin sodium

African lion	Cefovecin sodium Dose (4 mg/kg) Pharmacokinetic parameter
K_{el} (hr^{-1})	0.005
T_{max} (hr)	4
C_{max} (ug/ml)	9.71
AUC_{inf} (hr*ug/ml)	1045.77
$AUMC_{inf}$ (hr*hr*ug/ml)	182612.05
MRT (hr)	174.62
$T_{1/2}$(hr)	126.75

Conclusion

A successful HPLC method was developed that determined and quantified cefovecin sodium concentrations in African Lion's plasma and will be used in the future work in a pharmacokinetic study of cefovecin sodium on African lions. The assay is straightforward and easy to be validated according to FDA bioanalytical analysis requirements. The ease of use and accuracy of the assay method will provide an advantage for use in future studies with dangerous animals.

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