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# Determination of Ceftiofur and its Metabolites in Plasma Using Reverse Phase Liquid Chromatography

Sherry Cox
*University of Tennessee, Knoxville*

Molly White
*University of Tennessee, Knoxville*

Kristen Gordon
*University of Tennessee, Knoxville*

Joan Bailey
*University of Tennessee, Knoxville*

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Sherry Cox, Molly White, Kristen Gordon, Joan Bailey, University of Tennessee College of Veterinary Medicine, Department of Biomedical & Diagnostic Sciences, Knoxville, TN 37996

## ABSTRACT

A high performance liquid chromatography procedure for the determination of ceftiofur and all its desfuroylceftiofur metabolites in plasma has been developed and validated using reverse phase liquid chromatography. Following a derivatization method that converts ceftiofur and all desfuroylceftiofur metabolites to desfuroylceftiofur acetamide, separation was attained on a Symmetry $C_{18}$ column and quantification occurred using UV detection at 265 nm. The mobile phase was a mixture of (A) 0.1% trifluroracetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile. The mixture was pumped at a starting gradient of 90% A and 10% B and was adjusted to 75% A and 25% B over 25 min, and back to initial conditions over 3 min. with a flow-rate of 1.0 ml/min. The procedure produced a linear curve over the concentration range of 0.1 – 100 µg/mL with a lower limit of quantification of 0.1 µg/mL. Intra-assay variability ranged from 0.7 to 4.5% and inter-assay variability ranged from 3.6 to 8.8% for desfuroylceftiofur acetamide, respectively, and the average recovery was 99%. This method could be useful to those investigators dealing with small sample volumes, particularly when conducting pharmacokinetics studies which require multiple sampling from the same animal.

## INTRODUCTION

Ceftiofur is a broad spectrum, third-generation cephalosporin antibiotic used in a variety of gram-positive, gram-negative, and anaerobic infections in domestic animal species including many species of *Pasterurella, spp*., *Streptococcus spp*., *Staphylococcus spp*., *Salmonella spp*., and *Escherichia coli*. Ceftiofur is cleaved into furoic acid and desfuroylceftiofur, an active metabolite. Like most cephalosporin's, it is bactericidal and acts by inhibiting cell wall synthesis. The sodium salt of ceftiofur is approved for use in the US for a variety of domestic species including cattle, swine, sheep, goats, horses, dogs, and poultry. The hydrochloride salt of ceftiofur is approved for use in cattle and swine. Ceftiofur crystalline-free acid (CCFA, Excede) is a long-acting preparation of ceftiofur labeled for use as a single dose in cattle and swine for treatment of certain bacterial pathogens. CCFA is also labeled for treatment of lower respiratory infections in horses and is used off label in many non-domestic species, including birds kept as companion animals or in zoological collections.

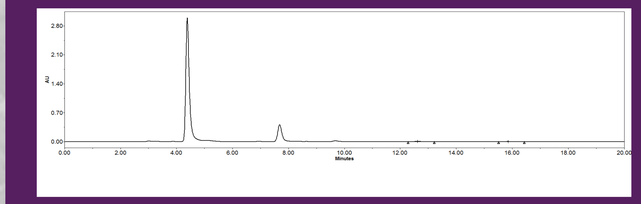
## MATERIALS AND METHODS

### Reagents and Standards

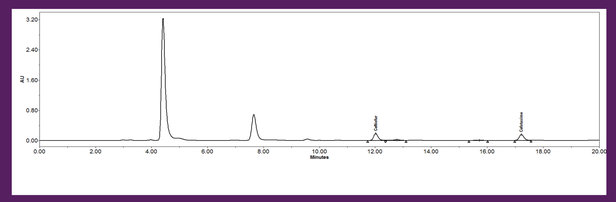
Ceftiofur and cefotaxime were purchased from U.S. Pharmacopeia. All other regent grade chemicals and solvents were purchased from Fisher Scientific. Stock solutions of ceftiofur and cefotaxime (100 µg/ml) were prepared in methanol. Dilutions in methanol were prepared to produce 1 and 10 µg/ml working stocks. The standards were stored at 4° C and were stable for a minimum of 6 months. For preparation of calibration standards and quality control samples, appropriate aliquots of the working stock solutions were added to untreated plasma. The final concentrations were 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 µg/ml for calibration standards and 0.35, 3.5, 35 and 75 µg/ml quality control samples. Calibration standards and control samples were treated the same as test samples. Linearity was assessed by linear regression analysis. The calibration curve had to have a correlation coefficient of 0.99 or better. The acceptance criterion for each back calculated standard concentration was 15% deviation from the nominal value except lower limit of quantification (LLOQ), which was set at 20%.

## Figure 1

A: Blank calf plasma sample




B: Spiked 5 µg/ml standard in calf plasma




C: Calf plasma sample 4 h after CCFA administration

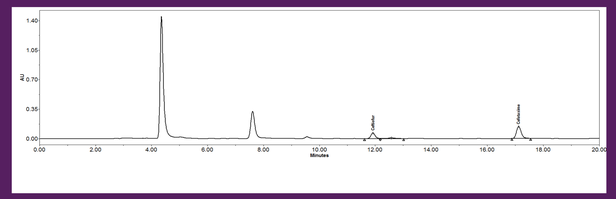



**Table 1. Intra-assay accuracy and precision for ceftiofur in plasma. (n=5)**

| Concentration Added (µg/mL) | Concentration Measured (µg/mL) (Mean ± SD) | CV (%) | Accuracy (%) |
|---|---|---|---|
| 0.35 | 0.35 ± 0.02 | 4.5 | 97 |
| 3.5 | 3.5 ± 0.12 | 3.4 | 100 |
| 35 | 35 ± 1.33 | 3.8 | 100 |
| 75 | 75 ± 0.55 | 0.73 | 100 |

SD: standard deviation; n: number of samples; CV: coefficient of variation

**Table 2. Inter-assay variability and recovery for ceftiofur in plasma. (n=5)**

| Concentration Added (µg/mL) | Concentration Measured (µg/mL) (Mean ± SD) | CV (%) | Accuracy (%) | Recovery (%) |
|---|---|---|---|---|
| 0.35 | 0.35 ± 0.01 | 3.6 | 100 | 100 |
| 3.5 | 3.3 ± 0.29 | 8.8 | 94 | 95 |
| 35 | 35 ± 1.6 | 4.7 | 100 | 98 |
| 75 | 71 ± 5.5 | 7.7 | 95 | 104 |

SD: standard deviation; n: number of days; CV: coefficient of variation

## Chromatographic conditions

The system consisted of a 2695 separations module and a 2487 UV detector (Waters). Separation was attained on a Symmetry $C_{18}$ column (4.6 x 250 mm, 5 µm) with a Symmetry guard column. The mobile phase was a mixture of (A) 0.1% trifluroracetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile. The mixture was pumped at a starting gradient of 90% A and 10% B and was adjusted to 75% A and 25% B over 25 min, and back to initial conditions over 3 min. The drug was quantified using UV detection at 265 nm and the flow rate was 1.0 ml/min.

### Sample Treatment

Ceftiofur was extracted from plasma samples using a derivatization method that converts ceftiofur and all desfuroylceftiofur metabolites to desfuroylceftiofur acetamide. Previously frozen plasma samples were thawed and vortexed and 100 µl were transferred to a clean test tube then 15 µl of internal standard (100 µg/ml cefotaxime) added. Seven milliliters of 0.4 % dithioerythritol in borate buffer was added then tubes were placed in a 50˚ C water bath for 15 min. The tubes were removed and allowed to cool to room temperature then 1.5 ml of iodoacetaminde buffer was added. The solution was then passed through a prewet Oasis HLB extraction column. Samples were eluted with a 5% glacial acetic acid in methanol which was then evaporated to dryness with nitrogen gas. Samples were reconstituted in 200 µl of mobile phase and 50 µl were injected into the HPLC system.

## RESULTS AND DISCUSSION

Blank plasma samples were prepared in the same manner as study samples and no endogenous plasma components interfered with the elution of the peaks of interest. Fig. 1 shows chromatograms of a (A) blank plasma sample, (B) a 5 µg/ml spiked calf plasma standard and (C) a calf plasma sample 4 hours after administration of a 6.6 mg/kg subcutaneous dose of ceftiofur. Retention times were 11.97 min for ceftiofur and 17.15 min for cefotaxime. For specificity testing six different blank plasma samples were used in the pre-validation process.

The plasma peak ratio (area of ceftiofur divided by the IS area) versus the concentration was plotted and produced a linear curve, over the concentration range of 0.1 – 100 µg/ml, with resulting correlation coefficients of >0.999. A typical equation for the calibration curve was $y = 0.0939x + -0.669$, where $y$ represents the peak area ratios of ceftiofur to internal standard and $x$ represents the plasma concentration of ceftiofur in µg/mL. Intra and inter-assay RSD for plasma spiked with specific concentrations of ceftiofur ranges from 0.7 to 7.7% (Tables 1 and 2). The precision was below the set ± 15% for all quality control samples.

The recovery (Table 2) for ceftiofur from spiked plasma was determined by comparing extracted areas with directly injected analyte areas and ranged from 95% to 104%. The relative standard deviation was less than 10% for all values. The recovery of cefotaxime was 98% and its relative standard deviation was 3.3%. The addition of cefotaxime as an internal standard corrects for intra- and inter-day assay variability in the assay. The lower limit of quantification (LOQ) was 0.1 µg/ml and represents a peak approximately five times baseline noise.

## CONCLUSIONS

In conclusion, this analytical procedure was validated in terms of recovery, linearity, LLOQ, precision, and accuracy. The limit of quantification and recovery are more than adequate for use in pharmacokinetic studies. Our results indicate that this HPLC procedure is a reproducible method that provides consistent quantification of ceftiofur and all its desfuroylceftiofur metabolites in plasma. The addition of cefotaxime as an internal standard also allows for the correction of intra- and inter-assay variability. This method has been used successfully to determine ceftiofur concentrations in plasma samples at this institution.