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Short communication

Determination of ceftiofur in bovine plasma by HPLC-DAD

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Abstract

Ceftiofur sodium is a third generation broad-spectrum cephalosporin, formulated as an intramuscular injection, which is used to treat respiratory diseases in swine, ruminants and horses. The thioester bond on ceftiofur is rapidly cleaved to give desfuroylceftiofur which is further metabolized to a disulfide dimer and various desfuroylceftiofur-protein and amino acid conjugates. Methods of analysis of ceftiofur rely on cleavage by dithioerythritol to produce desfuroylceftiofur, which is further stabilized by derivatization to desfuroylceftiofur acetamide using iodoacetamide. Previous analytical methods have extracted derivatized analyte from plasma and tissue using solid-phase extraction clean-up steps followed by HPLC analysis with results reported as ceftiofur-free acid equivalents (CFAE). The simplified method presented here involves direct HPLC injection of a cleaved and derivatized sample following a protein precipitation step with calibration by external standardization and selectivity achieved based on chromatography and diode-array detection (DAD). The assay was linear over the calibration range 0.4–40 μg/ml in plasma. Intra-batch reproducibility R.S.D. was 10.3% and intra-batch sample repeatability R.S.D. was 2.1% at the 5 μg/ml level. The mean accuracy over the range of the calibration curve was −4.2% and the detection limit was 0.15 μg/ml. The assay was successfully applied to bovine plasma following intramuscular injection of ceftiofur sodium. This simplified method is suitable for pharmacokinetic applications involving ceftiofur at normal therapeutic levels.

Keywords: Ceftiofur; Desfuroylceftiofur; Plasma; HPLC

1. Introduction

Ceftiofur sodium (Fig. 1) is a third generation broad-spectrum cephalosporin, formulated as an intramuscular injection (Excenel®; Pharmacia, Sydney, NSW, Australia) which is used to treat respiratory diseases in swine, ruminants and horses [1]. The thioester bond on ceftiofur is rapidly cleaved to give desfuroylceftiofur which is further metabolized to a disulfide dimer and various desfuroylceftiofur-protein and amino acid conjugates [2,3]. Free desfuroylceftiofur is an active metabolite with the intact cephalosporin part of the molecule responsible for biological activity.

Methods of analysis of ceftiofur rely on freeing conjugated desfuroylceftiofur by cleavage of disulfide and thioester bonds using dithioerythritol. The sulfhydral group is further stabilized by derivatization to desfuroylceftiofur acetamide using iodoacetamide [2]. The advantage of this method is that both free and conjugated ceftiofur is analysed, with results reported as ceftiofur-free acid equivalents (CFAE).

Previous analytical methods have extracted derivatized analyte from plasma using a single solid-phase extraction step with Oasis HLB cartridges [4], double solid-phase extraction clean-up steps with C18 and SAX cartridges [2,5], and from tissue using triple solid-phase extraction clean-up steps with C18, SAX and SCX cartridges [3], followed by HPLC analysis with results reported as ceftiofur-free acid equivalents (CFAE). The simplified method presented here involves direct injection of a cleaved and derivatized sample, a protein precipitation step, and HPLC analysis using diode-array detection (DAD) with external standardization.

2. Experimental

2.1. Chemicals

Dithioerythritol and sodium iodoacetate were obtained from Sigma-Aldrich (Sydney, NSW, Australia). Trifluoracetic acid

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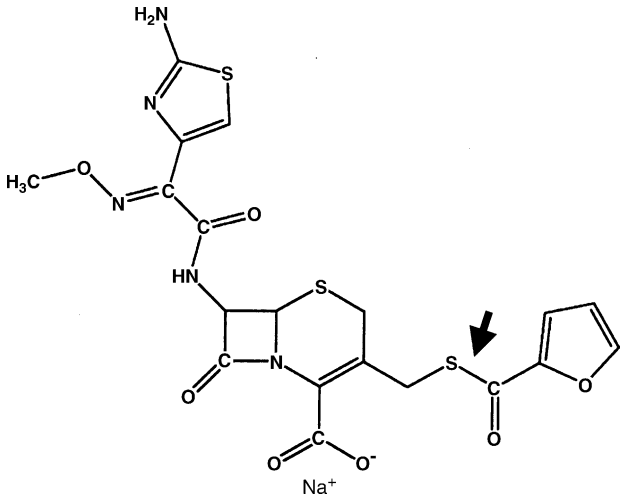

Fig. 1. Structure of ceftiofur sodium showing the thioester bond which is cleaved to yield desfuroylceftiofur.

(TFA), orthophosporic acid, borax and ammonium dihydrogen phosphate buffer were analytical grade and acetonitrile and distilled water were HPLC grade.

2.2. Ceftiofur standard

Ceftiofur sodium standard was prepared from Excenel® (Pharmacia, Sydney, Australia) formulation. A solution of ceftiofur sodium was prepared by dissolving 20 mg Excenel® powder in 2 ml of water. One millilitre of this solution was injected twice into an HPLC system (Rheodyne injector; Cotati, CA, USA and Varian 9010 solvent delivery system, Varian 9050 UV detector; Varian Inc., Melbourne, Australia) consisting of a mobile phase of 20% acetonitrile in 0.05 M ammonium dihydrogen orthophosphate water with a flow rate of 5 ml/min, a 1 ml fixed loop and a preparative Prep Nova-Pak HR C18 6 μm 25 × 10 mm column (Waters, Sydney, Australia). Eluent monitoring was performed at 254 nm and fractions collected during elution of the ceftiofur.

Fractions were then combined into a round bottom flask and the acetonitrile from the mobile phase evaporated using a rotary evaporator with the flask heated at 40 °C. Aliquots of this solution (approximately 5 ml) were then passed through pre-conditioned 60 mg OASIS HLB solid-phase extraction cartridges (Waters, Sydney, Australia), washed with 5 ml of water, and eluted with 2 ml of acetonitrile. Extracts were combined into a round bottom flask and evaporated to dryness using a rotary evaporator with the flask heated at 40 °C.

Extracted ceftiofur sodium was recovered from the round bottom flask, oven dried, and confirmed by elemental analysis and NMR. The resulting product was a cream-white powder compared to the tan-brown coloured Excenel®. Elemental analysis found a N, C, H, S, O composition of 12.98, 41.77, 3.03 and 17.46% matching the calculated content of 12.83, 41.77, 3.11 and 17.59%, respectively. NMR spectroscopy (1H) confirmation was performed on approximately 1 mg of sample dissolved in D_2O using a 400 MHz Inova Wide Bore spectrometer (Varian, Palo Alto, USA), a Z-gradient inverse 5 mm probe at 25.0 ± 0.1 °C, with sodium TSP internal standard (3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt).

2.3. Protein cleaving and derivatization to desfuroylceftiofur

Borax (sodium tetraborate) buffer bulk solution (0.05 M) was prepared by adding 1.91 g/100 ml water. Bulk working solutions of dithioerythritol (130 mM) were prepared by adding 25 mg of dithioerythritol to 1.25 ml of borax buffer per sample. Bulk iodoacetate working solutions (200 mM) were prepared by adding 50 mg sodium iodoacetate to 500 μl of 25 mM pH 7 phosphate buffer per sample.

Two hundred and fifty microliters of plasma were added to disposable 10 ml plastic sample tubes with caps. Cleavage from conjugated metabolites to give unconjugated desfuroylceftiofur was achieved using the dithioerythritol working solution (1.25 ml added), tubes capped, then vortex mixed for 5 s and incubated in an orbital water bath for 15 min at 45 °C with moderate mixing speed.

Derivatization was then performed by adding 500 μl of the iodoacetate working solution to the test tubes containing the cleaved desfuroylceftiofur, tubes capped, and vortexed for 5 s, then incubated for 30 min at room temperature on a rocker-mixer (moderate speed) and protected from light by covering in aluminium foil. Final clean-up was achieved by adding 75 μl of 20% phosphoric acid in water, vortex mixing for 5 s, then centrifuging for 10 min at 3000 rpm. The supernatant (approximately 2 ml) was transferred to autosampler vials via filtration using 0.45 μm nylon syringe filters and then refrigerated prior to analysis.

All steps in the method involving liquid additions were performed accurately using adjustable volume pipettors as the HPLC injection relies on fixed loop injection with quantitation by external standardization.

2.4. HPLC system

The analytical HPLC system consisted of a Prostar 210 solvent delivery system, Prostar 330 UV-DAD and Prostar 410 autosampler with column oven set at 40 °C (Varian Inc, Melbourne, Australia) with a Waters Novapak C18 column (150 × 4.6 mm; Waters, Sydney, Australia) and 200 μl fixed loop injection. Detection and quantitation was performed at wavelength of 265 nm. In addition to retention time, diode-array detection was used to confirm peaks based on wavelength with neighbouring peaks possessing significantly different UV spectra.

Mobile phase conditions consisted of 100% solvent A (0.1% TFA in water) decreasing linearly to 75% solvent A, 25% solvent B (0.1% TFA in acetonitrile) at 25 min with a flow rate of 1.0 ml/min. The retention time of the derivatized desfuroylceftiofur was 16.5–17.5 min, with a total run time of 25.0 min, and equilibration time of 2.0 min.

2.5. Calibration and measurement as ceftiofur-free acid equivalents (CFAEs)

Determinations of ceftiofur rely on firstly cleaving of ceftiofur metabolite disulfide bonds using dithioerythritol, then derivatizing the free desfuroylceftiofur to desfuroylceftiofur acetate using sodium iodoacetate. Quantitation of samples by external standardization was performed using a calibration curve based on ceftiofur-free acid equivalents (CFAEs) calculated from peak areas of cleaved and derivatized calibration samples of blank plasma spiked with ceftiofur standard.

Calibration samples were prepared from a working solution of ceftiofur 1 mg/ml in water, which was further diluted 1:2, 1:4, 1:8 and 1:40 with water. Ten microliters of the 1 mg/ml solution and each dilution was added to 240 μl of plasma to give spiked concentrations equivalent to 40, 20, 10, 5 and 1 μg/ml in plasma. Samples were prepared as outlined in Section 2.3 and analyzed by HPLC as outlined in Section 2.4.

2.6. Assay validation

Intra-batch reproducibility was assessed by spiked plasma samples ($n = 5$) at the 5 μg/ml level. The samples were fortified in plasma in the same manner as the 5 μg/ml calibration sample from Section 2.5, prepared as outlined in Section 2.3 and analyzed by HPLC as outlined in Section 2.4.

Recovery was assessed by spiked water samples at the 5 μg/ml level representing 100% theoretical recovery. The samples were fortified in water in the same manner as the 5 μg/ml calibration sample in plasma from Section 2.5, prepared as outlined in Section 2.3 and analyzed by HPLC as outlined in Section 2.4. Plasma samples spiked at 5 μg/ml and analyzed in the same manner were used to calculate recovery as a percent of theoretical recovery.

Intra-assay accuracy was expressed as mean percentage error ([found concentration − spiked concentration]/spiked concentration × 100) over the range of the spiked plasma samples (1, 5, 10, 20 and 40 μg/ml) as well as at the 5 μg/ml plasma level. Intra-batch sample repeatability was investigated by multiple injections of the same sample at the 5 μg/ml plasma level. The lower limit of quantification (LLoQ) was estimated from the lowest concentration with an R.S.D. of less than 15% by analyzing replicate samples ($n = 5$) at 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml in plasma. Detection limit was estimated from a signal-to-noise ratio of 3.

2.7. Application of the assay in bovine plasma

The assay was applied to blood samples from two calves administered ceftiofur by intramuscular and subcutaneous injection at 1 mg/kg bodyweight (2 ml/100 kg). Plasma was separated from blood samples collected from the tail vein. Blood was collected using an 18-gauge needle and 10 ml lithium heparin tube. Calves were sampled at pre-treatment and 1, 4, 8, 12 and 24 h after injection. Plasma samples were stored at −20 °C until analysis.

3. Results

The calibration curve for CFAE in plasma was linear over the range 0.4–40 μg/ml with $r^2 = 0.995$. Intra-batch reproducibility R.S.D. was 10.3% ($n = 5$) at 5 μg/ml CFAE in plasma. Mean recovery of CFAE expressed as a percent of theoretical recovery from a cleaved and derivatized spiked water sample containing 5 μg/ml ceftiofur was 99.9% with a R.S.D. of 10.2% ($n = 5$).

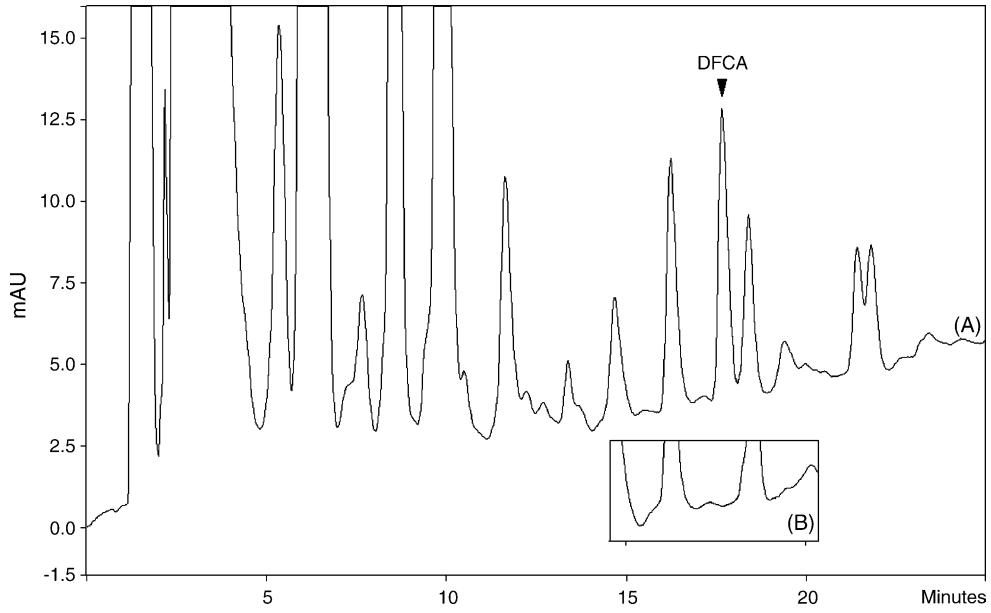

Fig. 2. Chromatogram (A) of a calf plasma sample showing the desfuroylceftiofur acetate peak (DFCA) with 4.0 μg/ml ceftiofur-free acid equivalents (CFAE) after subcutaneous injection of ceftiofur (1 mg/kg). Inset (B) shows a plasma blank.

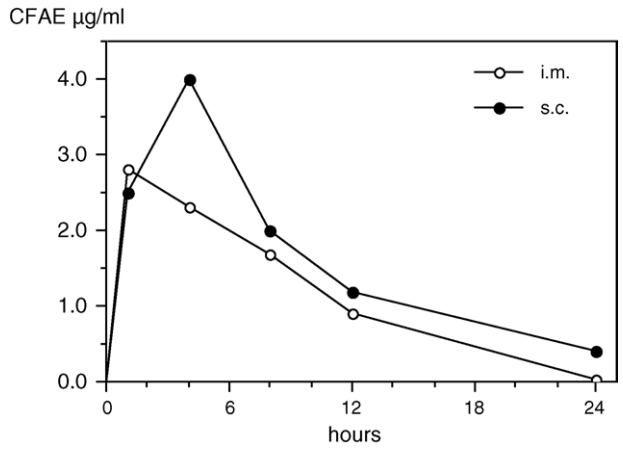

Fig. 3. Ceftiofur-free acid equivalents (CFAEs) µg/ml in calf plasma after subcutaneous and intramuscular administration of ceftiofur 1 mg/kg bodyweight.

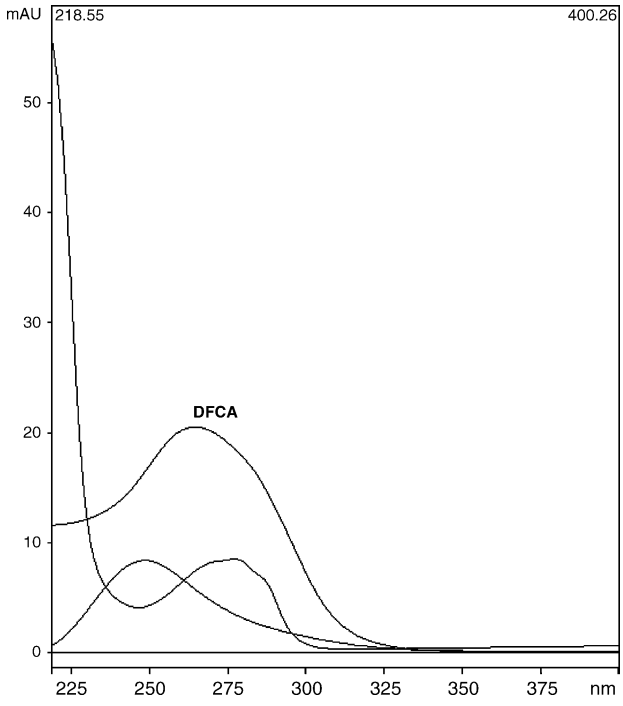

Fig. 4. UV spectra of desfuroylceftiofur acetate (DFCA) with λ_{max} = 264 nm and neighboring peaks at 16.3 min (λ_{max} = 278 nm) and 18.5 min (λ_{max} = 248 nm).

Mean accuracy from cleaved and derivatized spiked plasma samples was −4.2% (n = 5) over the range of the calibration curve and −11.4% (n = 5) at the 5 µg/ml level. The intra-batch sample repeatability R.S.D. was 2.1% (n = 5). LLoQ was 0.4 µg/ml with a R.S.D. of 12.6% and the detection limit was estimated at 0.15 µg/ml.

A chromatogram of a bovine plasma sample containing 4 µg/ml is shown in Fig. 2. The results of the application of this assay are shown in Fig. 3 illustrating bovine plasma levels of CFAE following administration of ceftiofur sodium. Selectivity based on UV spectra of neighboring peaks is shown in Fig. 4.

4. Discussion

The results of this assay have demonstrated that ceftiofur can be analyzed in plasma without time consuming solid-phase extraction procedures normally employed for this analyte. This selectivity has been achieved by a combination of chromatographic separation and detection using UV spectra confirmation with a diode-array detector. Selectivity with regard to other cephalosporins in plasma was not investigated but given the derivatization step and resolution afforded by the long solvent program, co-elution would be considered unlikely. While it can be expected that there will be minor HPLC retention time shifts from sample to sample under the conditions utilized in this asay, it was found that the UV spectrum for desfuroylceftiofur was distinctly different to the neighboring peaks under the present solvent conditions.

While this assay is less sensitive than previously reported assays [2], it can be performed using external standardization (requiring fixed volumes throughout the sample preparation) and without the need for solid-phase extraction. It has been previously noted in a multi-laboratory trial for determining ceftiofur-related residues in bovine and swine kidney, muscle, and bovine milk that solid-phase extraction cartridge performance must be carefully evaluated before samples are processed [6]. This is particularly relevant given existing assays for plasma ceftiofur reported in the literature do not utilize an internal standard and intra-batch variability cannot be easily controlled for in the analysis.

The cleavage, derivatization and phosphoric acid satisfactorily precipitate unwanted protein conjugates after centrifugation without the need for further clean-up. Unwanted guard column and chromatography effects consistent with injection of complex sample matrices were not observed and this assay was successfully employed for a batch analysis of over 100 samples.

5. Conclusion

The assay presented here is modified from that reported by Jaglan et al. [2] which has formed the basis of ceftiofur assays over the last decade. By utilizing diode-array detection and omitting solid-phase extraction, the current plasma assay is simpler to perform than previously published methods while retaining the performance to satisfy common pharmacokinetic applications.

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