Journal of Chromatography B, 898 (2012) 62–68

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A determinative and confirmatory method for ceftiofur metabolite desfuroylceftiofur cysteine disulfide in bovine kidney by LC–MS/MS

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ARTICLE INFO

Article history:
Received 6 December 2011
Received in revised form 16 April 2012
Accepted 18 April 2012
Available online 25 April 2012

Keywords:
Ceftiofur
Desfuroylceftiofur cysteine disulfide
Bovine kidney
LC–MS/MS

ABSTRACT

Ceftiofur is a cephalosporin β-lactam antibiotic widely used for treating certain bacterial infections in beef and dairy cattle. The regulatory HPLC–UV method for ceftiofur residues in animal tissues is time consuming and non-specific. Additionally, because the regulatory method involves chemical reactions to convert the metabolites into a single moiety, it is virtually impossible to incorporate the procedure into a multi-residue method. Ceftiofur residue violations in beef and dairy cattle have been frequently reported and therefore an improved method is needed. Herein we report a rapid and sensitive LC–MS/MS method for the determination and confirmation of ceftiofur metabolite, desfuroylceftiofur cysteine disulfide (DCCD), in bovine kidney tissue. The new method utilizes a simple extraction with phosphate buffer followed by SPE cleanup. A deuterated internal standard was synthesized and used for quantitation. The matrix-based calibration curve was linear from 25 to 2000 ng/g. The average accuracy for control kidney samples from six different sources fortified at 50–1000 ng/g was 97.7–100.2% with CV ≤ 10.1%. The limit of confirmation was 50 ng/g.

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1. Introduction

Ceftiofur is a third generation cephalosporin β-lactam and an effective broad spectrum antibiotic. Presently, several injectable and intramammary dosage forms have been approved for treating certain respiratory diseases in cattle or clinical mastitis in lactating dairy cattle in the United States (US) [1]. Ceftiofur is quickly metabolized in cattle to the reactive desfuroylceftiofur (DFC) which can then form a free unbound metabolite desfuroylceftiofur cysteine disulfide (DCCD), or condense with itself to form a dimer, bind to glutathione, or to macromolecules [2].

In the US, the Department of Agriculture's Food Safety and Inspection Services (USDA FSIS) conducts sampling and testing of suspected beef products to prevent animals containing violative levels of drug residues from entering into the food market [3]. Ceftiofur residue violations have been frequently detected according to a recent FSIS report [4]. Currently, ceftiofur residue is detected using three methods [3,4]: a 7-plate bioassay for screening, a regulatory HPLC–UV method approved by the US Food and Drug Administration (FDA) for determination of the concentration, and a LC–MS/MS for confirmation.

The regulatory HPLC–UV method involves two chemical reactions: cleavage of the disulfide and/or thioester bond from various unspecified metabolites with dithioerythritol (DTE) to release DFC (the marker residue) and the subsequent derivatization with iodoacetamide. These treatments convert those metabolites containing an intact β-lactam structure to a single stable derivative of DFC, desfuroylceftiofur *S*-acetamide (DCA) which is analyzed by a HPLC–UV procedure [2,5,6]. In cattle, tolerances of 0.4 ppm in kidney (the target tissue), 2 ppm in liver, 1 ppm in muscle, and 0.1 ppm in milk have been set based on this method [7]. However, the method is not specific, very time consuming, expensive to perform and generates large volumes of chemical and biological waste. The tissue extracts have to be cleaned up by three solid phase extraction (SPE) steps before the HPLC analysis. Furthermore, because the analyte DCA is derived through chemical reactions, it is virtually impossible to develop a multi-residue method that involves DCA. Recently, a modified version of this method has been reported [8] based on the same chemical principles, but uses LC–MS/MS detection. The modified version is faster because it reduces the number of SPE steps from 3 to 2 and involves a significantly shorter LC run time.

Since the late 1990s, several studies have proposed a different way of detecting ceftiofur residues by monitoring the major unbound ceftiofur metabolite, DCCD, in bovine tissues. In 1998, Moats et al. reported the identification of DCCD in fractionated beef tissue extracts using HPLC–UV and suggested DCCD can be used as a "marker residue" to confirm positive samples screened by microbial inhibition screening tests [9–11]. In 2003, Fagerquist and Lightfield reported a confirmatory method for multiple β-lactam antibiotics in kidney by LC/electrospray ionization selective reaction monitoring ion trap tandem mass spectrometry (LC/ESI-SRM-MS^n) [12].

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1570-0232/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2012.04.020

DCCD was included in the method with a limit of confirmation of 500 ng/g. The method was not suitable for quantitation because of linearity and reproducibility problems. In 2005, the same authors published a confirmatory and quantitative method using a triple quadrupole LC–MS/MS system combined with dispersive SPE for multiple β-lactam antibiotic residues [13]. Among all the analytes, DCCD showed the lowest recovery of ~58%. A streamlined procedure published in 2008 improved the recoveries of all the β-lactam analytes except for DCCD, which remained essentially the same (~60%) [14].

DCCD is one of the ceftiofur metabolites that contribute to the total concentration of ceftiofur residues as measured by the HPLC–UV method. Because the tolerance of ceftiofur residues is tied to the HPLC–UV method, a quantitative relationship between DCCD and DFC (measured as DCA) must be established in order to use DCCD as a surrogate marker residue. In the absence of such a relationship, DCCD can only be used for qualitative confirmatory and/or screening purposes. For practical reasons, it is of great interest to use DCCD not only for confirmation, but also for the determination of the concentration of ceftiofur residues. Due to the poor accuracy of the existing methods for DCCD, our main objective is to first develop an improved quantitative method and then bridge it to the regulatory HPLC–UV method through incurred animal studies.

Herein, we report the development and validation of a determinative and confirmatory method for DCCD in bovine kidney by LC–MS/MS. The improvement is mainly due to a more efficient extraction procedure as well as the utilization of a deuterium labeled DCCD as an internal standard. This is the first report on such an approach for DCCD detection.

2. Methods and materials

2.1. Chemicals and reagents

Reference standard material of DCCD (96% purity) was purchased from Toronto Research Chemicals (Toronto, Canada) and was used for the method validation except for the stock and working solution stability testing. [$^2H_3$]cysteine-DCCD (d_3-DCCD) was synthesized in our laboratory (see Section 2.2 for detail). Samples of ceftiofur hydrochloride and DCCD were received as gifts from Pfizer Animal Health (Kalamazoo, MI, USA) and were used for synthesis and stability testing, respectively. Stock solution of DCCD was prepared in water at approximately 1 mg/mL. An intermediate stock solution of 20.0 μg/mL was prepared from either a freshly prepared stock solution or a freshly thawed vial of stock solution which was stored at −80 °C. Dilutions of the intermediate stock solution with water gave working standard solutions at 0.1, 0.2, 0.4, 0.6, 1.0, 2.0, 4.0 and 8.0 μg/mL (equivalent to 25, 50, 100, 150, 250, 500, 1000, and 2000 ng/g in 0.4 g of kidney sample). Both stock and working solutions were found stable in amber glass vials after 12 months of storage at −80 °C.

2,2′,3,3,3′3′-d_6-DL-Cystine (d_6-cystine, 99.5 at.% D) was purchased from CDN Isotopes Inc. (Quebec, Canada). Preparative silica gel thin layer chromatography (TLC) plates (20 cm × 20 cm, 1000 μm) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water was generated using a Milli-Q water purification system from Millipore (Danvers, MA, USA) to give resistivity > 18 MΩ and was used for all reference to water. Methanol (MeOH) and acetonitrile (MeCN) were high purity grade from Burdick & Jackson (Muskegon, MI, USA). Formic acid was Optima grade for LC/MS from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were ACS grade reagents from Fisher Scientific.

2.2. Synthesis of d_3-cysteine-DCCD(d_3-DCCD)

Ceftiofur hydrochloride (11.5 mg) was added to a 10 mL solution of 0.1 M ammonium acetate (AA) buffer (pH 8.7), which contained 200 mg of DTE. The mixture was shaken in a water-bath at 50 °C for 30 min. After the mixture was cooled to room temperature (rt), it was loaded onto a Mega Bond-Elut C18 SPE cartridge (1 g, 6 mL; Varian, Palo Alto, CA, USA) pre-conditioned with 10 mL MeOH and 10 mL 0.1 M AA buffer (pH 8.7). The sample was washed with 2 × 5 mL of AA buffer, and then eluted with 2 × 7.5 mL of MeOH. The combined eluate was evaporated to dryness under nitrogen (N_2) at rt, which was dissolved in 10 mL of AA buffer. To this solution, 100 mg of d_6-cystine was added and the resulting suspension was stirred vigorously at rt for 1 h. The mixture was centrifuged at 4100 rpm at rt for 10 min and the supernatant was cleaned up using the same SPE procedure as mentioned above. The eluate was evaporated under N_2 at 40 °C until it reached a level of ~2 mL. The crude material was purified on preparative TLC plates (developed with isopropanol: 0.1% acetic acid (75:25, v/v)). A broad band detected under a UV lamp that had a retention factor (R_f) of approximately 0.74–0.82 was scrapped off. The still wet silica gel was extracted with water (3 × 20 mL). The combined water extract was divided into 4 portions and passed through pre-conditioned Mega Bond-Elute C18 SPE cartridges. The eluate from the 4 cartridges was combined. The d_3-DCCD (>99.5% isotopic purity) was identified using LC–MS/MS. The concentration was determined to be 86 μg/mL by comparing the peak area to that of a non-labeled DCCD stock solution using HPLC–UV (264 nm). A working solution of 1.5 μg/mL used for kidney extraction was prepared by dilution with water, and stored at −80 °C until use.

2.3. LC–MS/MS conditions

The LC instrumentation used was a Waters Acquity UPLC system (Milford, MA) which consisted of a temperature controlled sample manager, a binary solvent manager and a heated column compartment. The autosampler temperature was kept at 10 °C. Chromatographic separation was performed using a Phenomenex Kinetex C18 column (50 mm × 2.1 mm, 2.6 μm particle size, 100 Å pore size), coupled to a Phenomenex KrudKatcher Ultra HPLC in-line filter (0.5 μm depth × 0.004 in ID) (both from Phenomenex; Torrance, CA, USA). The column compartment temperature was kept at 30 °C. The gradient mobile phases (A, 0.1% formic acid in water and B, 0.1% formic acid in MeCN) were: 0–1 min, 0% B; 1–4 min, 30% B; 4–4.1 min, 95% B and held for 2 min; 6.1–6.2 min, 0% B and then held for 1.8 min (the total run time was 8 min). The flow rate was 0.3 mL/min. The mobile phases should be used within one week after preparation. The injection volume was 10 μL in the full-loop mode.

A Micromass Quattro Micro mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operated in positive ion mode was used. Optimized operating parameters were: capillary voltage, 2.5 kV; extractor voltage, 2.00 V; RF lens, 0.1 V; source temperature, 120 °C; desolvation temperature, 375 °C; cone gas (N_2) flow, 20 L/h; desolvation gas (N_2) flow, 400 L/h; LM1 and LM 2 resolution, 13.0 and 14.0, respectively; HM1 and HM 2 resolution, 13.0 and 14.0, respectively; ion energy 1 and 2, 1.2 and 1.8, respectively. The compound dependent parameters including ion transitions for multiple reaction monitoring (MRM), cone and collision energy, and dwell time are listed in Table 1. The most abundant ion transitions were monitored for data acquisition. MassLynx V4.1 and TargetLynx V4.1 software (Waters) were used for data acquisition, processing and reporting.

Table 1
Compound dependent parameters for mass spectrometer.

Analyte	Precursor ion (Da)	Product ion (Da)	Cone (V)	Collision energy (eV)	Dwell time (s)
DCCD	548.8	183.0 (quantifying)	33	30	0.200
		241.0 (qualifying)	33	20	0.200
d_3-DCCD	552.1	183.1	32	30	0.200

2.4. Kidney samples

Control kidneys were obtained from either a local grocery store or freshly slaughtered steers that were never treated with ceftiofur. An incurred kidney sample was collected from a steer dosed with Naxcel® (ceftiofur sodium; PBS Animal Health, Massillon, OH, USA) and administered via intramuscular (IM) injection at 2.2 mg/kg for 5 times at 24-h intervals. The animal was slaughtered at 12 h after the last dose. After removing the fat tissue and outer layer of membrane, the kidney was flash frozen with dry ice and transferred to the laboratory for processing. During processing, each kidney was chopped into ~1/4 in. pieces before grinding into semi-liquid consistency with a blender. A second incurred sample was obtained by thoroughly mixing the above sample with a control kidney sample in a 1:3 ratio. The processed samples were divided into smaller portions and stored at −80 °C.

2.5. Sample extraction

After the samples were thawed to rt, approximately 0.4 g ± 0.008 g of test and control samples were weighed into 10-mL Falcon round-bottom polypropylene (PP) tubes (Fisher Scientific). Samples were centrifuged for 2 min at 1000 rpm to bring tissue to the bottom of the tubes. The matrix-based calibration samples and positive quality control samples were fortified with 100 μL of appropriate DCCD working standard solutions. Then 3 mL of 1% phosphate buffer (prepared using 8.0 g of KH_2PO_4 and 2.0 g of K_2HPO_4 in 1 L water; pH 6.2) was added to all samples. The samples were homogenized for 30 s using an Omni Prep homogenizer with disposable plastic probes (Omni International, Marietta, GA, USA), followed by centrifugation (Thermo IEC, Centra GPR8-R) at 4100 rpm at rt for 20 min. The supernatant was subjected to SPE (Phenomenex Strata X, 60 mg, 3 mL) cleanup.

The SPE cartridges were conditioned sequentially with 2 mL of MeOH, 2 mL of 0.01% EDTA in water, and 2 mL of 1% phosphate buffer. Samples were loaded onto the SPE cartridges and passed through under gravity. Cartridges were washed with 2 mL of water before eluting the analyte with 2 mL of MeCN/water (50:50, v/v). For most of the runs in this study the SPE procedure was carried out using a 5-module automated Zymark RapidTrace SPE workstation (steps listed in Table 2). Manual SPE manifold can also be used. If the RapidTrace SPE workstation was used, appropriately sized disposable glass tubes were used in order to fit into the racks. However, the eluate and a 0.5-mL water rinse of the glass tube that contained the eluate were transferred into 15-mL PP centrifuge tube (Fisher Scientific) in order to fit into the racks for the Zymark TurboVap (Hopkinton, MA) for evaporation. If SPE was carried out manually, conditioning, loading, washing, and eluting steps were done by gravity and the final eluate was collected directly into the 15-mL centrifuge tube. The eluate was evaporated under N_2 at 40 °C to a volume of ~1 mL. Then, 200 μL of d_3-DCCD working solution was added to each sample and the final volume was adjusted to 2 mL with water. A portion of the final extract (~1 mL) was then filtered using disposable PVDF syringe filters (0.2 μm pore size, 13 mm diameter; Fisher Scientific) directly into autosampler vials (2 mL amber glass vial with pre-slit PTFE/silicone septa; Waters) for LC–MS/MS analysis.

Table 2
SPE procedure using RapidTrace SPE workstation.

Step	Source	Destination	Volume (mL)	Flow (mL/min)
1 Purge-cannula	Water	Cannula	2.5	42
2 Condition	MeOH	Waste (Org)	2	42
3 Condition	0.01%EDTA	Waste (Aq)	2	42
4 Condition	Phosphate buffer	Waste (Aq)	2	42
5 Load	Sample	Waste (Aq)	2	2
6 Load	Sample	Waste (Aq)	2	2
7 Rinse	Water	Waste (Aq)	2	2
8 Purge-cannula	Water	Cannula	3	30
9 Collect	50%MeCN	Fract1	2	2
10 Purge-cannula	MeOH	Cannula	3	30
11 Purge-cannula	Water	Cannula	3	30

2.6. Method validation

Six independent sources of control kidney samples were used for method validation. Fortified samples at concentration levels between 50 and 1000 ng/g were prepared in those control samples. To accept a run, the coefficient of determination (r^2) for the calibration curve must be >0.99. The deviation of the calibration standards should be within 15% of its nominal value except for the lowest calibration point (assigned to be the limit of quantitation, LOQ) where the limit is ±20%. The calibration curve may be corrected if necessary by excluding no more than two outlying standards. If the lowest standard was excluded, the LOQ for that run was raised to the next higher standard. The analyte peak should be present with a signal-to-noise >10 for quantitation. Co-eluting interfering peaks in the negative control should have a response <20% of the response of the analyte in the lowest calibration standard.

The confirmation criteria described in the FDA Guidance for Industry No. 118 [15] was followed except a 2% acceptable range for retention time was used instead of 5% because of the reproducible chromatography.

3. Results and discussion

3.1. Method development

Previously, DCCD (along with other β-lactams) was first extracted from bovine kidney samples with a 4:1 MeCN-water mixture followed by either a conventional reverse-phase (RP) SPE clean up in a qualitative method or dispersive C18 SPE clean up in combination of hexane extraction of the fatty components in a quantitative method [12–14]. The dispersive SPE procedure, worked very well for most of the β-lactams included in the multi-residue methods, except for DCCD, which had an average recovery of ~60% (similar to that in the qualitative method). It was speculated that the low recovery was probably because of the instability of DCCD which could undergo thiol-disulfide exchange with proteins in the kidney tissue. We evaluated the 4:1 MeCN-water extraction combined with a polymer-based RP Strata-X SPE using 100% MeCN as eluting solvent. In our hands, the extraction efficiency was only 40–60% (calculated by comparing the responses of standards in pre-fortified and post-fortified control kidney samples). Because DCCD has very low solubility in MeCN as compared to in water, MeCN-water mixtures were tested as eluting solvents. We observed that DCCD could be completely eluted from the Strata-X SPE with as little as 10% MeCN in the mixture. A 50:50 (v/v) mixture was chosen in the final method because of its ability to elute other β-lactams. We also found that DCCD could be extracted with the phosphate buffer alone without increasing the background levels

or the matrix effect. Therefore MeCN was removed from the initial extraction solution, eliminating the need for an evaporation step prior to SPE cleanup. The overall extraction efficiency of the sample preparation procedure in this report was determined to be 78.2 ± 2.6% (n = 3), 75.5 ± 1.0% (n = 3), and 74.0 ± 1.0% (n = 3) at 50, 250, and 1000 ng/g, respectively.

The matrix effect was evaluated by comparing the ratio of the slopes of two external calibration curves: one was generated by standards post-fortified into the extracts of control samples and the other was generated by standards fortified into water. The ratio was 1.05 for the control kidney investigated, which indicates an average of 5% of matrix effect.

Because of the zwitterionic nature of DCCD, we also tested Strata X-C (a mixed mode SPE of RP and strong cation exchange) and Strata X-A (a mixed mode SPE of RP and anion exchange) following the general procedures recommended by the manufacturer. Results showed that the extraction efficiency was in the order of Strata-X > Strata-X-C ≫ Strata-X-A. Even though a MeOH washing step was included in the Strata X-C procedure, it did not result in cleaner chromatogram or reduced matrix effect. Other methods to help extract the analyte from sample matrix included using 30% trichloroacetic acid or 4% phosphoric acid in combination with RP SPE or mix-mode SPE were also tried. However, the simple extraction using 1% phosphate buffer combined with RP Strata-X produced the cleanest extracts.

After comparing several C18 columns, an ethylene bridged hybrid C18 column (3.5 μm) was used for nearly all the method development experiments. Even though the column performance in terms of peak shape, retention time reproducibility, and separation of the analyte peak from interfering peaks (those observable under the ion transitions monitored) was acceptable, a significant and variable matrix-related signal enhancement was observed. We later found that a 2.6 μm core-shell Kinetex C18 column performed better in terms of peak shape, and matrix effects were greatly reduced with this column. Therefore, the Kinetex C18 column was used in the final method.

Table 3
Accuracy and precision in 6 sources of matrix.

Level (ng/g)	n	Found (ng/g)	SD	CV (%)	Mean accuracy (%)
50	30	49.9	4.9	9.8	99.8
100	27	97.7	6.9	7.0	97.7
250	30	246	17.7	7.2	98.5
500	27	501	50.5	10.1	100.2
1000	30	991	76.0	7.7	99.1

During the method development, we first tried commercially available d_7-penicillin G (Toronto Research Chemicals; Toronto, Canada) as an internal standard. However, it was not useful because it had different extent of matrix effect than DCCD, making quantitation difficult. It was clear that using a stable isotope labeled DCCD as internal standard would improve the quantitation. Because the compound was not commercially available when this work was initiated, we synthesized d_3-DCCD by a two-step procedure. Ceftiofur was first hydrolyzed using a reducing agent DTE and the resulting DFC was treated with d_6-cystine which underwent a thiol-disulfide exchange reaction with DFC under slight basic condition to give d_3-DCCD [16,17]. The identity of the product was verified by comparing the MS/MS full scan spectrum with the unlabeled DCCD (Fig. 1) using a triple quadrupole mass spectrometer. Also, the labeled and unlabeled DCCD showed identical retention times under different chromatographic conditions. The molecular ions (MH^+) of DCCD and d_3-DCCD were m/z 549 and 552, respectively. Both were dissociated to main product ions at m/z 397, 366, 241, and 183. A MS/MS spectrum and rationalization of DCCD fragmentation were previously reported [12]. The only major difference between the

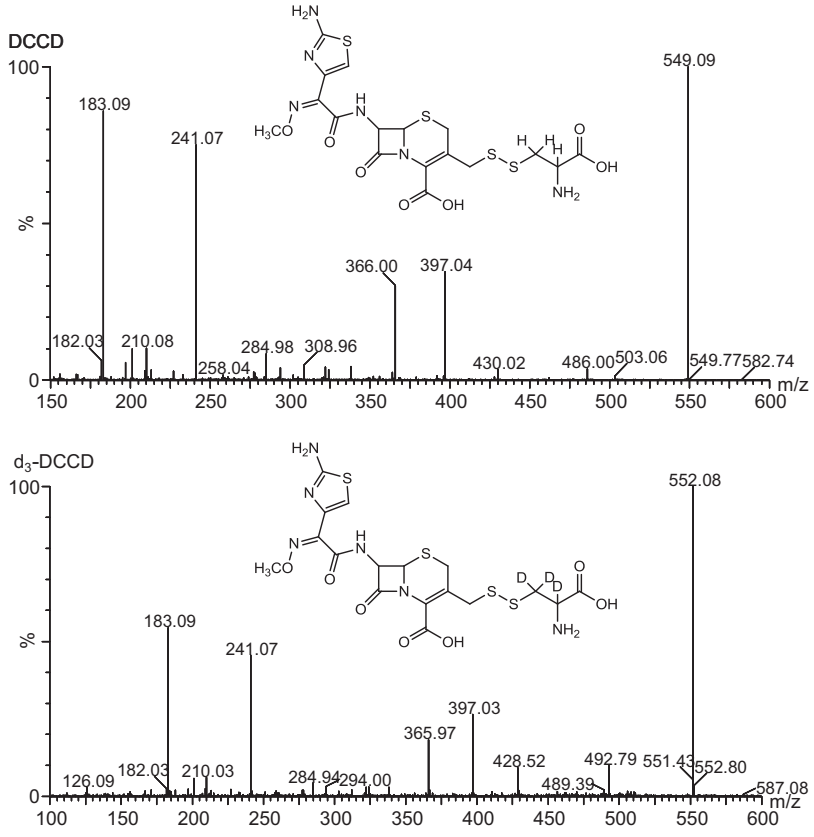

Fig. 1. MS/MS full scan mass spectra of DCCD and d3-DCCD.

Table 4
Comparison of calibration methods based on linear least-square regression.[a]

Run	Weighting factor	Slope[a]	Intercept	R^2	Mean residual (median; min to max)
	No	0.001741	0.02660	0.9990	−7.7 (−1.5; −39.1 to 9.0)
A	$1/x$	0.001772	0.01101	0.9980	0.0 (−2.1; −9.0 to 13.0)
	$1/x^2$	0.001807	0.00765	0.9940	0.0 (−0.05; −9.8 to 12.0)
	No	0.001911	−0.00506	0.9990	6.4 (2.3; −4.7 to 32.1)
B	$1/x$	0.001883	0.00924	0.9989	0.0 (−1.6; −4.1 to 6.0)
	$1/x^2$	0.001866	0.01083	0.9983	0.0 (−1.0; −3.4 to 6.4)
	No	0.002033	0.02383	0.9980	−9.6 (−5.3; −43.2 to 6.2)
C	$1/x$	0.002078	0.00094	0.9978	0.0 (−1.5; −6.9 to 5.7)
	$1/x^2$	0.002083	0.00050	0.9973	0.0 (−1.5; −6.9 to 5.6)

[a] The concentration of the internal standard was arbitrarily set as 1 for all data processing.

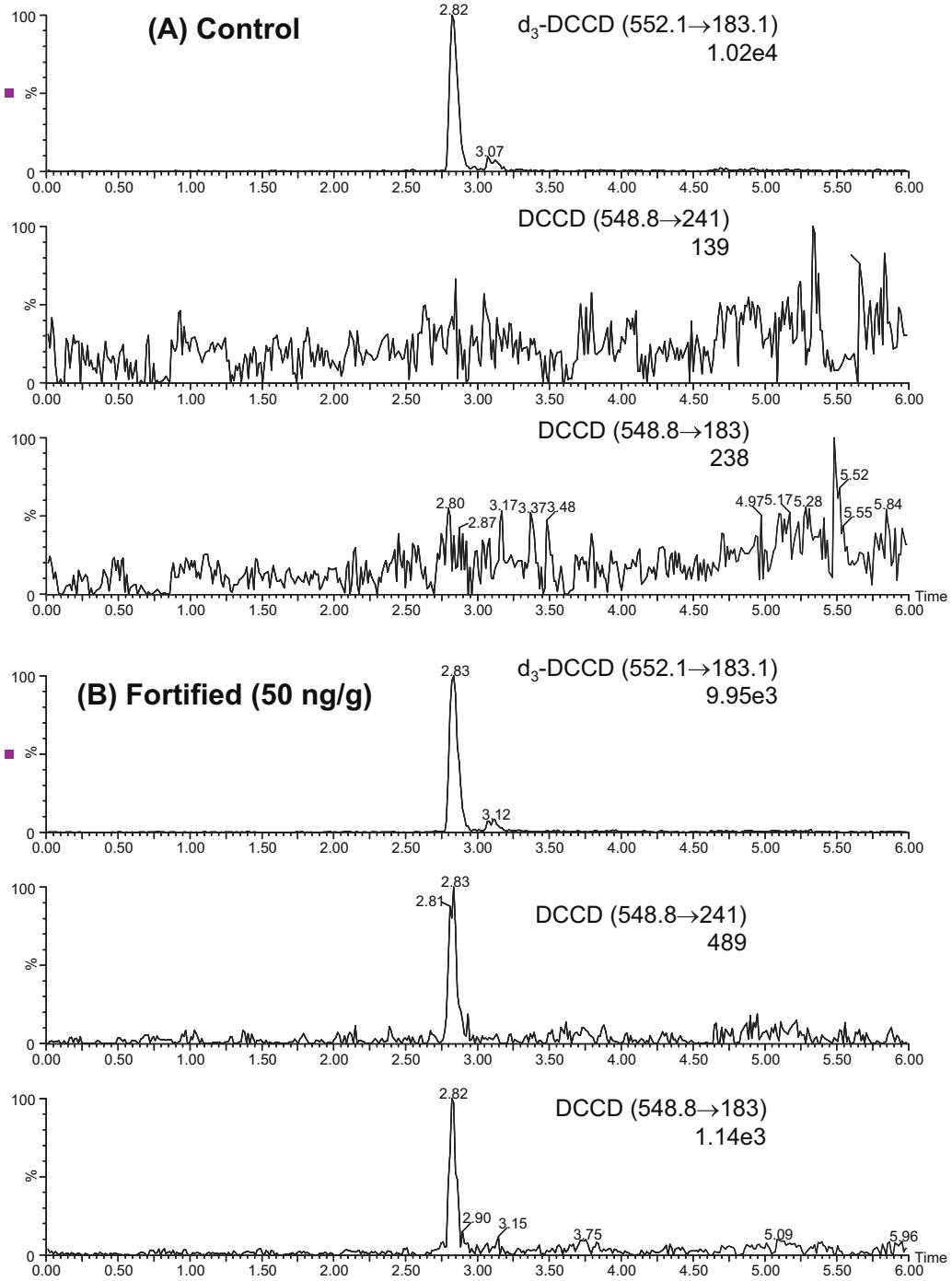

Fig. 2. Typical chromatograms: (A) a control kidney sample, (B) a fortified control kidney sample at 50 ng/g, and (C) an incurred kidney sample at 1450 ng/g.

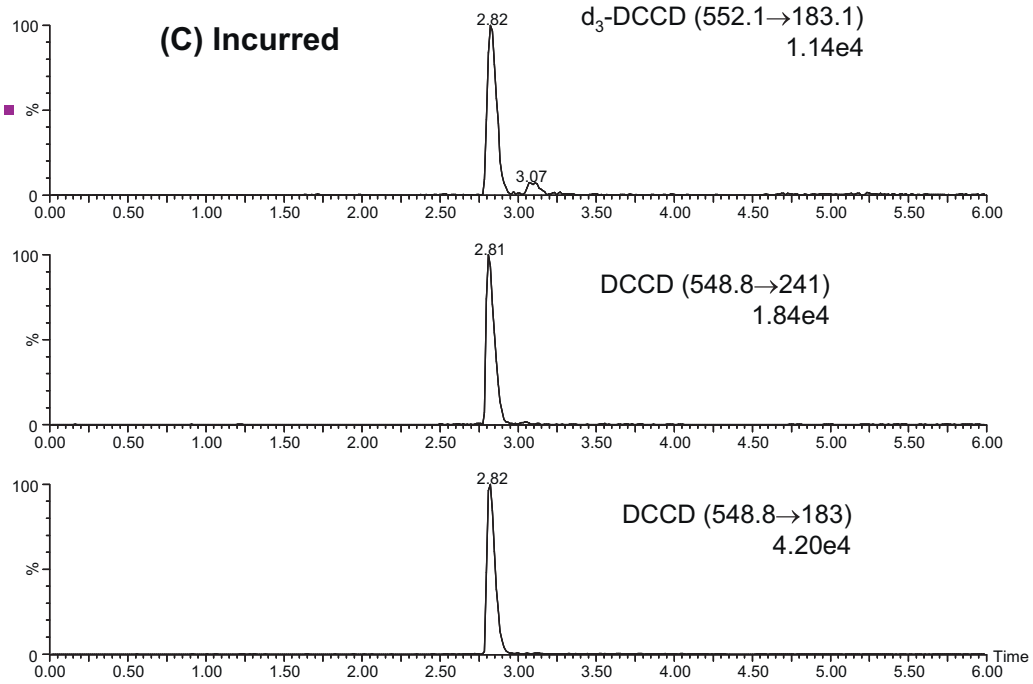

Fig. 2. (*continue*) Typical chromatograms:(C) an incurred kidney sample at 1450 ng/g.

present DCCD MS/MS spectrum and the previously reported one is in the relative ion abundance, probably due to the different types of instrument used (triple quadrupole versus ion trap). The ion transitions for DCCD monitored in our method are the same as in the method reported by Mastovska and Lightfield [14].

It was observed that ~1–1.5% of d_3-DCCD added to the control kidney samples was converted to unlabeled DCCD after going through the extraction procedure, presumably because of the thiol-disulfide exchange with free cysteine in the sample matrix. Therefore, we decided to add d_3-DCCD after the SPE cleanup in order to avoid such conversion. Due to the limitation of not being able to track the analyte loss during the extraction with d_3-DCCD, it was necessary to prepare the calibration curves in matrix.

3.2. Method validation

Chromatograms of control, fortified, and incurred samples are shown in Fig. 2. The retention times of DCCD and d_3-DCCD were both ~2.82 min. The average inter-day accuracy ranged from 97.7 to 100.2% and the inter-day precision (CV%) ranged from 7.0 to 10.1% (Table 3). Both accuracy and precision are considered acceptable [18]. The standard curves with $1/x$ weighting were linear from 25 to 2000 ng/g with $R^2 > 0.99$. The $1/x$ weighting was chosen after comparison with $1/x^2$ weighted and unweighted regression lines over three runs. As shown in Table 4, without weighting, R^2 values were the highest, but the residuals were too large. On the other hand, the residuals and distributions were very similar between $1/x$ and $1/x^2$. However, $1/x$ provided a slightly better R^2 value than $1/x^2$. The LOQ was 25 ng/g, at which the average accuracy was 96% with a CV of 7.8%.

In terms of method specificity, blank controls from six independent sources were analyzed and the interferences in those controls were less than 20% of the LOQ. Mixed standard solutions of 36 common veterinary drugs including sulfonamides, tetracyclines, penicillin G, macrolides, etc., were injected under the acquisition method and no interference was found.

The limit of confirmation (LOC), defined as the lowest concentration met all confirmation criteria including ion ratio, retention time, and signal to noise ratio. The LOC was 50 ng/g, at which the passing rate during the validation was 93.3% (28 out 30). The total passing rate in all fortified samples was 98.6% (142 out of 144). None of the controls were confirmed positive (0% false positive rate).

The two incurred samples were analyzed in 3 replicates each on 3 separate days ($n = 9$) and the DCCD concentrations were 1323.5 ± 143.6 (CV = 10.9%) and 406.3 ± 18.4 ng/g (CV = 4.5%), respectively.

Stability of DCCD in both matrix and processed extracts was evaluated by triplicate analyses of either incurred kidney samples or fortified samples. For matrix stability, the initial results of the incurred samples at above two levels were used as reference points. Changes after storage at −20 °C for 5 weeks, −80 °C for 5 months, and at refrigerated temperature for 12 h were within −3.9% to 6.9%, −3.4% to −11.0%, and −11.6% to 15.6%, respectively. However, the samples were less stable at rt with changes between −12.7% and −31.0% after 6 h. For extract stability, the initial results of fortified samples at 50 and 1000 ng/g were used as reference points. Changes after storage at refrigerated temperature for 2 days or at −20 °C for 38 days were less than ±10%.

Finally, the undiluted incurred kidney sample was also analyzed using the HPLC–UV method. The DFC (measured as DCA) concentration was found to be 2.55 ± 0.11 ppm ($n = 6$). Thus, the ratio between DCCD and DFC from this single sample is 0.52. The preliminary results suggest that DCCD may be a significant contributor to the total concentration of ceftiofur residues. Our next step is to obtain incurred samples through a controlled incursion study and investigate the relationship between these two measurements over a broad range of concentrations.

4. Conclusion

A LC–MS/MS method has been developed and validated for the determination and confirmation of DCCD, a ceftiofur metabolite, in bovine kidney. By using a simple extraction procedure, a labeled internal standard, and optimized instrumental conditions, a rapid, simple, and sensitive method was developed with a LOQ of 25 ng/g

and a LOC of 50 ng/g. Thorough validation was conducted to ensure the method performance.

Acknowledgments

We would like to thank Dr. Clifton K. Fagerquist and Mr. Alan R. Lightfield (USDA) for providing helpful information regarding DCCD stability and storage conditions with a coauthor (SF). We would also like to acknowledge a helpful communication between Pfizer Animal Health and a coauthor (PK) on this paper regarding the synthesis of unlabeled DCCD.

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