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Determination of ceftiofur and its desfuroylceftiofur-related metabolites in swine tissues by high-performance liquid chromatography

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Abstract

An HPLC method was developed and validated for the determination of ceftiofur-related metabolites that have the potential to be microbiologically active in swine muscle, kidney, liver and fat. Its performance was evaluated against incurred-residue swine tissues. This method is based on the cleavage of the disulfide and/or thioester bonds between the metabolites and their conjugate sulfur containing moiety using dithioerythritol to yield desfuroylceftiofur, and further stabilization to desfuroylceftiofur acetamide. The limit of quantitation was 0.1 μg ceftiofur equivalents/g tissue. The assay is specific for ceftiofur-related metabolites when evaluated against commercially available antibiotics for swine.

1. Introduction

Ceftiofur sodium (Naxcel/Excenel Sterile Powder, Upjohn) is a broad spectrum cephalosporin antibiotic which was approved by the FDA for intramuscular injection in the treatment of certain respiratory diseases in beef cattle, dairy cattle, day-old chickens and swine [1–3]. The metabolism of ceftiofur has been described in rats [4], dairy cattle [5] and swine [6]. A scheme on the metabolism of ceftiofur based on these data is shown in Fig. 1. Ceftiofur is rapidly metabolized to desfuroylceftiofur and furoic acid. Furoic acid will generate metabolites found in the normal urine of humans [7]. Desfuroylceftiofur is further metabolized to disulfides like desfuroylceftiofur cysteine disulfide (DFC-cysteine). It is also bound to macromolecules in plasma and tissues. Free desfuroylceftiofur (which contains an intact β-lactam ring) is the microbiologically active metabolite of ceftiofur. Since the binding of desfuroylceftiofur to other molecules is probably reversible [4], a method that determines both free and bound desfuroylceftiofur would be appropriate for the determination of ceftiofur-related metabolites in incurred animal tissues that have the potential to be microbiologically active.

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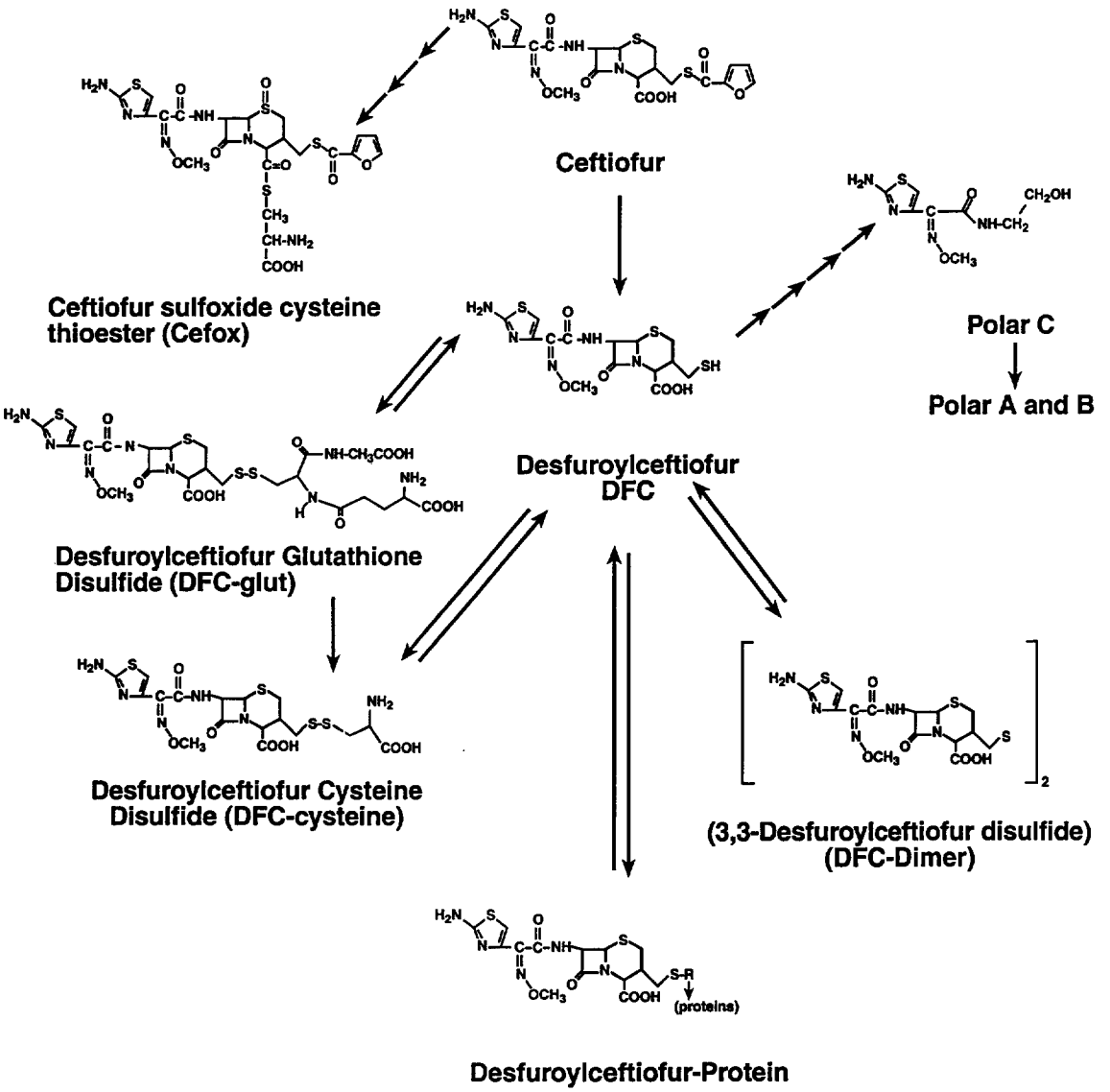

Fig. 1. Proposed metabolism of ceftiofur in rats, cattle and swine.

An HPLC assay developed for the determination of desfuroylceftiofur (DFC) in plasma [8] does not give satisfactory results when applied to animal tissues because of interference from endogenous compounds. This report describes a method that was developed for determining and quantifying ceftiofur and related metabolites containing the desfuroylceftiofur moiety in swine kidney, muscle, liver and fat.

2. Experimental

2.1. Analytical reference standard

Ceftiofur hydrochloride (ceftiofur syn-oxime, U-64,279A), lot No. Upjohn Control Reference Standard, Issue E, 893 μg/mg potency as ceftiofur free acid equivalents (CFAE), was supplied by the Upjohn Company.

2.2. Solvents, reagents and cartridges

Use distilled, deionized water and reagent grade solvents. The following solvents, reagents and cartridges were used: (a) Dithioerythritol (DTE), 99% (Aldrich, Milwaukee, WI, USA). (b) Iodoacetamide: 97% (Aldrich). (c) Borate buffer pH 9: weigh 19 g of sodium borate and 3.7 g of potassium chloride, dilute to 1000 ml with water. (d) Phosphate buffer (0.025 *M*, pH 7): weigh 3.4 g of potassium phosphate monobasic, add ca. 700 ml of water, adjust pH to 7 with potassium hydroxide and dilute to 1000 ml with water. (e) Extracting solution: 0.4% (w/v) DTE in borate buffer. (f) Iodoacetamide solution: 14% (w/v) in phosphate buffer. (g) Other solutions: sodium hydroxide, 0.01 *M*; sodium chloride, 0.1 *M*; calcium chloride, 0.1 *M*; phosphoric acid, 5%; and acetic acid, 5%. (h) C_{18} elution solution: acetonitrile–water (15:85, v/v). (i) SAX prewash solution: methanol–0.1 *M* sodium chloride (25:75, v/v). (j) SAX elution solution: acetonitrile–5% acetic acid in water (5:95, v/v). (k) SCX prewash solution: methanol–0.1 *M* calcium chloride (25:75, v/v). (l) SCX elution solution 1: acetonitrile–0.1 *M* sodium chloride (5:95, v/v). (m) SCX elution solution 2: acetonitrile–0.1 *M* sodium chloride (1:9, v/v). (n) Mobile phase A: 0.1% trifluoroacetic acid (TFA) in water. (o) Mobile phase B: 0.1% TFA in acetonitrile. (p) Mega Bond Elut C_{18} SPE cartridges 1 g 6 ml, Varian Sample Preparation Products. (q) Bond Elut LRC SAX SPE cartridges 500 mg 10 ml, Varian Sample Preparation Products. (r) Bond Elut LRC SCX SPE cartridges 100 mg 10 ml, Varian Sample Preparation Products.

2.3. HPLC system

Three HPLC systems were used. A Waters 600 E powerline multi-solvent delivery system, controller and pump equipped with a 717 Plus autosampler and a 996 photo diode array detector scanning a range of wavelengths centered at 266 nm. A Perkin-Elmer multi-solvent delivery system, controller and pump equipped with a Perkin-Elmer Iss-200 autosampler and a Perkin-Elmer tunable absorbance detector monitoring at 266 nm. Two Shimadzu pumps equipped with a Shimadzu mixing chamber, Waters Wisp 710B autosampler and a Waters 486 tunable absorbance detector monitoring at 266 nm.

2.4. HPLC conditions

A Keystone Scientific BDS Hypersil C_{18} column (5 μm, 250 × 4.6 mm) was used, equipped with a Keystone Scientific BDS Hypersil C_{18} Guard Column. The volume of injection was 500 μl.

For swine muscle and kidney the gradient was linear 0–35% B from 0 to 35 min. The flow-rate was 1.0 ml/min. The column was washed with 50% B for 15 min (1.5 ml/min) and equilibrated with starting conditions (0% B) for 20 min before the next injection.

For swine liver and fat the gradient was isocratic (15% B) from 0 to 5 min, it increased linearly to 25% B from 5 to 10 min. The flow-rate was 1.0 ml/min. The column was washed with 50% B for 15 min (1.5 ml/min) and equilibrated with starting conditions (0% B) for 20 min before the next injection.

2.5. Quantitative standard curve solutions

Standard stock solution preparation

An accurately weighed amount of ceftiofur hydrochloride was dissolved in 100 ml of 0.025 *M* phosphate buffer (pH 7) to give approximately 250 μg CFAE/ml stock solution. This solution was transferred into 1.5 ml aliquots and stored, for a period no longer than two months, at −20°C until use.

Calibration standards preparation

On each assay day three solutions of ceftiofur free acid were prepared by accurately diluting measured aliquots of the 1.5 ml of the 100 μg CFAE/ml stock solution to the appropriate volume of 0.025 *M* phosphate buffer (pH 7) to yield approximately 100, 10 and 1 μg CFAE/ml solutions. Six ceftiofur calibration standards were further prepared by diluting the above solutions to 15 ml of extracting solution or gram tissue

equivalent to give the following concentrations: 10, 5.0, 1.0, 0.5, 0.25, and 0.05 μg CFAE/g tissue equivalent.

2.6. Fortified tissue preparation

Tissue was collected from six independent sources (Pease Packing, Scotts, MI, USA) and was passed through the meat grinder twice. Two 10-g (±0.05) aliquots were taken from each animal tissue and analyzed for background response. The remaining tissue was frozen.

After assaying for background response, the tissues were thawed at 5°C overnight. Aliquots of 120 g of tissue from each animal were pooled. The combined samples were mixed well with a spatula, passed through the meat grinder three times and divided into 10 (±0.05) g aliquots which were stored in 50 ml polypropylene screw cap centrifuge tubes at −20°C.

On each assay day, the appropriate number of 10-g tissue aliquots were removed from the freezer, allowed to thaw and placed in separate Waring blender bowls (or centrifuge bottles for fat). Fortification of each aliquot with ceftiofur solutions was done directly on the tissues. The tissues were allowed to set at room temperature for approximately 5 min and then were extracted and processed as described below simultaneously with the calibration standards.

Quadruplicate control swine kidney samples were fortified with ceftiofur at concentrations of 0, 0.1, 0.5, 2 and 10 μg CFAE/g tissue, quadruplicate control swine muscle samples were fortified at 0, 0.03, 0.1, 2 and 10 μg CFAE/g tissue. The rest of the tissue samples were fortified in triplicate with ceftiofur at concentrations of 0, 0.1, 1 and 10 μg CFAE/g tissue.

2.7. Tissue extraction

Fat

Extracting solution (20 ml) plus hexane (20 ml) were added to a 250-ml centrifuge bottle and homogenized with the fat sample at a Waring blender speed of medium for 5 min. The homogenate was centrifuged at 3000 *g* for 10 min. One gram equivalent (2 ml) of the aqueous (bottom) layer was transferred to a 50-ml centrifuge tube. Extracting solution (13 ml) was added for a total volume of 15 ml or 1 g tissue equivalent.

Other tissues

A 140-ml aliquot of extracting solution was added to each bowl and tissues were homogenized as above. One 15-ml aliquot (1 g tissue equivalent) of homogenate from each blender bowl was transferred to a 50-ml centrifuge tube.

Standards and fortified tissues were processed simultaneously.

2.8. Cleavage of ceftiofur and metabolites containing an intact β-lactam ring to yield desfuroylceftiofur

Tubes containing 15 ml of the fortified extracting solution (1 g tissue equivalent) or tissue homogenate were capped and placed in a shaking water bath at 50°C for 15 min.

2.9. Derivatization

A 3-ml aliquot of the iodoacetamide solution was added to each 50-ml tube. The tubes were mixed well and were left at room temperature for 30 min (Fig. 2). The pH was adjusted to 2.5–2.6 with 5% phosphoric acid and the tissue samples were centrifuged at 48 000 *g* for 20 min at 4°C. The calibration standard solutions were placed in the refrigerator (4°C).

2.10. C_{18} column cleanup

The C_{18} cartridges were preconditioned with 4 ml of methanol followed by 5 ml of phosphate buffer. The supernatant was charged onto the cartridges using gravity feed. The cartridges were washed with 5 ml of phosphate buffer, followed by 3 ml of 0.01 *M* sodium hydroxide. New collection tubes were put in the manifold. A 3-ml aliquot of the C_{18} elution solution was added and cartridges were allowed to drain by gravity feed. Then, vacuum was used to drain the remaining solution in the cartridge. The collection tubes

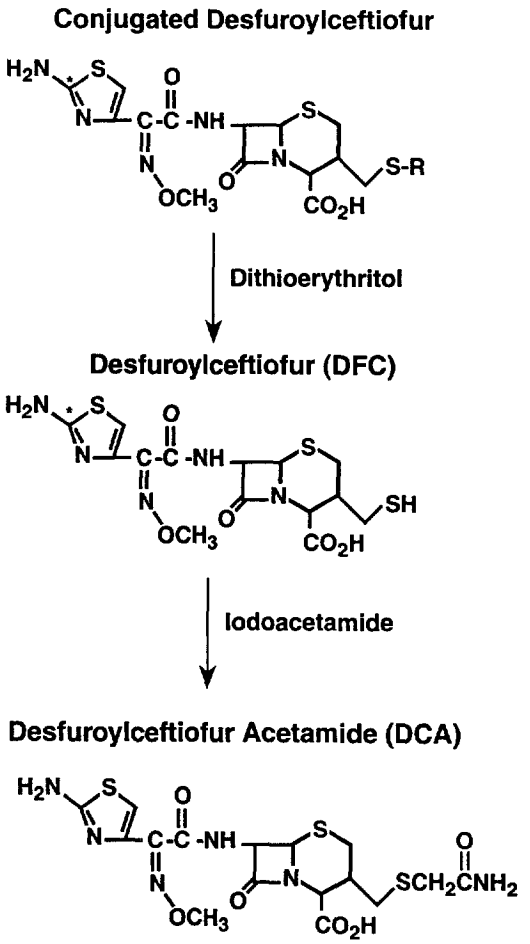

Fig. 2. Cleavage of desfuroylceftiofur (DFC) from the conjugated metabolites and stabilization by derivatization to desfuroylceftiofur acetamide (DCA).

were removed and 15 ml of water were added to each tube to give a total volume of 18 ml.

2.11. SAX column cleanup

The SAX cartridges were preconditioned with 2 ml of methanol, followed by 2 ml of SAX prewash solution and two times with 1 ml of water. The samples were transferred to the SAX cartridges, allowed to drain by gravity feed (vacuum was used to charge the fat samples at an approximate rate of 1 drop/s) and washed with 1 ml water. New collection tubes were placed in the manifold and the cartridge contents were eluted with 2.5 ml of SAX elution solution (gravity feed then emptied with vacuum). At this point, a 500-μl aliquot of this eluate was analyzed by HPLC for the fat samples and the corresponding calibration standards for fat. To all other collection tubes, 10 ml of water were added (for a total of 12.5 ml) and the tubes were mixed well.

2.12. SCX column cleanup

The SCX cartridges were preconditioned with 1 ml of methanol followed by 2 ml of SCX prewash solution and twice with 1 ml of water. The samples were transferred to the SCX cartridges and allowed to drain by gravity feed. The cartridges were then washed with 1 ml water.

2.13. Elution of the derivative

New collection tubes were placed on the manifold. Muscle and kidney samples and the corresponding standards were eluted with 2.5 ml of the SCX elution solution 1 (gravity feed then emptied with vacuum). Swine liver samples and the corresponding calibration standards were eluted with 2.0 ml of the SCX elution solution 2. An aliquot (500 μl) of this eluate was analyzed by HPLC.

In all cases, if columns plugged, positive pressure was applied to the cartridges with nitrogen to produce a flow-rate of ca. 1 drop/s.

2.14. Calculation of concentrations

A standard curve was generated from the DCA peak area vs. the ceftiofur concentration (μg CFAE/g tissue) of the standards. The accuracy of the regression (observed concentration/back-calculated concentration) was checked and recorded. Weighted regression (1/concentration^2) proved to be the best fit. Sample DCA concentrations were calculated as follows:

$$\textit{Concentration } (\mu g \textit{ CFAE } / g \textit{ tissue}) = \frac{\textit{DCA area} - \textit{intercept}}{\textit{Slope} \cdot 1 \textit{ g (equivalent sample weight)}}$$

2.15. Recovery

Recovery was determined by comparing measured concentrations of fortified tissue extracts with their theoretical concentrations.

2.16. Within-day and total (ignoring day) precision and accuracy of the method

The within-day precision was assessed using the coefficient of variation (relative standard deviation) calculated from the replicates measured for the same concentrations for that day. The total (ignoring day) precision was assessed using the coefficient of variation (relative standard deviation) calculated from all replicates obtained for the same concentrations during the study. The accuracy was calculated as the percent difference between the measured and the theoretical concentration.

2.17. Limit of detection (LOD), limit of quantitation (LOQ) and limit of determination/decision (LODe)

The LOD is defined as the lowest concentration of that residue in the sample which can be detected, but not necessarily quantified, under the prescribed experimental conditions, and the LODe is defined as the measure of the lowest predictable value of concentration for which the corresponding instrument response can be differentiated from the intercept with a certain statistical confidence [9]. These values could not be calculated using the traditional procedures available in literature [9,10], since for this method there was no background response from the control samples, the variances increased with increasing concentrations, and the variation of the calibration standards was different from the variation of the fortified matrices. Thus, the LOD and the LODe were calculated as 3 and 10 times the square root of the mean square error ($M.S.E.^{\frac{1}{2}}$) of the lowest fortified sample, respectively. This is a conservative approach since the estimate of variability from the matrix blank readings should be much less than the estimate of variability from the lowest fortification level used. Since extrapolation beyond the standard curve is not acceptable, for this study the LOD was the lowest calibration standard used.

The LOQ for this study was the lowest fortified sample for which precision and accuracy were determined and found acceptable [9].

2.18. Assay specificity

The specificity of the HPLC-DCA assay was evaluated against the following commercially available cephalosporins and other antibiotics: Cephapirin, sodium salt; Dihydrostreptomycin, sulfate; Neomycin, sulfate; Penicillin G, sodium; Spectinomycin, sulfate tetrahydrate; Tetracycline, hydrochloride; Cefquinome sulfate, Cefoperazone sodium, and Cephacetril, sodium. Stock solutions were prepared by dissolving approximately 0.05 g of the antibiotics in 20 ml of water.

(A) An aliquot of each stock solution was diluted to 2.5 ml with the SCX elution solution to give an approximate concentration of 4 μg antibiotic/ml SCX solution (which was equivalent to 10 μg antibiotic/g tissue). Aliquots (500 μl) of the unprocessed materials were analyzed by HPLC.

(B) Of the above stock solutions 0.1 ml were diluted to 25 ml with phosphate buffer. The resulting solutions (1 ml) were used to fortify 10 g of matrix and further processed by the HPLC-DCA method. Fortification levels in all cases were of at least 10 μg antibiotic/g tissue. Simultaneously, matrices fortified with 0 and 2.14 μg CFAE/g tissue were analyzed.

2.19. Animal phase

(A) Twelve castrated crossbred male swine and twelve female swine, weighing approximately 25–40 kg at the start of the study were randomly assigned to receive either 5.0 or 7.5 mg ^{14}C-ceftiofur free acid equivalents/kg body weight (bw). Each swine received three consecutive intramuscular doses in the neck at a 24-h interval of its assigned treatment. Twelve hours

after administration of the last dose, the animals were euthanized.

(B) Twelve castrated crossbred male swine and twelve female swine, weighing approximately 22–27 kg at the start of the study, received 3 mg ^{14}C-ceftiofur free acid equivalents/kg bw as intramuscular injections in the neck during three consecutive days. Animals were randomly assigned to be slaughtered at either 12, 72 or 120 h after the last dose.

After euthanitization the liver, kidneys, fat (500 g) and skeleton muscle (500 g) were harvested. Tissues were ground twice in a meat grinder fitted with a 5 mm face plate and divided into 10-g aliquots. Aliquots were frozen at −20°C until assayed. On each assay day, the appropriate number of 10-g tissue aliquots were removed from the freezer, allowed to thaw and placed in separate Waring blender bowls (or centrifuge bottles for fat) to be extracted.

2.20. Analytical methods for the animal phase

Determination of total ceftiofur-related residues

Total ceftiofur-related residues were determined by total ^{14}C radioactivity measurements as follows: triplicate weighed aliquots (about 0.5 g) of liver, kidney, fat and muscle were combusted in a Packard Tri-Carb Sample Oxidizer (Packard Instruments) and ^{14}C was counted as trapped CO_2 in a Packard Tri Carb Model 2000 Liquid Scintillation Counter (LSC).

Determination of ceftiofur-related metabolites containing an intact β-lactam ring

Ceftiofur-related metabolites containing an intact β-lactam ring were determined in the incurred tissues using the HPLC-DCA method described above.

3. Results and discussion

3.1. Method principle

An HPLC method (HPLC-DCA) developed for the determination of ceftiofur (syn-oxime) and desfuroylceftiofur-related metabolites that have the potential to be microbiologically active (contain an intact β-lactam ring) was evaluated in swine kidney, muscle, liver and fat. For evaluating this method, ceftiofur itself was used for fortification since the cleavage of its thioester bond by DTE parallels the reduction of the bonds between DFC and its conjugate, thus generating DFC in situ. All steps in this method are critical. Successful results can only be obtained by following closely all points considered in the methods section. Twelve samples can be prepared, in duplicate, by an experienced analyst in approximately 6–8 h, depending on the tissue. The assay makes use of HPLC equipment, col-

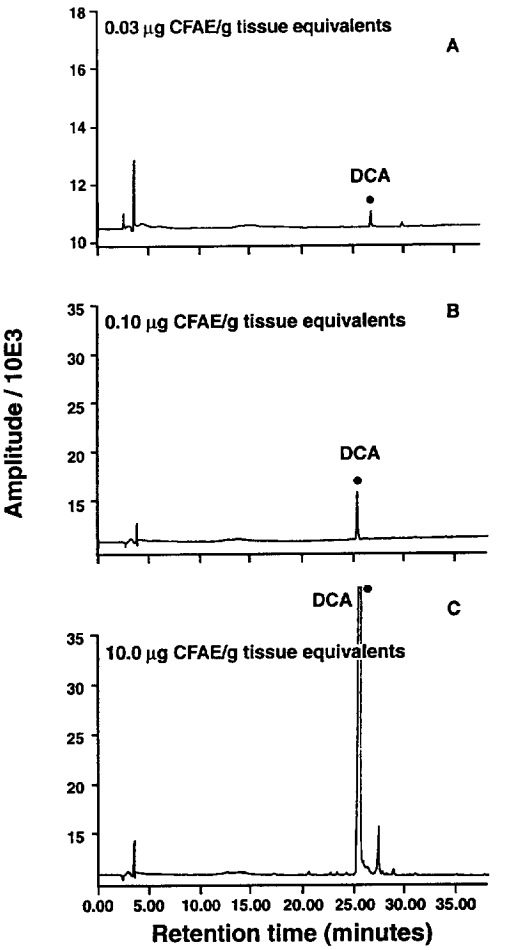

Fig. 3. Representative chromatograms from calibration standard solutions processed by the HPLC-DCA method (concentrations expressed as µg CFAE/g tissue equivalent).

umns and reagents that are commercially available to laboratories engaged in residue determination worldwide.

3.2. Calibration curves

The calibration curves were linear for the entire calibration range of 0.05–10 μg CFAE/g tissue. Calibration standard concentrations were back-calculated on each day of analysis using the corresponding regression line. Back-calculated values were in all cases within 10% of the theoretical value. Representative chromatograms obtained from the calibration standards are shown in Fig. 3.

3.3. Assay results

Recovery values of ceftiofur and desfuroylceftiofur-related metabolites containing an intact β-lactam ring from the different tissue matrices are presented in Tables 1–4. Average recovery values ranged from 70.37 to 85.01%, 74.72 to 88.0%, 88.27 to 94.90%, 85.44 to 89.40% for muscle, kidney, liver and fat, respectively, across concentrations. Representative chromatograms obtained from different control and fortified matrices are shown in Fig. 4.

The within-day precision and total precision (ignoring day) for the various fortification levels for each tissue are presented in Tables 1–4. For the 0.03 μg CFAE/g muscle fortification level,

Table 1
Accuracy (% recovery) of ceftiofur and related metabolites containing an intact β-lactam ring from fortified swine muscle

Fortified concentration (μg/g)	Day	n	Concentration recovered (μg/g)		C.V. (%)	Mean recovery (%)
			Mean	S.D.		
0.03	1	4	0.025	0.006	24.07	82.96
	2		0.028	0.005	18.36	94.82
	3		0.024	0.001	3.97	78.37
	4		0.025	0.003	10.71	81.93
	5		0.026	0.001	4.12	87.24
	Total	20	0.026	0.004	14.86	85.01
0.10	1	4	0.083	0.004	4.36	83.34
	2		0.087	0.008	9.23	87.38
	3		0.090	0.004	4.30	90.22
	4		0.073	0.008	11.16	72.55
	5		0.071	0.002	2.15	71.12
	Total	20	0.081	0.009	11.60	80.90
2.0	1	4	1.586	0.028	1.79	79.29
	2		1.445	0.016	1.08	72.25
	3		1.351	0.023	1.70	67.57
	4		1.347	0.065	4.81	67.33
	5		1.433	0.066	4.60	71.65
	Total	20	1.432	0.100	6.99	71.61
10.0	1	4	7.905	0.165	2.08	79.05
	2		7.191	0.361	5.03	71.91
	3		6.716	0.302	4.50	67.16
	4		6.574	0.133	2.03	65.74
	5		6.797	0.709	10.43	67.97
	Total	20	7.037	0.553	7.96	70.37

Table 2
Accuracy (% recovery) of ceftiofur and related metabolites containing an intact β-lactam ring from fortified swine kidney

Fortified concentration (μg/g)	Day	n	Concentration recovered (μg/g)		C.V. (%)	Mean recovery (%)
			Mean	S.D.		
0.10	1	4	0.103	0.014	13.91	103.38
	2		0.087	0.004	4.90	87.08
	3		0.069	0.009	12.54	68.54
	4		0.088	0.005	5.91	87.99
	5		0.093	0.005	5.84	93.00
	Total	20	0.088	0.014	16.08	88.00
0.50	1	4	0.423	0.007	1.75	84.65
	2		0.385	0.055	14.36	76.95
	3		0.429	0.014	3.19	85.82
	4		0.416	0.010	2.29	83.24
	5		0.416	0.006	1.51	83.30
	Total	20	0.422	0.038	9.03	83.10
2.0	1	4	1.555	0.106	6.84	77.76
	2		1.479	0.049	3.34	73.97
	3		1.693	0.032	1.90	84.65
	4		1.681	0.048	2.88	84.06
	5		1.549	0.018	1.17	77.43
	Total	20	1.591	0.100	6.26	79.57
10.0	1	4	7.886	0.339	4.30	78.86
	2		6.540	0.522	7.99	65.41
	3		7.739	0.186	2.40	77.39
	4		7.871	0.489	6.21	78.71
	5		7.327	0.545	7.43	73.27
	Total	20	7.472	0.650	8.70	74.72

the within-day coefficient of variation (C.V.) was larger than 15% on the first and second day of analysis (24.07% and 18.36%, respectively). For this same tissue and concentration level, the total (ignoring day) C.V. was 14.86%. For all remaining tissues and fortification levels analyzed the within-day and total (ignoring day) C.V. were less than 15%.

As explained in Section 2, the LOD for this study was the lowest calibration standard used. The LOQ was the lowest fortified sample for which the within-day and total precision were determined to be less than 15% and for which accuracy was determined to be on the average between 80 and 110% of the theoretical value. According to this LOQ criterion, the LOQ for the study was 0.1 μg CFAE/g tissue for all tissues. This concentration corresponded to the lowest fortification level analyzed in kidney, liver and fat, and to the second to lowest fortification level analyzed in muscle. The LOD and LOQ used in this study for the different tissues are shown in Table 5 together with the theoretical LOD and LODe calculated for this HPLC-DCA method with and without adjustment for recoveries. Given the low background response and the low variability observed with the method, low concentrations of ceftiofur and desfuroylceftiofur-related metabolites containing an intact β-lactam ring can be detected. The calculated LOD were 0.01, 0.03, 0.02 and 0.02 μg CFAE/g tissue for muscle, kidney, liver and fat,

Table 3
Accuracy (% recovery) of ceftiofur and related metabolites containing an intact β-lactam ring from fortified swine liver

Fortified concentration (μg/g)	Day	n	Concentration recovered (μg/g)		C.V. (%)	Mean recovery (%)
			Mean	S.D.		
0.10	1	3	0.088	0.002	1.81	88.13
	2		0.086	0.004	4.80	86.28
	3		0.088	0.010	11.24	88.43
	4		0.111	0.003	2.70	110.61
	5		0.101	0.008	7.89	101.07
	Total	15	0.095	0.011	11.69	94.90
1.0	1	3	0.951	0.018	1.91	95.06
	2		0.908	0.018	2.02	90.85
	3		0.880	0.025	2.85	88.00
	4		0.991	0.064	6.48	99.13
	5		0.883	0.015	1.68	88.28
	Total	15	0.923	0.053	5.69	92.26
10.0	1	3	9.299	0.234	2.52	92.99
	2		9.878	0.699	7.07	98.78
	3		7.095	0.141	1.98	70.95
	4		9.320	0.263	2.82	93.20
	5		8.544	0.161	1.89	85.44
	Total	15	8.951	0.962	10.75	88.27

respectively, after adjusting for recovery. The calculated LODe were 0.05, 0.10, 0.06 and 0.06 μg CFAE/g tissue for muscle, kidney, liver and fat, respectively, after adjusting for recovery.

3.4. Analysis of incurred tissues by the HPLC-DCA method

The average concentrations of ceftiofur-related metabolites containing an intact DFC moiety (i.e. an intact β-lactam ring) in the tissues of swine slaughtered at 12 h after the last of three daily doses at 7.5, 5.0 and 3.0 mg CFAE/kg bw were the following: for muscle, 0.70, 0.52 and 0.29 μg CFAE/g tissue; for kidneys, 4.16, 3.20 and 1.1 μg CFAE/g tissue; for liver, 1.29, 0.94 and 0.64 μg CFAE/g tissue; and for fat 1.00, 0.75 and 0.50 μg CFAE/g tissue. The concentration of metabolites containing an intact DFC moiety in swine tissues was proportional to the dose level. No ceftiofur-related metabolites containing an intact β-lactam ring were detected with this assay in the muscle and liver of swine dosed at 3.0 mg CFAE/kg bw and slaughtered at either 72 or 120 h after receiving the last dose. Only one kidney sample and one fat sample from the four animals dosed at 3.0 mg CFAE/kg bw and slaughtered at 72 h showed positive results for ceftiofur-related metabolites containing an intact β-lactam ring, while none were detected in animals slaughtered at 120 h after the last dose.

The high precision (low variability) of the HPLC-DCA assay is illustrated by the coefficient of variation between duplicate assays for each sample. The C.V. was less than 15% for all duplicate samples analyzed, and ranged from 0.06 to 4.95, 0.26 to 12.87, 0.21 to 12.33 and 0.11 to 12.08% for muscle, kidney, liver and fat, respectively. For each analysis day six quality control samples were analyzed. The recovery from the quality control samples averaged between 80 and 100% for each assay day.

Table 4
Accuracy (% recovery) of ceftiofur and related metabolites containing an intact β-lactam ring from fortified swine fat

Fortified concentration (μg/g)	Day	n	Concentration recovered (μg/g)		C.V. (%)	Mean recovery (%)
			Mean	S.D.		
0.10	1	3	0.092	0.004	4.48	91.58
	2		0.089	0.002	2.38	89.35
	3		0.109	0.004	3.99	108.70
	4		0.077	0.003	4.24	77.06
	5		0.080	0.009	11.30	80.32
	Total	15	0.089	0.013	14.21	89.40
1.0	1	3	0.971	0.123	12.70	97.06
	2		0.789	0.087	11.02	78.92
	3		0.736	0.027	3.62	73.57
	4		0.888	0.022	2.46	88.78
	5		0.889	0.033	3.70	88.90
	Total	15	0.859	0.104	12.10	85.44
10.0	1	3	8.569	0.454	5.29	85.69
	2		8.549	0.081	0.95	85.49
	3		8.056	0.835	10.37	80.55
	4		8.757	0.236	2.69	87.57
	5		9.491	0.136	1.43	94.91
	Total	15	8.694	0.632	7.27	86.84

3.5. Correlation between total residues and microbiologically active residues in incurred swine tissues.

The residues of ceftiofur in tissues include polar residues and microbiologically active metabolites (those containing an intact β-lactam ring). Both types of residues are detected as "total residues" by combustion of ^{14}C in tissues. In contrast, only microbiologically active metabolites of ceftiofur (those containing an intact β-lactam ring) are detected by the HPLC-DCA assay. Thus, total residue concentrations in tissues obtained by combustion analysis should be higher than residue concentrations detected by the HPLC-DCA method. As expected, all concentration measurements obtained by combustion analysis were higher than those obtained by the HPLC-DCA method in this study. Fig. 5 shows the plots of total residue concentrations determined by ^{14}C combustion vs. residue concentrations by the HPLC-DCA method for each tissue, together with the regression line and the corresponding confidence limits. The correlation coefficients between both assays were 0.98, 0.88, 0.89, and 0.84 for muscle, kidney, liver and fat, respectively. Total residue concentrations in tissues can be predicted from HPLC-DCA measurements that fall within the range of concentrations studied, using the regression equations.

3.6. Ruggedness of the method

Different laboratories should be able to implement an analytical method and obtain the same performance obtained by the laboratory where the assay was originally developed. The HPLC-DCA assay was evaluated using a minimum of triplicate samples at four different fortification levels for at least two days at CEPHAC Research Centre, France and at the Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa, Japan. In both laboratories, recoveries of ceftiofur from fortified tissues were greater than 80% with within-day and total (ignoring day) coefficient of variations smaller than 15%.

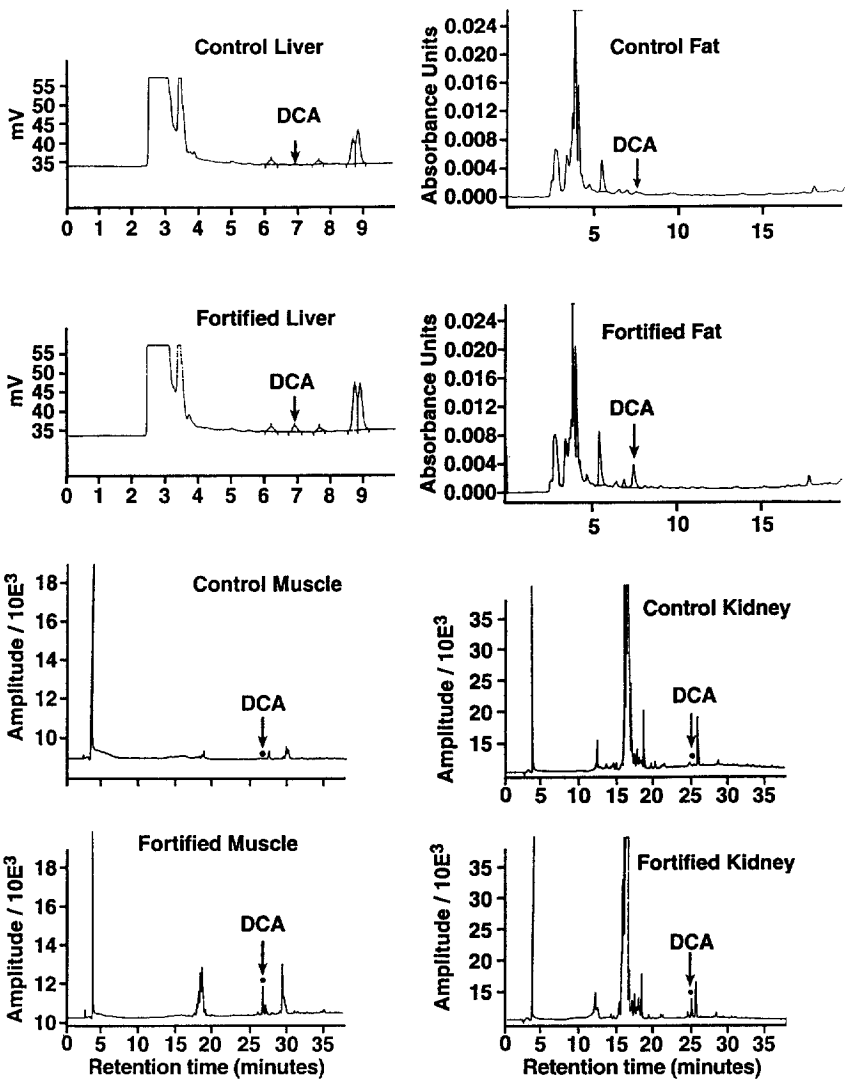

Fig. 4. Representative chromatograms obtained from various control (0 μg CFAE/g tissue) and fortified (0.1 μg CFAE/g tissue) matrices processed by the HPLC-DCA method.

3.7. Specificity of the assay against several commercially available antibiotics

No interference was observed with the non-cephalosporin antibiotics dihydrostreptomycin, neomycin and spectinomycin when they were chromatographed without derivatization or following processing by the HPLC-DCA method. The β-lactam antibiotic, penicillin G, absorbed weakly at 266 nm or had impurities which showed up in the chromatogram without derivatization. Tetracycline also absorbed at 266 nm when not derivatized, but at a considerably longer retention time than DCA. Once derivatized and processed by the HPLC-DCA method, both penicillin G and tetracycline no longer appeared in the chromatograms.

All of the cephalosporins, cephapirin, cefquinome, cefoperazone and cephacetril, when not derivatized, absorbed at 266 nm and were observed in the chromatograms. Only cefquinome had a retention time similar to DCA.

Table 5
Limit of detection (LOD), limit of quantitation (LOQ) and limit of determination/decision (LODe) of the HPLC-DCA method in different swine tissue matrices

Tissue	Study		Calculated		Recovery adjusted	
	LOD (μg CFAE/g)	LOQ (μg CFAE/g)	LOD (μg CFAE/g)	LODe (μg CFAE/g)	LOD (μg CFAE/g)	LODe (μg CFAE/g)
Muscle	0.03	0.10	0.01	0.04	0.01	0.05
Kidney	0.10	0.10	0.03	0.09	0.03	0.10
Liver	0.05	0.10	0.02	0.06	0.02	0.06
Fat	0.05	0.10	0.02	0.05	0.02	0.06

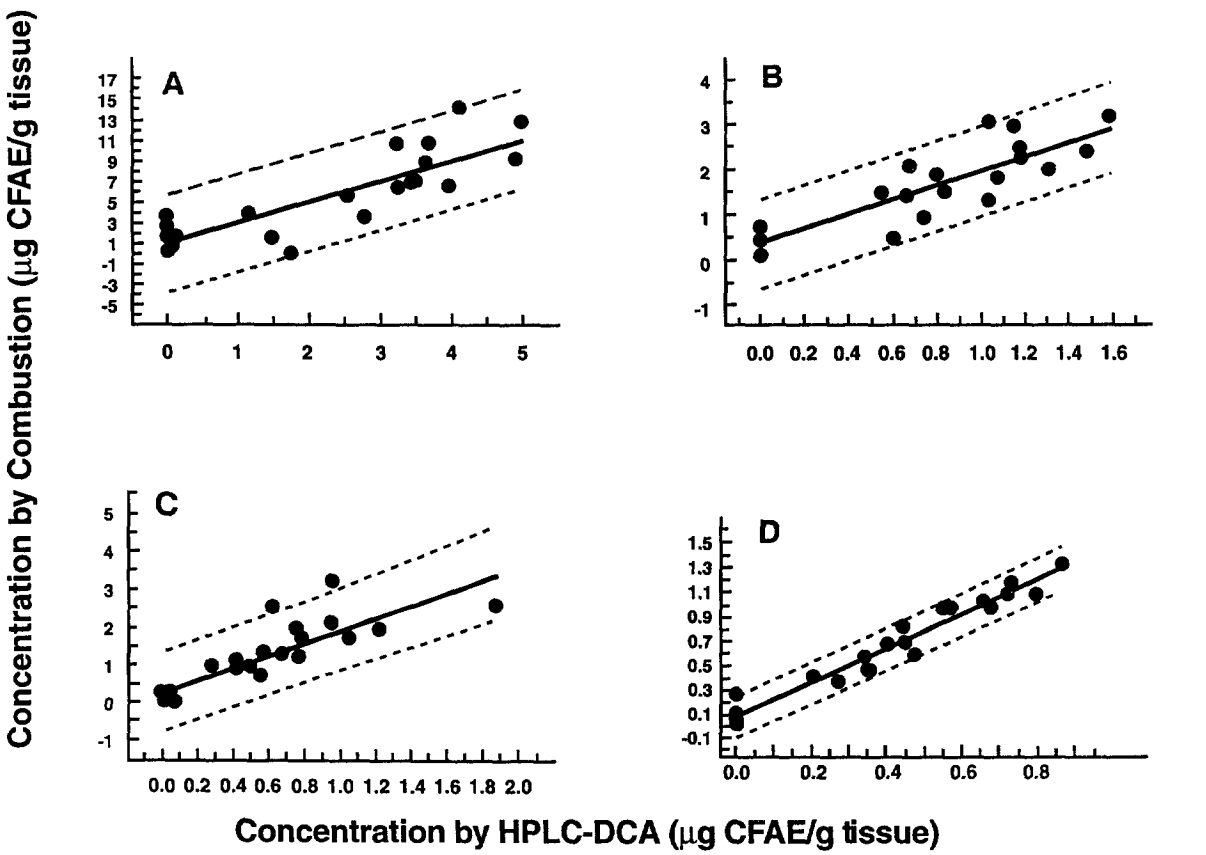

Fig. 5. Correlation between total ceftiofur residues and microbiologically active ceftiofur residues in incurred swine tissues. (A) Kidney: Tot (μg/g) = 1.22 + 2.01 HPLC-DCA (μg/g), $r^2 = 0.88$; (B) liver: Tot (μg/g) = 0.34 + 1.60 HPLC-DCA (μg/g), $r^2 = 0.89$; (C) fat: Tot (μg/g) = 0.22 + 1.79 HPLC-DCA (μg/g), $r^2 = 0.84$; (D) muscle: Tot (μg/g) = 0.08 + 1.38 HPLC-DCA (μg/g), $r^2 = 0.98$.

When these cephalosporins were subjected to the derivatization and purification process of the HPLC-DCA method, cefquinome, cephacetril and cefoperazone were completely removed and did not appear in the chromatograms. Cephapirin, however, appeared in the chromatograms after derivatization and purification by the HPLC-DCA method as a peak that eluted approximately 1 min earlier than DCA. Cephapirin should not interfere in the HPLC-DCA assay for ceftiofur metabolites because of its different retention time with DCA. In case of doubt cochromatography is recommended.

4. Conclusions

An HPLC method was developed and validated for the determination and quantitation of ceftiofur and desfuroylceftiofur (DFC)-related metabolites containing an intact β-lactam ring in swine muscle, kidney, liver and fat.

This HPLC-DCA method is based on the cleavage of the disulfide and/or thioester bonds between the metabolites and their conjugate sulfur containing moiety using DTE to yield desfuroylceftiofur, derivatization of DFC to the more stable derivative DCA, and concentration and purification by SPE columns. The HPLC method allows for determination and quantitation of ceftiofur-related metabolites containing an intact β-lactam ring in tissues at concentrations of 0.05–10 μg CFAE/g tissue with precision and accuracy. The resulting calibration curves are linear with correlation coefficients >0.99 over the range evaluated. The HPLC-DCA method makes use of HPLC equipment, columns and reagents that are commercially available to laboratories engaged in residue determination worldwide and has been successfully implemented in laboratories in France and Japan. It is specific for ceftiofur and related metabolites when evaluated against several commercially available antibiotics for swine. Total residue concentrations in swine muscle, kidney, liver and fat can be predicted with reasonable precision from HPLC-DCA measurements that fall within the range of concentrations, dose levels and slaughter times used in this study, which were 5 and 7.5 mg ceftiofur free acid equivalents (CFAE)/kg body weight, slaughtered at 12 h after the last dose, and 3 mg CFAE/kg bw slaughtered at 12, 72 or 120 h after the last dose with sample storage time of less than 6 months.

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