Assessment of Liquid Chromatography Tandem Mass Spectrometry Approaches for the Analysis of Ceftiofur Metabolites in Poultry Muscle

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Assessment of Liquid Chromatography Tandem Mass Spectrometry Approaches for the Analysis of Ceftiofur Metabolites in Poultry Muscle

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The use of cephalosporin antibiotics in veterinary practice is likely to play an important role in the development of β-lactam resistant bacteria. To detect off-label cephalosporin antibiotic usage an analytical method is needed that, besides the native compound, also detects active metabolites thereof. In this paper the applicability of three approaches for the quantitative analysis of ceftiofur using liquid chromatography coupled to tandem mass spectrometry is assessed, viz. (A) the analysis of ceftiofur, desfuroylceftiofur and/or desfuroylceftiofur cystein disulfide, (B) the derivatization of ceftiofur metabolites to desfuroylceftiofur acetamide and (C) the chemical hydrolysis using ammonia in order to produce a marker compound for ceftiofur. We found that approach A is not suited for quantitative analysis of total ceftiofur concentration nor for effectively detecting off-label use of ceftiofur. Approach B resulted in adequate quantitative results, but is considered to be a single compound method because it depends on the cleavage of a thioester group which is present in only a limited number of cephalosporin antibiotics. Approach C showed adequate quantitative results as well. In contrast to approach B, this approach is applicable to a range of cephalosporin antibiotics and therefore applicable as a broad quantitative screening of cephalosporin compounds in poultry tissue samples to indicate off-label use of cephalosporins in poultry.
breeding. Based on the research presented here, it is concluded that the multi-method following approach C is the most suited to detect off-label use of a range of cephalosporin antibiotics in the fight against emerging bacterial resistance.
Assessment of liquid chromatography tandem mass spectrometry approaches for the analysis of ceftiofur metabolites in poultry muscle

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The use of cephalosporin antibiotics in veterinary practice is likely to play an important role in the development of β-lactam resistant bacteria. To detect off-label cephalosporin antibiotic usage an analytical method is needed that, besides the native compound, also detects their active metabolites. In this paper the applicability of three approaches for the quantitative analysis of ceftiofur using LC-MS/MS is assessed, viz. (A) the analysis of ceftiofur, desfuroylceftiofur and/or desfuroylceftiofur cystein disulfide, (B) the derivatization of ceftiofur metabolites to desfuroylceftiofur acetamide and (C) the chemical hydrolysis using ammonia in order to produce a marker compound for ceftiofur. We found that approach A was not suited for quantitative analysis of total ceftiofur concentration nor for effectively detecting off-label use of ceftiofur. Approach B resulted in adequate quantitative results, but was
considered a single compound method because it depends on the cleavage of a thioester group, which is present in only a limited number of cephalosporin antibiotics. Approach C showed adequate quantitative results as well. In contrast to approach B, this approach is applicable to a range of cephalosporin antibiotics and therefore applicable as a broad quantitative screening of cephalosporin compounds in poultry tissue samples to indicate off-label use of cephalosporins in poultry breeding. Based on the research presented here, it was concluded that the multi-method following approach C is the most suitable to detect off-label use of a range of cephalosporin antibiotics.

**Keywords:** ceftiofur, metabolites, LC-MS/MS, poultry, cephalosporin antibiotics

**Introduction**

Cephalospirins are semi-synthetic β-lactam antibiotics, consisting of a six membered dihydrothiazine ring fused with a four membered β-lactam ring, which is responsible for the biological activity of the compounds. Nowadays, five generations of cephalosporins have been developed, reflecting their spectrum of activity, structural similarity and time of introduction (Hornish 2002). Ceftiofur (Figure 1a) is a third generation cephalosporin which is highly effective in the treatment of bacterial infections of the respiratory tract (Salmon 1995). Ceftiofur is registered for use in food-producing mammals (EU/37/2010) but occasionally off-label use occurs in poultry breeding. This is likely to contribute to the emergence of resistant bacteria like extended-spectrum β-lactamase (ESBL) producing bacteria (Hasman 2005, Pfeifer 2010, Chander 2011, Hur 2011, Persoons 2011). Cephalosporins are assigned as
critically important antimicrobials for human health (WHO 2007) and should therefore be used sparingly in veterinary practice to prevent the occurrence of bacterial resistance (Wittum 2007, EMEA 2009, Den Heijer 2010, Dutil 2010, Ewers 2010).

Ceftiofur is known to metabolize rapidly after intramuscular administration. Reported metabolites include desfuroylceftiofur (DFC), desfuroylceftiofur cysteine disulfide (DCCD), protein bound DFC and desfuroylceftiofur thiolacton (Jaglan 1989, Jaglan 1992, Gilbertson 1995, Berendsen 2009). Although no maximum residue limit (MRL) has been established for poultry muscle, it is important to realize that ceftiofur MRLs for other species are defined as the sum of all residues retaining the ß-lactam structure, expressed as DFC (EU/37/2010). This implies that all active metabolites should be included in the analysis of ceftiofur residues in food products.

Two main approaches for the analysis of ceftiofur residues in animal tissue and plasma have been reported. The first approach focuses on the analysis of one or more ceftiofur metabolites. LC-UV methods for the analysis of ceftiofur itself were reported by Navarre et al. (1999) and Nagata et al. (2004). An LC-UV method including ceftiofur and DFC was reported by Tyczkowska et al. (1993) and including ceftiofur, DCCD and DFC dimer by Moats et al. (1998). A very straightforward LC-MS/MS method monitoring only DCCD in kidney was reported by Mastovska et al. (2008).

The second approach includes an extraction and deconjugation of all protein bound DFC using dithioerythritol (DTE) followed by a derivatization of the resulting
free DFC using iodoacetamide to obtain desfuroylceftriaxone acetamide (DCA) as a marker residue (Figure 1e). This approach was first introduced for the analysis of plasma by Jaglan et al. (1990) in combination with UV detection and was optimized by Beconi-Barker et al. (1995) and Jiang et al. (2008) for the analysis of muscle. This second approach was validated by a multi-laboratory trial by Hornish et al. (2003) demonstrating its applicability. However, these methods are very laborious including several solid phase extraction (SPE) steps. Simplified methods applying the same approach were reported by De Baere et al. (2004) for the analysis of plasma and synovial fluid, by Drillisch et al. (2006) and Witte et al. (2010) for the analysis of several matrices among which serum and endometrial tissue.

Based on the results of our in vitro metabolism study (Berendsen 2009), it is expected that currently applied methods are likely to underestimate the residue levels of ceftiofur and cephapirin found in kidney samples because they do not include all active metabolites. Furthermore, it should be noted that cephalosporin multi-methods that include unstable cephalosporins (like ceftiofur and cefapirin) and are able to detect the active metabolites of these compounds, are still lacking. Therefore, a new approach for the analysis of ceftiofur including its metabolites was suggested (Berendsen 2009). This third approach is based on an alkaline hydrolysis of ceftiofur and its metabolites to produce 2-amino-α-(methoxyimino)-4-thiazoleacetamide (AMTA), Figure 1e) as a marker residue for the total amount of active ceftiofur metabolites. This approach also proved to be applicable for other cephalosporins, including cefcapene, cephapirin and cefquinome.

Based on incurred ceftiofur poultry muscle material a critical assessment of the three approaches is presented in this paper: (A) the analysis of ceftiofur, DFC and DCCD using the method reported by Mastovska et al. (2008), (B) the derivatization of
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ceftiofur metabolites to DCA as reported by Witte et al. (2010) and (C) a newly
developed method applying an alkaline hydrolysis of ceftiofur, of which the
preliminary concept was reported recently (Berendsen 2009).

Materials and methods

Reagents

HPLC grade methanol, HPLC grade acetonitrile, hexane (Biosolve, Valkenswaard,
The Netherlands), 25% ammonia, 99% acetic acid, sodium chloride, sodium
hydroxide, ammonium acetate, 85% phosphoric acid (Merck, Darmstadt, Germany),
disodium tetraborate, dithioethrytol (DTE) and iodoacetamide (IAA) (Sigma-Aldrich,
St. Louis, MO, USA) were used. Ceftiofur, DFC, DCCD and ceftiofur-d$_3$ were
obtained from Toronto Research Chemicals (North York, Ontario, Canada).

Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2
MΩ·cm (Millipore, Billerica, MA, USA). Bakerbond® Octadecyl 40 µm was obtained
from J.T. Baker (Phillipsburg, NJ, USA). The solid phase extraction (SPE) cartridges
used were Mega Bond-Elut C18 1 g / 6 mL, Bond Elut SCX 100 mg / 3 mL (Agilent
Technologies, Santa Clara, CA, USA) and Oasis HLB 60 mg / 3 mL (Waters, Milford,
MA, USA).

Borate buffer pH 9 was prepared by dissolving 10.0 g disodium tetraborate
and 29.2 g sodium chloride in 1 L of water. A 1 M ammonium acetate solution was
prepared by dissolving 7.71 g of ammonium acetate in 100 mL water. This was
diluted tenfold in water to obtain a 0.1 M ammonium acetate solution. A 17%
phosphoric acid solution was prepared by diluting 10 mL 85% phosphoric acid to 50
mL with water. A 2% acetic acid solution was prepared by diluting 10 mL 99% acetic
acid to 500 mL with water. A 5 M sodium hydroxide solution was prepared by
dissolving 20 g sodium hydroxide in 100 mL water. A 20 mg mL\(^{-1}\) DTE solution was prepared by dissolving 2.0 g of DTE in 100 mL borate buffer pH 9. A 0.2 M IAA solution was prepared by dissolving 3.7 g IAA in 100 mL 0.1 M ammoniumacetate buffer. Individual stock solutions of ceftiofur and ceftiofur-d\(_3\) were prepared in methanol and stock solutions of DFC and DCCD in water at 100 µg L\(^{-1}\).

**Methods**

Three sample preparation methods (described below) were implemented and tested at our laboratory for the analysis of ceftiofur and its metabolites in poultry muscle. All final extracts were analyzed using the LC-MS/MS system described.

**Method A: individual analysis of ceftiofur, DFC and DCCD**

This method focuses on the individual analysis of ceftiofur, DFC and DCCD and was based on Mastovska *et al.* (Mastovska 2008). One gram of poultry muscle was weighed into a centrifuge tube and ceftiofur-d\(_3\) was added as the internal standard. 2 mL of water and 8 mL of acetonitrile was added to the sample and the tube was shaken using a rotary tumbler (5 min). After centrifugation (3500 g, 15 min) the supernatant was decanted into a new centrifuge tube containing 0.5 g of octadecyl sorbent. After vortex shaking (30 s) and centrifugation (3500 g, 5 min) 5 mL of the extract was transferred into a 12 mm centrifuge tube and evaporated (45°C, N\(_2\)) until the volume was below 1 mL. The concentrated extract was transferred into an autosampler vial.

**Method B: derivatization to DCA**

This method includes a deconjugation and derivatisation of ceftiofur metabolites to DCA and was based on Witte *et al.* (Witte 2010). One gram of poultry muscle was
weighed into a centrifuge tube and ceftiofur-d$_3$ was added as the internal standard.

Deconjugation of DFC conjugates resulting in free DFC was carried out by adding 5 mL of DTE solution followed by shaking using a rotary tumbler (15 min). The extract was incubated in a water bath set at 50°C for 15 minutes and afterwards DFC was stabilized by adding 5 mL IAA solution followed by shaking using a rotary tumbler (15 min) resulting in DCA. The derivatization was completed by incubating the extract for 30 min at room temperature. After the derivatization the pH of the extract was adjusted to pH 3 by adding droplets of 17% phosphorous acid solution. After centrifugation of the extract (4000 g, 30 min) the supernatant was isolated and the pH was adjusted to pH 5 by adding droplets of a 5M sodium hydroxide solution. A 1g, 6 mL Mega Bond Elut C$_{18}$ SPE cartridge was conditioned using 5 mL of methanol followed by 5 mL 0.1M ammonium acetate solution. The entire extract was applied onto the SPE cartridge which was subsequently washed with 5 mL 0.1 M ammonium acetate solution and 5 mL 2% acetic acid in water. DCA was eluted from the SPE cartridge using 5 mL 2% acetic acid in water: acetonitrile (8:2, v/v). A 100 mg, 3 mL SCX SPE cartridge was conditioned with 3 mL of methanol followed by 3 mL 2% acetic acid in water. The eluent of the C$_{18}$ cartridge was applied onto the SCX cartridge which was subsequently washed with 2 mL of methanol. After drying the cartridge under vacuum for 5 min, DCA was eluted using 1 mL 1M ammonium acetate in water: acetonitrile (85:15, v/v). The extract was transferred into an autosampler vial.

**Method C: hydrolysis to AMTA**

This method includes an alkaline hydrolysis of ceftiofur metabolites to AMTA and was derived from Berendsen et al. (Berendsen 2009). 2.5 grams of poultry tissue was
weighed into a centrifuge tube and ceftiofur-d₃ was added as the internal standard. 10 mL of borate buffer pH 9 and 250 µl of 25% ammonia were added to the sample. After shaking using a rotary tumbler (5 min) the extract was incubated in a water bath of 60 °C for 20 hours. After hydrolysis, 10 mL hexane was added and the extract was shaken using a rotary tumbler (5 min) and centrifuged (3500 g, 15 min). A 60 mg, 3 mL Oasis™ HLB SPE cartridge was conditioned by subsequently 5 mL of methanol and 5 mL of water. 5 mL of the clear aqueous layer of the extract was transferred onto the SPE cartridge, which was subsequently washed with 3 mL of water. After drying the cartridge under vacuum for 5 min, the derivate AMTA was eluted using 5 mL methanol:acetonitrile (1:1, v/v). The eluent was evaporated until dryness (45°C, N₂) and the residue was re-dissolved in 500 µL of water, filtered using a 0.45 µm acrodisc® PVDF membrane filter (Pall corporation, Port Washington, NY, USA) and transferred into an autosampler vial.

**LC-MS/MS**

The LC instrumentation consisted of a vacuum degasser, autosampler and a binary pump (Acquity Waters, Milford, MA, USA) equipped with a Waters X-Bridge C₁₈ (3.5 µm) analytical column of 2.1 × 100 mm placed in a column oven at 50 °C. The gradient (solvent A, 0.2 % ammonia in water adjusted to pH 8 using acetic acid; solvent B, 0.2 % ammonia in water adjusted to pH 8 using acetic acid : methanol (1:9, v/v)) was: 0–1.0 min, 0 % B, 1.0–5.0 min, linear increase to 50 % B, 5.0–6.0 min, linear increase to 100 % B with a final hold of 0.5 min, operating at a flow rate of 0.4 mL min⁻¹. The injection volume was 20 µl.

Detection was carried out using a Waters Quattro Ultima mass spectrometer operating with electrospray ionization (ESI) in positive mode. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 20 V; source temperature,
120°C; desolvation temperature, 450 °C; cone gas flow, 200 L hr\(^{-1}\); and desolvation gas, 550 L hr\(^{-1}\). Ceftiofur, ceftiofur-d\(_3\), DFC, DCCD, DCA, DCA-d\(_3\), AMTA and AMTA-d\(_3\) were fragmented using collision induced dissociation (CID) with the settings presented in table 1. Data were acquired and processed using MassLynx 4.1 software (Waters).

[Insert Table 1 here]

**Controlled treatment study**

Following ethical approval, ceftiofur incurred poultry tissue samples were obtained from Schothorst feed research (Lelystad, The Netherlands). Fifteen one day old broilers were held under controlled conditions (water, feed and housing) for 22 days. At day 22, the broilers were weighed and 12 broilers were injected in the breast with a 4 mg mL\(^{-1}\) solution of Excenel (Pfizer Animal Health, New York, NY, USA). The other three animals were considered the untreated control group. The volume of the injected solution was adapted to the body weight in such a way that each animal received 3 mg ceftiofur per kg. The animals were euthanized via cervical dislocation, three animals each at 1, 2, 4 and 8 hours after injection. From each animal breast and thigh muscle, liver and kidneys were removed and stored at -80 °C. After transportation to the laboratory on dry ice, the samples were thawed, minced using a laboratory mincer and stored at -80 °C immediately afterwards to prevent degradation.

**Derivatization/hydrolysis yield**

For method B and C the efficiency of the conversion of ceftiofur, DFC and DCCD to DCA and AMTA respectively were tested. Ceftiofur, DFC and DCCD were each...
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added to six aliquots of blank poultry muscle at a concentration of 1000 µg kg⁻¹. The
poultry muscle samples were analyzed according to method B and C and the peak
areas of DCA and AMTA were determined. Peak areas of ceftiofur and DCCD were
corrected to 1000 µg kg⁻¹ DFC to facilitate a correct comparison of the results by: Y / 
M_DFC * M, in which Y is the response of DCA or AMTA of the samples spiked with
ceftiofur or DCCD, M_DFC is the molecular mass of DFC and M is the molecular mass
of ceftiofur or DCCD. The corrected areas of the samples fortified with the different
metabolites were compared using a Students’ t-test (α=0.05).

Quantitative analysis

First, the limit of detection (LOD) of the methods were determined by extrapolation
of the chromatogram of the lowest calibration point (10 µg kg⁻¹) of the least abundant
product ion monitored to a signal-to-noise ratio of 3. Next, all of the incurred poultry
breast muscle samples were analyzed in duplicate using the three described methods.
The samples were quantified using a matrix matched calibration line. For method A,
blank samples were spiked with 0 to 500 µg kg⁻¹ ceftiofur, DFC and DCCD. For
method B and C blank samples were spiked with 0 to 3000 µg kg⁻¹ ceftiofur only. For
all methods the total amount of ceftiofur metabolites was calculated and expressed in
µg kg⁻¹ DFC by X / M * M_DFC, in which X is the calculated concentration of ceftiofur
or DCCD in the samples, M is the molecular mass of ceftiofur or DCCD and M_DFC is
the molecular mass of DFC. For each animal the results obtained by applying
different methods were compared using a Students’ t-test (α=0.05).

Applicability to other matrices.
The applicability of the hydrolysis approach (method C) was additionally tested for
other matrices than poultry breast muscle by studying the concentration of total
Results and discussion

General considerations

Method A and B were adopted from literature and method C was based upon the concept of the alkaline hydrolysis of ceftiofur to AMTA as reported previously (Berendsen 2009). To study the applicability of this method C concept, a straightforward extraction and sample clean-up procedure was developed. We found it to be crucial to carry out the hydrolysis during extraction (in the presence of the muscle matrix) to obtain good hydrolysis yields for ceftiofur, DFC and DCCD. If ammonia in pure water (without addition of salts) is used as the extraction solvent, muscle proteins denature during incubation, resulting in extreme gelation of the extract. To prevent gelation, sodium borate buffer pH=9 and sodium chloride had to be used as the extraction solvent to facilitate alkaline hydrolysis in the presence of the muscle matrix.

Critical assessment of approaches

Linearity and detection limits

For method A, B and C a chromatogram of a blank poultry muscle sample, spiked at 10 µg kg⁻¹ with the relevant compounds and internal standard ceftiofur-d₃, is presented in Figure 2.

[Insert Figure 2 here]
In the range from 0 to 3000 µg kg$^{-1}$ good linearity ($r > 0.998$) was observed for all three methods indicating that they are suited for the quantification of ceftiofur and (the selected) metabolites in poultry muscle. Method A resulted in an LOD of 1 µg kg$^{-1}$ for ceftiofur, 2 µg kg$^{-1}$ for DFC and 3 µg kg$^{-1}$ for DCCD. Method B resulted in an LOD of 1 µg kg$^{-1}$ for the total ceftiofur content which is superior to previously reported LODs for this approach (10 µg kg$^{-1}$ for swine muscle (Beconi-Barker 1995), 100 µg kg$^{-1}$ for endometrial tissue (Witte 2010) and uterine tissue (Drillich 2006)). Method C resulted in an LOD of 0.5 µg kg$^{-1}$ for the total ceftiofur content indicating that this method is very adequate for detecting residues of ceftiofur metabolites.

**Derivatization/hydrolysis yield**

For a method that includes the production of one marker compound from several individual compounds, the conversion is important: each metabolite should be converted to the marker compound with the same yield to obtain a correct total concentration. The average response and the standard deviation of six poultry muscle samples spiked at 1000 µg kg$^{-1}$ with ceftiofur, DFC and DCCD individually, analyzed using both method B and C, were calculated. The results are presented in Figure 3a and b.

[Insert Figure 3 here]

From the results it is observed that, when using method B, the derivatisation of ceftiofur and DCCD to DCA occurs with the same yield. However, the derivatisation of DFC only results in 20% DCA compared to ceftiofur and DCCD. DFC is the most reactive metabolite having a free sulfide group and quickly degrades under the formation of e.g. protein bound DFC (Olson 1998) and DFC thiolactone (Berendsen...
2009) which might not be effectively derivatized to DCA. From this, it can be
concluded that when using DFC for preparation of matrix matched calibrants, an
incorrect quantification of the total amount of ceftiofur metabolites is obtained.

Furthermore, it can be seen from Figure 3b that the hydrolysis of ceftiofur,
DFC and DCCD results in the same amount of AMTA: Students’t-tests showed no
significant differences among the groups. It can be concluded that the yield of the
hydrolysis of various ceftiofur metabolites to AMTA is comparable and an accurate
quantification of the total amount of ceftiofur metabolites is realistic using the method
C approach.

Quantitative analysis of incurred poultry samples

The individual levels of ceftiofur, DFC and DCCD found in the incurred poultry
breast muscle samples using method A are presented in Table 2. It was observed that
a significant amount of ceftiofur was present 1 hour after administration, whereas it
was not detectable 4 hours after treatment. DFC is the main metabolite observed, but
also this metabolite is metabolised/excreted rapidly and from extrapolation it is
estimated that it will no longer be detectable 24 hours after administration of a single
dose of ceftiofur. DCCD, which is suggested to be a suitable biomarker for detecting
ceftiofur (Fagerquist 2003) is present at concentrations lower than DFC and is
excreted rapidly as well: it is no longer detectable approximately 24 hours after
administration.

[Insert Table 2 here]

[Insert Figure 4 here]
The determined levels of total ceftiofur metabolites, expressed as DFC, using method A, B and C are presented in Figure 4. Method A clearly results in the lowest concentration of ceftiofur metabolites for all broilers. It was previously reported that a significant amount of protein-bound metabolites are formed after ceftiofur administration (Gilbertson 1995, Beconi-Barker 1997, Olson 1998). This is in agreement with the observation that when using method A, which only detects selected metabolites, a lower total ceftiofur concentration was found compared to methods B and C, which do include protein-bound metabolites. It can be concluded that method A results in an underestimation of the total amount of ceftiofur metabolites. From this and the observation that ceftiofur cannot be detected approximately 24 hours after administration of a single dose, we conclude that the analysis of individual metabolites of ceftiofur is not a suitable approach for detecting the off-label use of ceftiofur in the poultry-breeding.

For 10 out of the 12 samples, method C resulted in an average higher total ceftiofur concentration compared to method B, however, except for animals number 4 and 8 no statistically significant difference was found between method B and C using a Students’ t-test. Note that method C also includes the minor metabolite desfuroylceftiofur thiolacton (Berendsen 2009). It can be concluded that method C is comparable to or better than method B regarding its quantitative aspect and that the method C approach is adequate for the quantitative analysis of ceftiofur metabolites in poultry muscle.

**Applicability to cephalosporin antibiotics other than ceftiofur**

The approach applied in method B depends on the cleavage of a thioester bond using DTE. None of the other registered cephalosporins (for use in cattle and swine breeding) contains the thioester bond and therefore it can be expected that method B
is only applicable to a very small range of cephalosprins. This was confirmed by Beconi-Barker et al. (1995) who demonstrated that cefquinome, cefacetrile and cefoperazone (Figure 5) were not detectable using method B, when applying UV detection, whereas a signal possibly corresponding to cefapirin (Figure 5b) was detected. In the present study, we tested this derivatization procedure for cefcapene, a cephalosporin antibiotic being an ester instead of a thioester (Figure 5a). After applying the derivatization procedure used in method B, neither cefcapene acetamide nor any other representative marker for cefcapene was found by direct infusion analysis. It is concluded that method B is not a suitable approach for detecting a broad range of cephalosporins as was also stated by Fagerquist et al. (Fagerquist 2005).

In theory, the approach used in method C is more generic and should be applicable to a broad range of cephalosporin antibiotics. Previously we reported that an AMTA analogue was detected after hydrolysis of cefapirin (Berendsen 2009). The broad applicability was further demonstrated by derivatisation of cefcapene in the presence of poultry muscle, resulting in the marker compound analogous to AMTA. A chromatogram of a blank poultry muscle sample spiked with 1 mg kg\(^{-1}\) of ceftiofur and cefcapene is presented in Figure 6. From this it is concluded that, in contrast to method B, the method C hydrolysis approach is applicable as a multi-cephalosporin method that includes active metabolites, provided that not only AMTA but also the AMTA analogues are recovered during the sample clean-up and detected by selected reaction monitoring (SRM).
The broad applicability of the new approach might also be considered as a weakness. Only a minor moiety of ceftiofur is used as the marker compound and therefore the selectivity of AMTA as a bio-marker for ceftiofur metabolites might be questioned. The Pubchem database (NCBI 2011) was searched for molecules containing the AMTA substructure resulting in many hits, amongst which 18 antibacterial substances, all being cephalosporines (eg. cefquinome, Figure 5c). This demonstrates that the new approach is not specific for ceftiofur. However, if the AMTA analogues are analyzed as well, the new approach is very useful as a broad quantitative screening of cephalosporin compounds in tissue samples to effectively indicate off-label use of cephalosporins in poultry breeding. Subsequently, for the determination of the identity of the actual cephalosporin administered, another method is needed monitoring intact cephalosporins or metabolites thereof.

Application to other matrices

The applicability of the method C hydrolysis approach was tested for other matrices than poultry breast muscle. This was done by studying the concentration of total ceftiofur residues in the thigh muscle, kidney and liver samples obtained from the ceftiofur treated broilers. For each of the matrices a calibration line having a coefficient of correlation above 0.991 was obtained, indicating that the quantitative aspect of the method is adequate for the analysis of different kinds of tissue matrix.

The total amount of ceftiofur metabolites, expressed as DFC, in thigh muscle, kidney and liver, determined using method C, is presented in Table 3. The results indicate that ceftiofur metabolites are rapidly distributed throughout the body. Levels
of ceftiofur metabolites in the breast muscle are equal to the concentrations in liver following intramuscular breast injection. Levels in the thigh muscle were lower, whereas the highest concentrations were detected in the kidneys. This is in agreement with previous findings after intramuscular treatment of swines (Gilbertson 1995, Beconi-Barker 1997) and subcutaneous injection of ducks (Chung 2007). It is concluded that, from an analytical point of view, poultry kidney is potentially better suited for detecting off-label use of ceftiofur. However, only a very limited amount of kidney (approximately 5 g per broiler) is available complicating its use for monitoring purposes. From the results it is concluded that the hydrolysis approach is applicable to different kinds of poultry tissues illustrating its versatility and robustness.

Conclusions

Three LC-MS/MS approaches for the analysis of ceftiofur and its active metabolites in poultry muscle were critically compared. The first approach focussed on the analysis of ceftiofur, DFC and DCCD. The detection limits of this method are 1.0 µg kg\(^{-1}\) for ceftiofur, 2 µg kg\(^{-1}\) for DFC and 3 µg kg\(^{-1}\) for DCCD. Although this approach was the simplest one to apply, it resulted in a serious underestimation of the total ceftiofur residue concentration and off-label use of ceftiofur was only detectable for approximately 24 hours after a single dose treatment. Therefore, it was concluded that for the analysis of ceftiofur, DFC and/or DCCD the method is inadequate for detecting off-label use of ceftiofur in the poultry-breeding sector. The second approach included a derivatization to produce DCA, as a marker for ceftiofur and its metabolites. The LOD of this method was 1 µg kg\(^{-1}\) and this approach resulted in significantly higher total ceftiofur concentrations compared to the first approach. However, this second method was applicable to the detection of ceftiofur and its
metabolites only and should be considered as only a single compound method. The third approach included a hydrolysis using ammonia to produce AMTA as a marker for ceftiofur including its metabolites. The detection limit was 0.5 µg kg\(^{-1}\), and this approach included protein-bound ceftiofur and the minor metabolite desfuroylicingefur thiolactone, resulting in comparable or slightly higher total ceftiofur concentrations compared to the second approach. Only the third approach was applicable to a broad range of cephalosporin antibiotics. It is fair to state that the third approach was not entirely specific for ceftiofur because some chemicals, other than ceftiofur, might yield the same AMTA marker. Nevertheless, the method can be very useful as a broad quantitative screening of any cephalosporin compound in poultry tissue samples to indicate off-label use of cephalosporins in poultry breeding. It can be expected that such a multi-method that includes the active metabolites of all unstable cephalosporins will help to detect off-label use of cephalosporin antibiotics.

Acknowledgements

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References


Table 1. Precursor ions, product ions and collision energy of ceftiofur, DFC, DCCD, DCA, AMTA and the internal standards.

Table 2. Concentration of ceftiofur, DFC and DCCD expressed as DFC detected in poultry breast muscle samples from a single dose controlled treatment using method A. Data between brackets: standard deviation (n=2).

Table 3. Concentration of total ceftiofur, expressed as DFC, detected in poultry thigh muscle, kidney and liver samples from a single dose controlled treatment using method C. Data between brackets: standard deviation (n=2), not duplicate value available for kidney because of the lack of sample material.

Figure 1. Molecular structure of (a) ceftiofur, (b) desfuroylceftiofur (DFC), (c) desfuroylceftiofur cysteine disulfide (DCCD), (d) desfuroylceftiofur acetamide (DCA) and (e) 2-amino-α-(methoxyimino)-4-thiazoleacetamide (AMTA).

Figure 2. A blank poultry muscle sample spiked at 10 µg kg$^{-1}$ with (a) ceftiofur, DFC, DCCD and ceftiofur-d$_3$ analyzed using method A, (b) ceftiofur and ceftiofur-d$_3$ analyzed as DCA and DCA-d$_3$ using method B and (c) ceftiofur and ceftiofur-d$_3$ analyzed as AMTA and AMTA-d$_3$ using method C.

Figure 3. Average peak area including standard deviation (n=6) of poultry muscle samples spiked at 1000 µg kg$^{-1}$ with ceftiofur, DFC and DCCD individually, analyzed using (a) method B and (b) method C.
Figure 4. The determined levels of total ceftiofur metabolites (expressed as DFC) in poultry breast muscle samples from a single dose controlled treatment using method (■) A, (■) B and (■) C (n=2).

Figure 5. Molecular structure of (a) cefcapene, (b) cefapirin, (c) cefquinome, (d) cefacetrile and (e) cefoperazone. The AMTA analogue is indicated by the dotted square.

Figure 6. A blank poultry muscle sample spiked at 1 mg kg\(^{-1}\) with ceftiofur and cefcapene analyzed using method C. Cefcapene* indicates the hydrolysis product of cefcapene.
Table 1. Precursor ions, product ions and collision energy of ceftiofur, DFC, DCCD, DCA, AMTA and the internal standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftiofur</td>
<td>524</td>
<td>40</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>241</td>
</tr>
<tr>
<td>DFC</td>
<td>430</td>
<td>45</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>241</td>
</tr>
<tr>
<td>DCCD</td>
<td>549</td>
<td>25</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>241</td>
</tr>
<tr>
<td>DCA</td>
<td>487</td>
<td>25</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>241</td>
</tr>
<tr>
<td>AMTA</td>
<td>201</td>
<td>23</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>126</td>
</tr>
<tr>
<td>Ceftiofur-d$_3$</td>
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<td>17</td>
<td>244</td>
</tr>
<tr>
<td>DCA-d$_3$</td>
<td>490</td>
<td>20</td>
<td>244</td>
</tr>
<tr>
<td>AMTA-d$_3$</td>
<td>204</td>
<td>15</td>
<td>126</td>
</tr>
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Table 2. Concentration of ceftiofur, DFC and DCCD expressed as DFC detected in poultry breast muscle samples from a single dose controlled treatment using method A. Data between brackets: standard deviation (n=2).

<table>
<thead>
<tr>
<th>Broiler no.</th>
<th>Time after administration (h)</th>
<th>Ceftiofur (µg kg⁻¹)</th>
<th>DFC (µg kg⁻¹)</th>
<th>DCCD (µg kg⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>167 (2)</td>
<td>386 (31)</td>
<td>246 (22)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>267 (42)</td>
<td>561 (147)</td>
<td>187 (10)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>157 (41)</td>
<td>349 (58)</td>
<td>115 (36)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2 (1)</td>
<td>78 (68)</td>
<td>79 (47)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>13 (2)</td>
<td>121 (61)</td>
<td>69 (32)</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>4 (1)</td>
<td>53 (13)</td>
<td>70 (2)</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>ND</td>
<td>26 (5)</td>
<td>14 (0)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>ND</td>
<td>41 (3)</td>
<td>12 (3)</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>ND</td>
<td>24 (17)</td>
<td>34 (7)</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
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<td>11 (1)</td>
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<td>11</td>
<td>8</td>
<td>ND</td>
<td>15 (6)</td>
<td>6 (2)</td>
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<td>12</td>
<td>8</td>
<td>ND</td>
<td>11 (6)</td>
<td>15 (4)</td>
</tr>
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</table>

ND = Not detected
Table 3. Concentration of total ceftiofur, expressed as DFC, detected in poultry thigh muscle, kidney and liver samples from a single dose controlled treatment using method C. Data between brackets: standard deviation (n=2), not duplicate value available for kidney because of the lack of sample material.

<table>
<thead>
<tr>
<th>Broiler no.</th>
<th>Time after administration (h)</th>
<th>Thigh muscle (μg kg⁻¹)</th>
<th>Kidney (μg kg⁻¹)</th>
<th>Liver (μg kg⁻¹)</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>488 (20)</td>
<td>3042</td>
<td>1906 (97)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>595 (9)</td>
<td>2722</td>
<td>1755 (286)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>458 (27)</td>
<td>2894</td>
<td>1855 (24)</td>
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<tr>
<td>4</td>
<td>2</td>
<td>442 (77)</td>
<td>1802</td>
<td>821 (1)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>545 (10)</td>
<td>2240</td>
<td>796 (44)</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>476 (8)</td>
<td>2111</td>
<td>797 (28)</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>475 (22)</td>
<td>1277</td>
<td>524 (109)</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>300 (20)</td>
<td>854</td>
<td>285 (39)</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>392 (5)</td>
<td>1123</td>
<td>356 (91)</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>145 (8)</td>
<td>705</td>
<td>221 (8)</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>196 (29)</td>
<td>942</td>
<td>317 (77)</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>222 (5)</td>
<td>939</td>
<td>358 (3)</td>
</tr>
</tbody>
</table>
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ceftiofur metabolites (µg kg⁻¹ DFC)

Animal number and time after administration

http://mc.manuscriptcentral.com/tfac  Email: fac@tandf.co.uk
AMTA: 201 > 126
17100000

AMTA: 201 > 125
5730000

Cefcapene*: 198 > 181
2960000

Cefcapene*: 198 > 153
1800000