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Development of an analytical method to detect metabolites of nitrofurans Application to the study of furazolidone elimination in Vietnamese black tiger shrimp (*Penaeus monodon*)

Caroline Douny [a,*], Joëlle Widart [b], Edwin De Pauw [b], Frédéric Silvestre [c], Patrick Kestemont [c], Huynh Thi Tu [d], Nguyen Thanh Phuong [d], Guy Maghuin-Rogister [a], Marie-Louise Scippo [a]

[a] University of Liège, CART (Center for Analytical Research and Technology), Analysis of Foodstuffs of Animal Origin, Faculty of Veterinary Medicine, Liège 4000, Belgium
[b] University of Liège, CART (Center for Analytical Research and Technology), Mass Spectrometry Laboratory, Chemistry Department, Liège 4000, Belgium
[c] University of Namur (FUNDP), Unit of Research in Organismic Biology (URBO), Namur, Belgium
[d] College of Aquaculture and Fisheries, University of Can Tho, 3/2 Str., Campus II, Can Tho City, Viet Nam

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ABSTRACT

Nitrofurans, banned antibiotics in the European Union (EU), have often been found in imported aquaculture products in the past and are still found nowadays according to the Rapid Alert System for Feed and Food (RASFF) of the European Commission. A quantitative method based on liquid chromatography coupled to isotopic dilution tandem mass spectrometry (LC–IDMS/MS) was developed for the determination of the residues of four nitrofuran antibiotic residues in shrimps. The experimental protocol consisted of an acid-catalysed release of protein-bound metabolites, followed by derivatisation with 2-nitrobenzaldehyde (NBA). Then, a double liquid–liquid extraction with ethyl acetate was performed before LC–IDMS/MS analysis by positive electrospray ionisation (ES+) with multiple reaction monitoring (MRM) of two transitions per compound. An "in-house" validation of the method for shrimp analysis was conducted according to the EU criteria for the analysis of veterinary drug residues in foods. The decision limits (CCalpha) were 0.08–0.36 μg kg^{-1} and the detection capabilities (CCbeta) were 0.12–0.61 μg kg^{-1}, which are both below the minimum required performance limit (MRPL) set at 1 μg kg^{-1} by the EU. The developed method was applied to evaluate the elimination of furazolidone residues in shrimp muscles after a contamination experiment. After 28 days of decontamination, a concentration of 115 μg kg^{-1} of furazolidone metabolite 3-amino-2-oxazolidinone (AOZ) was still measured in the shrimp muscle.

1. Introduction

There are several important concerns with regard to the use of antibiotics and chemotherapeutic agents in aquaculture, such as food safety, aquaculture product quality, and environmental impacts related to the dispersion of chemicals and drugs into water and sediments, as well as their effects on aquatic communities. The lack of toxicological and elimination kinetics data obtained for cultured organisms and within the aquaculture system itself, and the lack of alternative for chemical application are a reality. Very few studies of antibacterial drug depletion in farmed fish and shrimp are available. In the literature, the pharmacokinetics of furazolidone and its metabolite 3-amino-2-oxazolidinone (AOZ) are reported only for some fish species such as Nile tilapia (*Oreochromis niloticus*) (Xu et al., 2006), orange-spotted grouper (*Epinephelus coioides*) (Guo et al., 2007), or *Carassius Aurats* (Han et al., 2009).

The fishery and aquaculture sectors have become the leading export sectors in Vietnam, with marine shrimp farming having the most important activity in aquaculture. Tiger shrimps (e.g., *Litopenaeus vannamei* and *Penaeus monodon*) are the main contributors to shrimp production, accounting for 70–80% of produced shrimps. The expansion of shrimp culture led to the increased use of chemicals (pesticides) and drugs (antimicrobials) from which residue levels are affecting or threatening export acceptability of products. Within a research programme involving Belgian and Vietnamese universities, confirmatory methods for pesticide and antimicrobial residue measurements in shrimps are under development.

From early shrimp production practice investigations, it appeared that the nitrofurans are potentially among the most commonly used antimicrobials in extensive modes of production. In Vietnam, these antibiotics are used during the culture of shrimp, mainly to prevent (prophylactic use) and treat (therapeutic use) bacterial diseases (Tu et al., 2006, 2009).

Nitrofurans are synthetic broad spectrum antimicrobial agents that have been used in the past for breeding or in animal production (McCracken and Kennedy, 1997a; McCracken et al., 1995). They are

* Corresponding author. Tel.: +32 43664043; fax: +32 43664054.
E-mail address: cdouny@ulg.ac.be (C. Douny).

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effective against Gram-negative and Gram-positive bacteria. They also have anti-protozoal activity (Gräslund and Bengtsson, 2001). Nitrofurans have been extensively used in fish and shrimp farming (GESAMP, 1997).

In 1993, the European Union decided to ban the use of nitrofurans (furaltadone, nitrofurazone, nitrofurantoin and furazolidone) because of the potential mutagenic and carcinogenic effects of their metabolites (3-amino-5-morpholinomethyl-1,3-oxazolid-2-one (AMOZ), semicarbazide (SEM), 1-aminohydantoin (AHD) and 3-amino-2-oxazolidinone (AOZ), respectively) (Anonymous, 1993; van Koten-Vermeulen et al., 1993). Once administered, nitrofurans are quickly transformed into DNA and protein covalently-bound metabolites, complicating their quantitative analysis. However, the metabolite–protein bond can be hydrolysed to permit the extraction of the free residues (Horne et al., 1996). In 2003, the Commission Decision 2002/657/EC fixed a minimum required performance limit (MRPL) of 1 μg kg^{-1} for each free residue of nitrofurans in aquaculture products and in poultry meat (Anonymous, 2002).

There is serious concern about the consumption of shrimps containing forbidden substances such as nitrofuran metabolites as they are still found in imported aquaculture products nowadays according to the Rapid Alert System for Feed and Food (RASFF) of the European Commission. Indeed, for the last ten years, 74 alerts and 96 border rejections concerning nitrofuran metabolites in crustaceans and associated products have been reported in the RASFF database. For banned substances, these MRPL values are used as a limit of acceptability for imported products coming from various countries in the EU, according to regulation 2005/34/EC (Anonymous, 2005). For this reason, in this work, we shall present a quantitative method of analysis of the sum of free and bound nitrofuran residues using liquid chromatography combined with tandem in space mass spectrometry (LC–MS/MS) and its validation performed according to the Decision of the Commission 2002/657/EC (Anonymous, 2002). The developed method was applied to shrimp muscles after a contamination/decontamination experiment with furazolidone to evaluate the amount of nitrofuran metabolite that could be found in shrimps exported from Vietnam.

2. Experiment

2.1. Chemicals and solvents

1-Aminohydantoin·HCl (AHD·HCl, purity: >95%), 3-amino-5-morpholinomethyl-1,3-oxazolid-2-one (AMOZ, purity: >95%), d_2-aminohydantoin·HCl (AHD-d_2·HCl, purity: >95%), 5-(morpholinomethyl)-amino-2-oxazolidinone-d_5 (AMOZ-d_5, purity: >95%), and 3-amino-2-oxazolidinone-4,4,5,5-d_4 hydrochloride salt (AOZ-d_4·HCl, purity: 98%) were purchased from Chemical Synthesis Services (United Kingdom). Semicarbazide hydrochloride (SEM·HCl, purity: >95%) and 3-amino-2-oxazolidinone (AOZ, purity: >99%) were purchased from Aldrich (St-Louis, MO, USA). Semicarbazide hydrochloride [1,2-$^{15}N_2$, ^{13}C] (SEM-^{15}N–^{13}C·HCl, purity: >99%) was purchased from WITEGA (Berlin, Germany). The three deuterated compounds and ^{15}N–^{13}C labelled SEM were used as internal standards in the quantitative analysis. HiPersolV HPLC water was obtained from BDH Laboratory supplies (Poole, England). LC–MS-grade acetonitrile, LC–MS-grade methanol and HPLC-grade ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). 2-nitrobenzaldehyde (NBA) (98%) was purchased from Sigma (St-Louis, MO, USA). Acrodisc® 25 mm syringe filters (with 5 μm Versapor® membrane) were purchased from Pall Life Sciences, MI, USA.

2.2. Standard solutions

Eight individual stock solutions (1 mg/ml) were prepared by dissolving 5–15 mg of each nitrofuran standard in HPLC grade water. Eight intermediate solutions (10 μg/ml) were obtained by diluting 50 μl of each stock solution with 4.95 ml of HPLC grade water. A pool containing AHD·HCl, AMOZ, SEM·HCl and AOZ at a concentration of 0.01 μg/ml was prepared by diluting in a 100 ml volumetric flask 100 μl of each of the four 10 μg/ml solutions with 99.6 ml of HPLC grade water. A second pool containing AMOZ-d5, AOZ-d4·HCl and SEM-^{15}N–^{13}C·HCl at a concentration of 0.05 μg/ml and AHD-d2·HCl at a concentration of 0.1 μg/ml was prepared by diluting 50 μl of the AMOZ-d5, AOZ-d4·HCl and SEM-^{15}N–^{13}C·HCl 10 μg/ml solutions and 100·μl of the AHD-d2·HCl 10 μg/ml solution with 9.75 ml of HPLC grade water. All of the standard solutions were kept for maximum of 6 months at +4 °C.

2.3. Samples used for the validation of the method

For the validation of the method, four types of shrimps were bought in a Belgian supermarket in Spring 2004: tails of Vietnamese shrimps, two kinds of medium Indonesian shrimps and Bangladesh shrimps. A pool of the shrimps of each type, typically 100 g, was created by peeling and blending the shrimps until homogeneous in a mechanical blender (Moulinex, Germany). Then, each type of the samples was labelled and stored at −25 °C until analysis.

Those shrimp samples were checked, using the developed method, as "blank" regarding their nitrofuran metabolites content. They were then used as blank samples and spiked quality control (QC) samples for validating the developed method and for the calibration curves (see Section 2.6).

2.4. Sample preparation

One gram of shrimp was homogenised and spiked with a solution containing the four internal standards (as described in Section 2.1) at a concentration corresponding to 2 μg kg^{-1}. Hydrolysis of the metabolite–protein bond was performed using 5 ml of 0.2 M HCl and a derivatisation was performed by adding 50 μl of 0.1 M 2-nitrobenzaldehyde. Hydrolysis and derivatisation took place at the same time during an overnight incubation period of 16 h at 37 °C. After cooling the solution to room temperature, 500 μl of 2 M NaOH and 1 ml of 0.5 M phosphate buffer were added to adjust pH of the solution to a value included between 6.3 and 7. Nitrofuran derivatives were extracted with 2×4 ml of ethyl acetate followed by evaporation of the solvent to dryness under nitrogen and the addition of 300 μl of HPLC grade water. Finally, the solution was filtered through an Acrodisc® filter and transferred into an injection vial.

2.5. LC–MS/MS analysis

A 2690 Alliance Separation Modules (Waters, Milford, MA, USA) integrated autosampler, solvent delivery system and column heater coupled to a Quattro Ultima Platinum triple–quadrupole mass spectrometer (Micromass, Manchester, UK) were used for LC/MS–MS analysis. The LC column used was a Symmetry C_{18} (2.1×150 mm, 3.5 μm), with a Symmetry C_{18} guard column (2.1×10 mm, 3.5 μm), both from Waters Corporation, Milford, MA, USA. The mobile phase was acetonitrile (solvent A) and water containing 0.1% acetic acid (solvent B). The gradient elution conditions were: from 10% to 80% of solvent A between 0 and 17 min; then, conditions were held for one minute and the contribution of solvent A was decreased to 10% over 10 min. The oven temperature was set at 40 °C and the injection volume was 20 μl. The flow rate was 0.4 ml/min, with a split of 1:1 prior to the MS source.

The mass spectrometer was equipped with an electrospray ionisation (ESI) interface, used in positive ionisation mode. Derivatised native and deuterated standard solutions were synthesised in our laboratory and were infused in the mass spectrometer to optimise the MS tune parameters: capillary: 3.2 kV, source temperature: 125 °C, desolvation temperature: 250 °C, cone gas flow: 50 l/h, desolvation