Research article

Drug Testing and Analysis

Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.1362

Monitoring of florfenicol residues in fish muscle by HPLC-UV with confirmation of suspect results by LC-MS/MS

Rodrigo H. M. M. Granja,[a]* Andreia C. de Lima,[a] Raj K. Patel,[b] Alessandro G. Salerno[a,c] and Amarylis C. B. A. Wanschel[c]

Florfenicol, a derivative of thiamphenicol in which the hydroxyl group at C-3 has been replaced with fluorine, is listed by the World Health Organization as an antibacterial agent for human medicine that is critically important in risk management strategies for non-human use. AOAC International has also identified it as an important molecule for the development of effective methods for the seafood sector. Following inspection missions from the European Union and United States of America, it was introduced in the Brazilian Residues Control Program to fulfill export and internal national requirements with a Maximum Residue Limit of 800 μg/kg. A high performance liquid chromatography method with ultraviolet detection at a wavelength of 230 nm (λ = 230 nm) for the detection of florfenicol in fish muscle was developed and validated according to the Brazilian Regulation 24/2009 (equivalent to European Union Decision 2002/657/EC). Fish samples were extracted with ethyl acetate and hexane followed by C18 solid phase clean-up and chromatographic separation on a reversed-phase C18 LC column with acetonitrile:water as mobile phase. The method results were also was compared with those obtained using liquid chromatography-tandem quadrupole mass spectrometry. The method meets the Brazilian regulatory requirements with a decision limit (CCα) of 840 μg/kg and detection capability (CCβ) of 879 μg/kg. This method is easy to use and has been implemented into Brazil's residue control program, with liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmation of any suspect samples using the same method. © 2012 John Wiley & Sons, Ltd.

Keywords: high performance liquid chromatography; residues; florfenicol; fish; liquid chromatography-tandem mass spectrometry

Introduction

Aquaculture production has notably increased in the last decade, mainly due to intensive fish farming. Together with market globalization, this has given rise to the spreading of several fish diseases, increasing the demand for the use of veterinary drugs for aquatic species. Many classes of antibiotics are commonly used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish.[1] The potential hazards associated with the presence of antibiotics in edible tissues from aquaculture include allergies, toxic effects, and acquisition of drug resistance by pathogens in the human body[2] as well as their potential carcinogenicity.[3]

Florfenicol (FFC) (Figure 1) is a broad-spectrum antibiotic and widely used in veterinary products, since the compounds of the same family, chloramphenicol and thiamphenicol were banned because of the risk aplastic anemia in humans[4–6] and the development of high bacterial resistance,[7,8] respectively. Residues of these compounds in foods of animal origin, including those raised in aquaculture intended for human consumption are of toxicological and regulatory concern. These concerns have led to the establishment of tolerance levels for residues remaining in the tissue and treatment schemes that take into account withdrawal periods for treated fish. In this sense, the Committee for Veterinary Medicinal Products of Europe recommended that florfenicol be included in Annex III of Council Regulation (ECC) No 2377/90[9] with a maximum residue level (MRL) of 1000 μg/kg in fish muscle (sum of florfenicol and its metabolites measured as florfenicol amine).

Scientific studies for the determination of florfenicol and its metabolites, such as florfenicol amine in fish, using high performance liquid chromatography (HPLC),[10–12] gas chromatography (GC),[13] gas chromatography–mass spectrometry (GC-MS),[14] liquid chromatography-mass spectrometry (LC-MS),[15] and ELISA[16] have been published. But many of the methods cannot simply be extrapolated for use in Brazil due to different species and environmental conditions. FFC in fish muscle in Brazil has established a maximum residue limit (MRL) of 800 μg/kg;[17] however, florfenicol amine, the main metabolite of florfenicol metabolism in some fish species, has not been included in the scope of analytes to be monitored for fish muscle in the national plan for the control of residues contaminants (PNCRC).

* *Correspondence to: Rodrigo H. M. M. Granja, Microbioticos Laboratories, Av. Santa Isabel 2120, Campinas, SP, 13084–471, Brazil. E-mail: rodrigo@microbioticos.com*

a *Microbioticos Laboratories, Campinas, SP, Brazil*

b *International Atomic Energy Agency, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria*

c *State University of Campinas, Campinas, SP, Brazil*

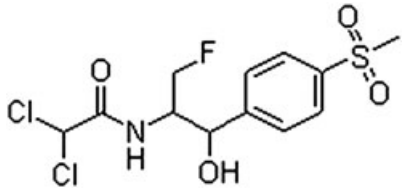

Figure 1. Florfenicol molecular structure.

These limits require the development of sufficiently sensitive and specific methods for the determination of florfenicol residues in fish muscle. The aim of this study was to develop a simple, rapid, and reliable high performance liquid chromatography method with ultraviolet detection (HPLC/UV) for the determination of florfenicol in fish muscle. The eventual identity of florfenicol found in routine samples can be confirmed by LC-MS/MS. The method has been validated in accordance with the Brazilian Regulation 24/2009[18] (equivalent to European Union Decision 2002/657/EC[19]) and in accordance with ISO/IEC 17025:2005[20] guidelines. Parameters such as decision limit (CCα), detection capability (CCβ), precision, recovery, uncertainly and accuracy were also determined.

Materials and methods

Chemicals and equipments

All reagents and solvents were analytical or HPLC grade and provided by Merck (Darmstadt, Germany), Carlo Erba (Milano, Italy) and Synth (São Paulo, Brazil). Florfenicol standard was obtained from Sigma-Aldrich (St Louis, MO, USA). The analysis was performed on a Waters Alliance 2695 Separation Module (Waters, Watford, Hertfordshire, UK), comprising a quaternary pump, automatic injector with capability of 120 vial tubes, a degasser and a Waters 486 UV detector. The confirmatory LC-MS/MS system was composed by an Agilent 1200 HPLC system and an Agilent 6410 triple quadrupole mass spectrometric detector. Mass spectrometric analysis operating conditions to suspect results are summarized in Table 1.

Preparation of standard solutions

A stock solution of 0.5 mg/ml florfenicol was prepared in methanol and kept in a freezer at −20°C. A 5 μg/mL florfenicol standard solution was prepared by diluting 1.0 ml of stock solution with 100 ml of methanol.

HPLC/UV conditions

A 250 x 4.5 mm NovaPak C-18 5 μm column (Waters, Milford, MA, USA) was used at 50°C. The mobile phase (pH 4.5) consisted of a mixture of acetonitrile: acidic water (60:40). The detection was performed at a fixed wavelength of 230 nm.

Sample preparation

Five g of fish muscle were accurately weighed into a 50-ml centrifuge tube. The analyte extraction was performed with 10 ml of ethyl acetate with vortexing (1 min), followed by centrifugation (1851 x *g*) for 10 min. The supernatant was transferred to another centrifuge tube and evaporated using a water bath at 55°C under a gentle steam of nitrogen. 2.0 ml of hexane was added and the solution was agitated using a vortex mixer for 30 s. Five millilitres (5 ml) of water was added, followed by centrifugation (1851 x *g*) for 5 min. The supernatant was removed and discarded. Subsequently, the sample was applied into a SPE C-18 cartridge pre-conditioned with 10 ml of ethyl acetate: methanol: water mixture and rinsed with methanol: water mixture. The analyte was eluted with 4 mL of ethyl acetate. The extract was evaporated under nitrogen at 40°C. The residue was re-suspended in 500 μl of methanol. The solution was filtered through a 0.45 μm filter (PTFE; 13 mm; Millipore, Bedford, MA - USA) into HPLC vials. Injection volume was set at 20 μl for the HPLC/UV analysis and 10 μL for the LC-MS/MS analysis.

Results and discussion

A validation study was carried out to determine parameters such as calibration curve, decision limit (CCα), detection capability (CCβ), precision, recoveries, uncertainty and accuracy. A calibration curve was prepared at the following concentrations of 250, 500, 1000, 1500, and 2000 μg/kg in triplicates (n = 3). The resulting correlation coefficients (r) were greater than 0.99 for all curves.

The CCα is the lowest concentration at which a method can discriminate with a statistical certainty of $1-\alpha$ that the analyte is present above the MRL. In the case of florfenicol with an established MRL, CCα was established by analyzing blank samples spiked around the Brazilian MRL level. The concentration at the MRL plus 1.64 times the corresponding standard deviation (SD) is defined as CCα (α = 5%). The CCβ is the concentration at which the method is able to detect truly contaminated samples with a

Table 1. LC-MS/MS operating conditions for florfenicol analysis in fish muscle

HPLC		Mass Spectrometry (MS)			
Parameters	Conditions	Parameters	Conditions		
Column	Zorbax 5 μm C_{18} (150x2.1 mm)	Ionization	ESI, positive		
		Drying gas	Ar (520 L/h,250°C)		
Mobile phase (isocratic mode)	Acetonitrile: H_2O: Ammonium Formate	V-cap	3000 V		
Flow rate	0.2 mL/min	Collision gas	N_2, 25 L/h		
Oven temp.	50°C	Dwell time	0.2 s		
Injection vol.	10 μL	Compound	Cone voltage (V)	Transition	Collision energy (V)
		FFC	45	355.8 > 336.0 (qualification),	39
			45	355.8 > 185.0 (quantification)	50

statistical certainty of $1-\beta$. CCβ was calculated by using the signal at CCα plus 1.64 times the standard deviation of the within laboratory reproducibility of spiked samples at the MRL level (β = 5%). The CCα and CCβ of the method were determined to be at 840 μg/kg and 879 μg/kg, respectively.

The precision of the method was determined as the relative standard deviation (RSD_r) and inter-day repeatability (RSD_R) by performing tests on six replicates (n = 6) of fish muscle samples spiked at 500, 800 and 1500 μg/kg levels corresponding to 0.625 x MRL, 1.0 x MRL and 1.875 x MRL, respectively, on three different days. Recoveries were determined at the same 3 concentrations (Table 2). The method uncertainty was calculated around to be ~ 6% and was determined considering the quantitative applications of the method, applying a top-down approach.[21] It was considered that the uncertainty contributions arising from all the possible sources of both type A and type B errors associated with the analytical procedure, such as weighing, sample preparation and preparation of solutions, were included in the standard deviations of replicate analyses performed during method validation. This method can be applied to procedures where no trends are observed.[22] The uncertainty estimations were carried out with spiked samples at 0.625, 1.0, and 1.875 times the Brazilian MRL level I, II, and III respectively. The combined uncertainty (CU) of the method was expressed as:

$$C.U = \sqrt{(C.V.\ \text{level I})^2 + (C.V.\ \text{level II})^2 + (C.V.\ \text{level III})^2}\ /3 \quad (1)$$

As the final step of the validation procedure, samples were prepared, coded and given to the analysts to analyse using the described method (Table 3). Typical chromatograms are shown in Figures 2 and 3.

Statistical analysis performed on the routine batches of analysis using Shewhart control chart on analyte recovery and calibration curve parameters were also performed. The parameters evaluated included retention time, recoveries, correlation coefficient, slope, and linear coefficient. The criteria used for the acceptability of such data were: (1) no more than 9 consecutive points above or below the main line; (2) no more than 7 consecutive points ascending or no more than 7 consecutive points descending; (3) no more than one point above or below the control limits, and (4) no more than 3 consecutives points above the warning limits (± 2) deviations from the main line.[23] The success of such control is an indicator of the ruggedness of the method. The ruggedness 2002/657/EC parameters were obtained on a more realistic basis considering the analytical behaviour of routine batches of samples. This suggests that the method is robust because it has not shown susceptibility to day-to-day laboratory variations. It is a reliable and a cost effective method to determine the robustness of the method, once if a non-controlled situation appears, the Shewhart control chart will promptly indicate it. In addition, the Youden test is suggested at 2002/657/EC Decision but the Shewhart control chart has been used successfully to monitor emergent non-controlled parameters and this method is not complicated and does not require much computational skills. And it covers day-to-day laboratory variations.

These parameters obtained on the validation studies qualify the HPLC/UV method developed to be used in regulatory programmes for monitoring florfenicol residues in fish muscle. The study demonstrated that this is a cost effective, sensitive and rapid method, for the determination of florfenicol residues in fish muscle. In conclusion, this methodology may be considered as a suitable method for the reporting of florfenicol levels in fish. Any suspect results obtained from the HPLC/UV analysis can then be confirmed immediately by injecting the samples onto a LC-MS/MS system under the conditions expressed in Table 1.

Table 2. Repeatability and recoveries for florfenicol

FFC levels (μg/Kg)	Conc. found (μg/Kg)	RSD_r (%)	RSD_R (%)	Recovery (%)
500	498.70	5.9	5.6	99.74
800	810.88	3.8	5.2	101.36
1500	1504.65	1.8	2.8	100.31

Table 3. Verification of the suitability of the method for the determination of concentration of florfenicol in unknown fish muscle samples

Unknown Coded Sample	Concentration of FFC found in Samples (μg/kg)
1	489 **[500]**
2	631 **[650]**
3	843 **[820]**
4	801 **[785]**
5	1091 **[1050]**
6	NA **[NA]**

These samples were prepared by the laboratory Quality Assurance (QA) and analyzed by the described method. The numbers in square brackets represents the coded concentrations provided by the QA officer after the samples had been analyzed.

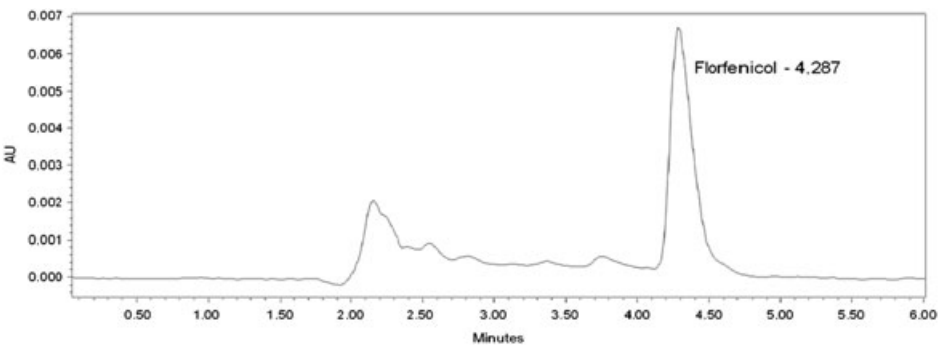

Figure 2. HPLC/UV chromatogram of a blank fish muscle sample spiked with 800 μg/kg of florfenicol.

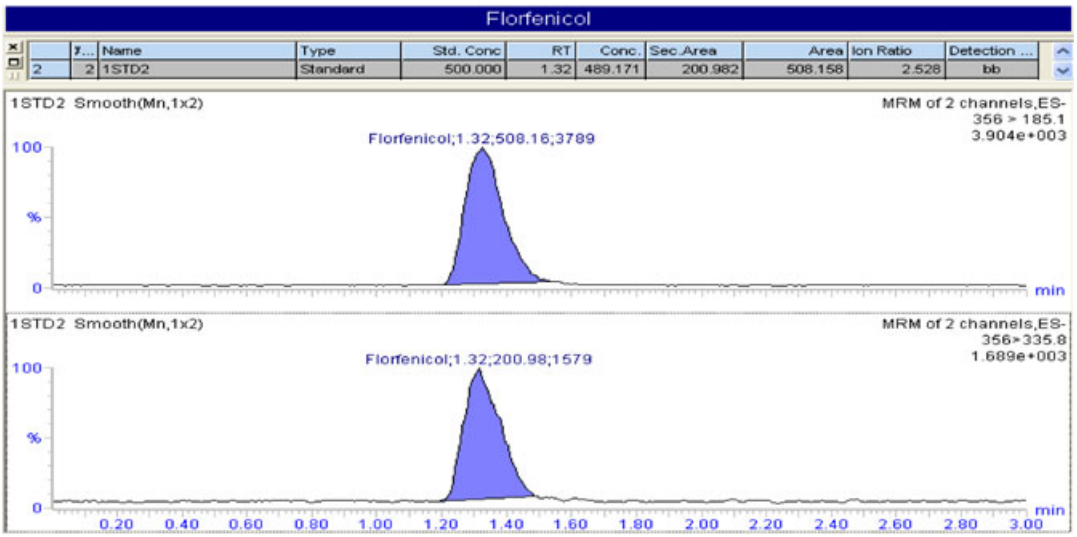

Figure 3. LC-MS/MS chromatogram of a blank fish muscle sample spiked with 500 μg/kg of florfenicol.

Florfenicol amine is described as the main metabolite of florfenicol metabolism in certain fish species such as Atlantic salmon and Korean catfish, it is also known that it has not been detected in metabolism studies conducted in several other fish species samples. The lack of metabolism in such species may explain the slower elimination of florfenicol in certain types of fish.[24] Currently, Brazilian authorities have not added florfenicol amine to the scope of testing for amphenicols in fish muscle or other animal tissues, but in the next step of this project, it is planned to extend this method to include florfenicol amine.

Acknowledgements

The authors want to thank the Food and Agriculture Organization (FAO) and International Atomic Energy Agency (IAEA) Joint Division of Nuclear Techniques in Food and Agriculture for the financial support of this project under the D5-20-36 framework.

Conflicts of interest

The authors have no conflicts of interest to declare.

References

[1] G. Rigos, G.M. Troisi. Antibacterial agents in Mediterranean finfish farming: A synopsis of drug pharmacokinetics in important euryhaline fish species and possible environmental implications. *Rev. Fish Biol. Fish* **2005**, *15*, 53.
[2] A.A.M. Stolker, U.A.T. Brinkman. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals - A review. *J. Chromatogr. A* **2005**, *1067*, 15.
[3] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzar, M. Sergi. Confirmatory analysis of sulfonamide antibacterials in bovine liver and kidney: Extraction with hot water and liquid chromatography coupled to a single- or triple-quadrupole mass spectrometer. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1146.
[4] A.A. Yunis, A.M. Miller, Z. Salem, M.D. Corbett, G.K. Arimura. Nitroso-chloramphenicol: Possible mediator in chloramphenicol-induced aplastic anemia. *J. Lab. Clin. Med.* **1980**, *96*, 36.
[5] A.A. Yunis. Chloramphenicol: Relation of structure to activity and toxicity. *Annu. Rev. Pharmacol.* **1988**, *28*, 83.
[6] J.E. Roybal. *Chloramphenicol and related drugs, Analytical Procedures for Drug Residues in Food of Animal Origin*, (Eds: S.B. Turnipseed, A.R. Long), Science Technology System: Sacramento, CA, **1998**, pp. 227–260.
[7] D. Becker, J. Katz, J. Metz. Comparative effects of thiamphenicol and chloramphenicol on haemopoiesis and lymphocyte transformation *in vitro* in man. *Postgrad. Med. J.* **1974**, *50*, 88.
[8] E.H. Abdennebi, R.J. Sawchuk, C.M. Stowe. Thiamphenicol pharmacokinetics in beef and dairy cattle. *J. Vet. Pharmacol. Ther.* **1994**, *17*, 365.
[9] Committee for Veterinary Medicinal Products (CVMP). The European Agency for the Evaluation of Medicinal Products, *Florfenicol (extension to fish)*, Summary Report, EMEA/MRL/760/00-FINAL, **2000**.
[10] T. Nagata. Chemical analysis of chloramphenicols, in *Chemical Analysis of Antibiotics in Agriculture*, (Eds: H. Oka, H. Nakazawa, K. Harada, J.D. MacNeil), AOAC International: Arlington, VA, USA, **1995**.
[11] V. Hormazabal, I. Steffenak, M. Yndestad. Simultaneous extraction and determination of florfenicol and the metabolite florfenicol amine in sediment by high-performance liquid chromatography. *J. Chromatogr. A* **1996**, *724*, 364.
[12] C.L. Wrzesinski, L.S. Crouch. Determination of florfenicol amine in channel catfish muscle by liquid chromatography. *J. AOAC Int.* **2003**, *86*, 515.
[13] S.X. Zhang, F.Y. Sun, J.C. Li, L.L. Cheng, J.Z. Shen. Simultaneous determination of florfenicol and florfenicol amine in fish, shrimp, and swine muscle by gas chromatography with a microcell electron capture detector. *J. AOAC Int.* **2006**, *89*, 1437.
[14] T. Nagata, H. Oka. Detection of residual chloramphenicol, florfenicol, and thiamphenicol in yellowtail fish muscles by capillary gas chromatography mass spectrometry. *J. Agric. Food Chem.* **1996**, *44*, 1280.
[15] J.M. van de Riet, R.A. Potter, M. Christie-Fougere, B.G. Burns. Simultaneous determination of residues of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in farmed aquatic species by liquid chromatography-mass spectrometry. *J. AOAC Int.* **2003**, *86*, 510.
[16] J.E. Wu, C. Chang, W.P. Ding, D.P. He. Determination of florfenicol amine residues in animal edible tissues by an indirect competitive ELISA. *J. Agric. Food Chem.* **2008**, *56*, 8261.
[17] Brazilian Official Gazette, Normative Instruction IN-14/2009, 25 May 2009 pp. 30. SDA - Ministry of Agriculture and Supply, 28 May **2009** (published date).
[18] Brazilian Official Gazette, Normative Instruction IN-24/2009, 14 July 2009. pp. 7–15. SDA - Ministry of Agriculture and Supply, 22 July **2009** (published date).
[19] European Commission. Commission Decision 2002/657/EC of 12 August 2002: implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Comm.* **2002**, *L221*, 8–36.
[20] ISO/IEC. *ISO/IEC 17025: 2005* - General requirements for the competence of testing and calibration laboratories, 2nd ed, ISO Copyright Office: Geneva, Switzerland, Reference Number ISO/IEC 17025:2005(E), **2005**.

[21] R.H.M.M. Granja, A.M. Montes Niño, F. Rabone, R.E. Montes Niño, A. Cannavan, A.G. Salerno. Validation of radioimmunoassay screening methods for β-agonists in bovine liver according to Commission Decision 2002/657/EC. *Food Addit. Contam.* **2008**, *25*, 1475.

[22] AOAC, *Measurement Uncertainty in the Testing Laboratory (2004)*, AOAC INTERNATIONAL TRAINING COURSE, Gaithersburg: Maryland, USA.

[23] R.H.M.M. Granja, A.M. Montes Niño, R. Zucchetti, R.M. Niño, R. Patel, A.G. Salerno. Determination of erythromycin and tylosin residues in honey by LC-MS/MS. *J. AOAC Int.* **2009**, *92*, 975.

[24] J.H. Wan Lim, M.S. Kim, Y.H. Hwang, I.B. Song, B.K. Park, H.I. Yun. Plasma and tissue depletion of florfenicol in olive flounder (*Paralichthys olivaceus*) after oral administration. *Aquaculture* **2010**, *307*, 71.