DETERMINATION OF FLORFENICOL IN FISH MUSCLE BY LIQUID CHROMATOGRAPHY

Date: 2014-04-18

PRINCIPLE

Florfenicol (FFC) is a broad spectrum antimicrobial agent used to treat bacterial diseases in fish and the corresponding MRL in fish muscle is 1000 μgkg⁻¹. Samples are extracted in ethyl acetate, cleaned-up by SPE and analysed by HPLC with ultra violet detector (UV).

SCOPE

The method is suitable for analysis of FFC in fish muscle at the LOQ of 500 μgkg⁻¹.

MATERIALS

The following consumables are required:

FFC analytical standard; MeCN (*), MeOH (*), Hexane(*), Ultrapure H₂O; Ethyl acetate, all HPLC grade.

(*) The solvents used during the clean-up process should be analytical or chromatographic (HPLC) grade with a high degree of purity. If a solvent does not meet this specification, it should be filtered using at least 0.45 μm membrane filters. Primary Standard Solution (SNI)-FFC Solution, 100.10³ μgL⁻¹; Secondary Standard Solution (SNII) - FFC solution.

Standards and solutions

a) Standard stock solution of FFC (SNI) 100.10 ³ μgL⁻¹

• Accurately weigh 10.0 mg (± 0.1 mg) FFC into a 100 mL volumetric flask and dissolve in ~80 mL of HPLC grade MeCN.
• Shake and after complete dissolution, fill to the 100 mL mark with MeCN.
• The stability of this solution is 3 mn in a freezer.

b) Working standard solution 10.10³µgL⁻¹

• Pipette 5 mL of the SNI FFC solution into a 50 mL flask, fill to the mark with MeCN and mix thoroughly.
• The stability of this solution is 7 days in a refrigerator.
• Keep in the dark.

c) MeOH:H₂O (10:90, v/v)

Mix 9 mL of H₂O with a 1 mL of MeOH in a 10 mL tube.

1 COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS. A summary report on Florfenicol in Fish. The European Agency for The Evaluation of Medicinal Products, Veterinary Medicine Evaluation Unit. EMEA/MRL/251/97-Final, July 2997
**Apparatus**

The following are necessary:

Vortex mixer; Pasteur pipette; Evaporation system; Conical plastic tubes 15 mL; Flask; Water bath; SPE cartridges (C-18 500 mg/3 mL clean-up material); Vacuum manifold HPLC-UV; Micropipette; Dispenser; Disposable 3 mL syringes; Vials with inserts; Analytical balance (0.0001 g); Beakers; 0.45 μm filter unit.

**PROCEDURE**

(a) Weigh 5.0 g (± 0.009) of the ground sample in 50 mL plastic conical tubes
(b) Weigh 6 portions of 5.0 g (± 0.009) of blank tissue ground in a 50 mL conical plastic tubes before transfer to a lab for analysis
(c) The preparation of the matrix calibration curve:
   (i) Fortify blanks with 10 μg/mL SNII according to the concentrations described in TABLE 6
   (ii) Use the remaining blank sample as a control. Using a dispensor, add 10 mL of ethyl acetate to weighed samples in test tubes
(d) Vortex for 1 min
(e) Centrifuge at 2000 rpm for 5 min
(f) Transfer supernatant to another 15 mL plastic tube
(g) Evaporate supernatant using nitrogen, in a water bath at 50-55 °C until an oily residue remains
(h) Add 2 mL of hexane to the tube containing the extract to remove fat
(i) Vortex for 15 sec
(j) Add 5 mL of water to the tube and shake for 30 sec
(k) Centrifuge at 2000 rpm for 5 min; remove the top layer (hexane) and if necessary repeat this step to defat
(l) Evaporate any remaining hexane under nitrogen in a water bath at 50-55 °C for 2 min
(m) Start SPE extraction as follows:
   (i) Prepare the vacuum manifold with the necessary amount of SPE (C18 500 mg/3 mL) cartridges
   (ii) Conditioning the cartridges with 5.0 mL of ethyl acetate, 10 mL of MeOH and 10 mL of H₂O.
   (iii) Load samples to the extraction cartridge; transfer the samples in the tubes to the cartridge, wash the tubes with 3 mL of H₂O and transfer to the reservoir; Elute the sample and allow the cartridge to dry
   (iv) Sample washing: Add 10 mL of MeOH:H₂O (1:9, v/v), then add 10 mL of H₂O, with a dispenser
   (v) Sample elution: Place the cartridge on 15 mL tapered plastic tubes to collect the sample eluted. Elute samples; Add to the cartridge 5.0 mL of ethyl acetate and dry the cartridge
(n) Remove the column C-18 cartridges and evaporate the solution in the tube (step m) to dryness in water bath at 50-55 °C under nitrogen.
(o) Add 1 mL of MeCN:H₂O (40:60, v/v) to the residue and mix well.
(p) Press content (step o) through a 0.45 μm filter into an HPLC vial
(q) Inject into HPLC for analysis using the chromatographic conditions described in TABLE 7.
TABLE 1. SAMPLE FORTIFICATION PROCEDURE

<table>
<thead>
<tr>
<th>Standard concentration</th>
<th>Vol (µL) of SNII concentration 1000 µgkg⁻¹</th>
<th>Mass (µg) added 5.0 g sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>125</td>
<td>125.10³</td>
</tr>
<tr>
<td>500</td>
<td>250</td>
<td>250.10³</td>
</tr>
<tr>
<td>1000</td>
<td>500</td>
<td>500.10³</td>
</tr>
<tr>
<td>1500</td>
<td>750</td>
<td>750.10³</td>
</tr>
<tr>
<td>2000</td>
<td>1000</td>
<td>1000.10³</td>
</tr>
</tbody>
</table>

TABLE 2. CHROMATOGRAPHIC CONDITIONS

<table>
<thead>
<tr>
<th>Chromatographic parameters</th>
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<tbody>
<tr>
<td>Detector: UV</td>
</tr>
<tr>
<td>Column: C-18 250 x 4.6 mm, 5 µm</td>
</tr>
<tr>
<td>Method set: FFC-UV</td>
</tr>
<tr>
<td>Column Temperature: 50 ºC</td>
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<tr>
<td>Wavelength (λ): 230 nm</td>
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<tr>
<td>Mobile phase: H₂O: MeCN (60:40, v/v)</td>
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<tr>
<td>Run time: 6 min</td>
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<tr>
<td>Injected volume: 20 µL</td>
</tr>
<tr>
<td>Flow rate: 1 mLmin⁻¹</td>
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<tr>
<td>RT: FFC 4.5 min</td>
</tr>
</tbody>
</table>

CRITICAL CONTROL

Ensure that:

(a) The analytical column is conditioned for up to 1 h 30 min prior to sample injection and analysis.

(b) After each run wash the column with MeOH at a low flow rate (~0.1-0.2 mLmin⁻¹) for at least 4 h.

ACCEPTABILITY CRITERIA

For method suitability, the ranges in TABLE 8 are recommended.

<table>
<thead>
<tr>
<th>Spike concentration (µgkg⁻¹)</th>
<th>Recovery interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 1</td>
<td>50 to 120%</td>
</tr>
<tr>
<td>1 to 10</td>
<td>70 to 110%</td>
</tr>
<tr>
<td>Over 10</td>
<td>80 to 110%</td>
</tr>
</tbody>
</table>

MEASUREMENT UNCERTAINTY

The estimated expanded measurement uncertainty is 5.4%.

LOQ

The method LOQ for FFC in fish is 500 µgkg⁻¹.

CC ALPHA AND CC BETA

- The CCα for FFC in fish is 840 µgkg⁻¹
The CCβ is 879 μgkg\(^{-1}\).

**SUMMARY OF THE SAMPLE PREPARATION PROCEDURE**

(a) Weigh 5.0 g of sample in a 50 mL test tube  
(b) Prepare matrix matched curve using standard concentrations 250, 500, 1000, 1500, 2000 μgkg\(^{-1}\).
(c) Add 10 mL of ethyl acetate and vortex for 1 min  
(d) Centrifuge at 2000 rpm for 5 min  
(e) Transfer the supernatant to a clean 15 mL test tube  
(f) Evaporate the supernatant under nitrogen in a H\(_2\)O bath at 50-55 °C  
(g) Add 2 mL of hexane to the tube containing the extract and shake for 15 sec  
(h) Add 5 mL of deionized H\(_2\)O and shake for 30 sec  
(i) Centrifuge at 2000 rpm for 5 min  
(j) Discard the upper layer (hexane)  
(k) Evaporate residual hexane using a stream of nitrogen and H\(_2\)O bath set at 50-55 °C, for 2 min  
(l) Condition SPE column with 5 mL of ethyl acetate, 10 mL of MeOH and 10 mL of deionized H\(_2\)O  
(m) Transfer the contents of the 15 mL tube (step e) containing the sample to a reservoir in the SPE clean up chamber  
(n) Rinse the sample tube with 3 mL of H\(_2\)O and transfer content to the reservoir  
(o) Elute the sample at a flow rate of 2-3 drops per sec  
(p) Add 10 mL of MeOH:H\(_2\)O (1:9, v/v) to the reservoir and elute the SPE column  
(q) Wash the column with 10 mL of H\(_2\)O  
(r) Dry the SPE column for 1 min  
(s) Use clean 15 mL tubes to collect eluted sample  
(t) Elute the sample with 5 mL of ethyl acetate at a flow of 1-2 drops per sec  
(u) Remove the SPE column and dry the samples under nitrogen in a H\(_2\)O bath at 50-55 °C  
(v) Reconstitute residue with 1.0 mL of MeCN:H\(_2\)O (40:60, v/v) and vortex for 1 min  
(w) Filter reconstituted residue through a polytetrafluoroethylene (PTFE) membrane, 0.45 μm into a vial before injecting into the HPLC-UV.