Original: Japanese
Provisional translation

Analytical Method for Florfenicol (Animal and Fishery Products)

1. Analytes

Florfenicol

Metabolite $FFNH_2$ [(1*R*,2*S*)-1-(4-methylsulfonylphenyl)-2-amino-3-fluoro-1-propanol] (hereinafter referred to as florfenicol amine, which includes compounds that are converted by hydrolysis.)

2. Application

Animal and fishery products

3. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

4. Reagents

Use the reagents listed in Section 3 of the General Rules, except the following.

Sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (150 mg): A polyethylene tube of 12–13 mm inside diameter packed with 150 mg of sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer or a cartridge equivalent to the specified one in separation capability.

Reference standard of florfenicol: Contains not less than 98% of florfenicol.

Reference standard of florfenicol amine: Contains not less than 97% of florfenicol amine.

5. Procedure

1) Hydrolysis and extraction

Add 20 mL of 6 mol/L hydrochloric acid to 10.0 g of sample, seal tightly, and heat at 100°C for 3 hrs. Allow to cool, add 10 mL of water and 40 mL of *n*-hexane, shake, and centrifuge at 3,000 rpm for 5 min. Discard the *n*-hexane layer, add 2 g of diatomaceous earth to the aqueous layer, mix, and filter with suction. Wash the container and the residue on the filter paper with 10 mL of acetone and water (1:1) mixed solution twice, and filter the washing with suction. Combine the filtrates, and add water to make exactly 100 mL.

2) Clean-up

i) Porous diatomaceous earth column chromatography

Take exactly a 2.5 mL aliquot of the solution obtained in 1), add 1 mL of 7.5 mol/L sodium hydroxide solution and 1 g of sodium chloride, mix well, and inject it into a porous diatomaceous earth cartridge (for 5 mL volume). After leaving the cartridge for 30 min, inject 30 mL of ethyl acetate, concentrate the eluate at below 40°C and remove the solvent. Dissolve the residue in 1 mL of 0.1 vol% acetic acid and methanol (1:1) mixed solution.

ii) Sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer column chromatography

Inject 2 mL of methanol and 3 mL of 0.1 vol% acetic acid and methanol (1:1) mixed solution into a sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (150 mg) sequentially and discard each effluent. Transfer the solution obtained in i) to the cartridge, add 3 mL of 0.1 vol% acetic acid and methanol (1:1) mixed solution and 2 mL of methanol sequentially, and discard each effluent. Then, add 5 mL of ammonia solution and methanol (1:99) mixed solution, concentrate the eluate at below 40°C, and remove the solvent. Dissolve the residue in a mixed solution of 0.1 vol% acetic acid and methanol (9:1) to make exactly 1 mL, and use this solution as the test solution.

6. Calibration curve

Prepare florfenicol amine standard solutions (0.1 vol% acetic acid and methanol [9:1]) of several concentrations, inject each solution into LC-MS/MS and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the concentration of florfenicol amine in the test solution corresponding to 0.01 mg/kg in the sample is 0.0025 mg/L.

7. Quantification

Inject the test solution into LC-MS/MS, and calculate the concentration of florfenicol amine from the calibration curve prepared in **6**.

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

(Example)

Column: Octadecylsilanized silica gel, 2.1 mm inside diameter, 150 mm in length and 3 μm in particle diameter

Column temperature: 40°C

Mobile phase: A mixed solution of acetonitrile and 0.1 vol% acetic acid (1:99)

Ionization mode: ESI (+)

Major monitoring ions (*m/z*): Precursor ion 248, product ions 230, 130

Injection volume: 5 μL

Expected retention time: 5 min

10. Limit of quantification

0.01 mg/kg

11. Explanatory note

1) Outline of analytical method

The method consists of adding hydrochloric acid to the sample, heating it, hydrolyzing florfenicol and its metabolites to florfenicol amine, washing with *n*-hexane, cleaning up with a porous diatomaceous earth cartridge and a sulfonate-modified divinylbenzene-*N*-

vinylpyrrolidone copolymer cartridge and quantifying and confirming using LC-MS/MS.

2) Notes

i) For the hydrolysis procedure, confirm that hydrolysis has been carried out sufficiently using a reference standard of florfenicol.

ii) In hydrolysis, the solution should be heated until all sample lumps have disappeared and the color has turned dark brown to black.

iii) If the filtration velocity is slow during suction filtration, glass fiber filter paper is recommend to use.

iv) If bubbles are generated and the volume cannot be adjusted properly during suction filtration, centrifuge at 3,000 rpm for 5 min.

v) Suction filtration is not required if no suspended solids are observed after washing with *n*-hexane. The procedure without suction filtration is as follows.
Discard the *n*-hexane layer, add 10 mL of acetone to the aqueous layer, and add water to make exactly 100 mL.

vi) Mix gently by inverting when adjusting the volume since some samples may foam if shaken vigorously.

vii) In sulphonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer column chromatography, when water remains during concentration, it is recommended to concentrate to about 0.5 mL at below 40°C, then concentrate again by adding about 2 mL of ethanol.

viii) Sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer column chromatography may be omitted when clean-up is sufficient using only porous diatomaceous earth column chromatography.

ix) In the spike recovery test with the spiking level at MRL during the development of the analytical method, sulphonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer column chromatography was omitted.

x) When the analytical method for florfenicol amine using LC-MS/MS was developed, the following monitoring ions were used:
for quantitative ions (*m/z*): precursor ion 248, product ion 230
for qualitative ions (*m/z*): precursor ion 248, product ion 130

xi) The cartridge shall be washed by increasing the acetonitrile concentration in the mobile phase after eluting florfenicol amine to reduce the influence of matrix carryover in the sample in LC-MS/MS measurements.

xii) The measurement conditions with LC-MS/MS for florfenicol used in developing the analytical method are as follows:
Column: Octadecylsilanized silica gel, 2.1 mm inside diameter, 150 mm in length and 3 μm in particle diameter
Column temperature: 40°C
Mobile phase: Initially, a mixed solution of acetonitrile and 0.1 vol% acetic acid

(1:99) for 6 min, followed by a linear gradient (1:99) to (19:1) in 6 min and hold at (19:1) for 5 min.

Ionization mode: ESI (−)

Major monitoring ions

for quantitative ions (*m/z*): precursor ion 356, product ion 336

for qualitative ions (*m/z*): precursor ion 356, product ion 185

Injection volume: 5 μL

Expected retention time: 11 min

xiii) Food items used to develop the analytical method: cattle muscle, cattle fat, cattle liver, and eel.

12. Reference

Determination and Confirmation of Florfenicol. CLG-FLOR 1.04. Revision 04. United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, 2010.

13. Type

C