

Analytical Method for Avilamycin (Animal Products)

1. Analytes

Avilamycin

Dichloroisoevernic acid (Including compounds that are converted to dichloroisoevernic acid by hydrolysis.)

2. Application

Animal 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.

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

Reference standard of dichloroisoevernic acid: Contains not less than 98% of dichloroisoevernic acid.

5. Procedure

1) Extraction

Add 50 mL of acetone to 10.0 g of sample, homogenize, centrifuge at 3,000 rpm for 5 min, and collect the supernatant. Add 25 mL of acetone to the residue, homogenize, and centrifuge as described above. Combine the resulting supernatants and add acetone to make exactly 100 mL.

2) Hydrolysis

Take a 10 mL aliquot of the solution obtained in 1), concentrate at below 40°C, and remove the solvent. Add 4 mL of 1 mol/L sodium hydroxide solution to the residue and heat at 70°C for 2 hrs. Allow to cool, add 10 mL of water and 10 mL of ethyl acetate, and shake. Discard the ethyl acetate layer, add 2 mL of phosphoric acid to the aqueous layer, and extract with shaking twice with 15 mL of ethyl acetate. Combine the extracts, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 2 mL of formic acid, ethyl acetate and *n*-hexane (1:100:900) mixed solution.

3) Clean-up

Inject 10 mL of formic acid, ethyl acetate and *n*-hexane (1:100:900) mixed solution into a silica gel cartridge (1,000 mg) and discard the effluent. Transfer the solution obtained in 2) to the cartridge, add 8 mL of formic acid, ethyl acetate and *n*-hexane (1:100:900) mixed solution, and discard the effluent. Then, add 15 mL of formic acid, ethyl acetate and *n*-hexane (1:300:700) mixed solution, concentrate the eluate at below 40°C, and remove the solvent.

Dissolve the residue in methanol to make exactly 1 mL and use this solution as the test solution.

6. Calibration curve

Prepare dichloroisoeverninic acid standard solutions (methanol) 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 dichloroisoeverninic acid in the test solution corresponding to 0.01 mg/kg in the sample is 0.01 mg/L.

7. Quantification

Inject the test solution into LC-MS/MS, and calculate the concentration of dichloroisoeverninic acid 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 μ m in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from a mixed solution of acetonitrile and 0.01 vol% formic acid (1:9) to (7:3) in 8 min.

Ionization mode: ESI (–)

Major monitoring ions (m/z):

Precursor ion 249, product ion 190

Precursor ion 251, product ion 192

Injection volume: 5 μ L

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 extracting avilamycin and its metabolites from sample using acetone, converting to dichloroisoeverninic acid by hydrolysis under alkaline conditions, washing with ethyl acetate, transferring it into ethyl acetate for re-dissolution under acidic conditions using phosphoric acid, cleaning up with a silica gel cartridge, and quantifying and confirming using LC-MS/MS.

2) Notes

- i) For the hydrolysis operation, confirm that hydrolysis has been carried out sufficiently using the reference standard of avilamycin.
- ii) If an emulsion was formed during washing with ethyl acetate and transferring into ethyl

acetate for re-dissolution under acidic conditions after hydrolysis, centrifuge at 3,000 rpm for 5 min.

iii) When the analytical method for dichloroisoeverynic acid using LC-MS/MS was developed, the following monitoring ions were used:

for quantitative ions (m/z): precursor ion 249, product ion 190

for qualitative ions (m/z): precursor ion 251, product ion 192

iv) In LC-MS/MS measurements, the cartridge should be washed by increasing the acetonitrile concentration in the mobile phase after dichloroisoeverynic acid is eluted in order to reduce sample matrix carryover effects.

v) Food items used to develop the analytical method: pig muscle, pig fat and pig liver.

12. Reference

None

13. Type

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