Analytical Method for Tulathromycin
(Animal Products)

1. Analytes

Tulathromycin A

Tulathromycin B

Metabolite M1:

[(2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-2-ethyl-3,4,10,13-tetrahydroxy-3,5,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xyro-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecane-15-one]

(including metabolites which are converted to metabolite M1 by hydrolysis)

Isomer of metabolite M1:

[(2*R*,3*R*,6*R*,8*R*,9*R*,10*S*,11*S*,12*R*)-2-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-8,11-hydroxy-3,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-4-azacyclotridecan-13-one]

(including metabolites which are converted to metabolite M1 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.

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

20 mmol/L acetic acid buffer (pH 4.7)

The first solution: Weigh 1.54 g of ammonium acetate, dissolve in water to make 1L.

The second solution: Weigh 1.20 g of acetic acid, dissolve in water to make 1L.

Mix the first solution and the second solution to adjust pH to 4.7.

Reference standard of tulathromycin A: Contains not less than 90% of tulathromycin A.

Reference standard of Metabolite M1: Contains not less than 97% of Metabolite M1.

5. Procedure

1) Extraction and hydrolysis

Weigh 10.0 g of sample, add 25 mL of ethyl acetate, and homogenize. Add 25 mL of 2 mol/L hydrochloric acid, and homogenize again. Centrifuge at 3,000 rpm for 5 minutes, discard the

ethyl acetate layer, and collect the aqueous layer. Add 10 mL each of ethyl acetate and 2 mol/L hydrochloric acid to the residue, and homogenize. Centrifuge at 3,000 rpm for 5 minutes, and discard the ethyl acetate layer. Combine the aqueous layer and the residue to the previously collected aqueous layer, wash the container for the previously obtained aqueous layer with 5 mL of 2 mol/L hydrochloric acid, and combine the washing. Heat the obtained solution and the residue at 60℃ for 30 minutes. Allow to cool, add 2 g of diatomaceous earth and mix, then filter with suction. Wash the residue in the container and on the filter paper with 5 mL of 2 mol/L hydrochloric acids twice, and filter the washing with suction. Combine the filtrates, and add water to make exactly 100 mL.

2) Clean-up

Add 2 mL of methanol and 5 mL of water to Sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge (150 mg) sequentially, and discard the effluent. Take exactly 5 mL of the solution obtained in **1)** and transfer to the cartridge, eluate with 5 mL of methanol, and discard the effluent. Transfer 4 mL of ammonia water and methanol (1:49, v/v), add ammonia and water (1:49, v/v) to the eluate to make exactly 5 mL, and use this solution as the test solution.

6. Calibration curve

Prepare standard solutions by dissolving reference standard of Metabolite M1 in 20 mmol/L acetic acid buffer (pH 4.7) and methanol (1:1, v/v), and prepare several diluted solutions at appropriate concentration range using ammonia water and methanol (1:49, v/v). Inject each standard solution into LC-MS/MS, and make calibration curves by peak-height or peak-area method, using the sum of peak-height or peak-area of Metabolite M1 and the isomer of Metabolite M1. When the test solution is prepared following the above procedure, the sample containing 0.01 mg/kg (equivalent to tulathromycin) gives the test solution of 0.001 mg/L (equivalent to tulathromycin) in concentration.

7. Quantification

Inject the test solution into LC-MS/MS, and calculate the sum of concentrations of Metabolite M1 and the isomer of Metabolite M1 from the calibration curves made in **6**, and obtain the concentration of tulathromycin (including metabolites which are converted to Metabolite M1 or the isomer of Metabolite M1 by hydrolysis) using the following equation:

The concentration (ppm) of Tulathromycin (including Metabolite M1 and metabolites which are converted to Metabolite M1 or the isomer of Metabolite M1 by hydrolysis)

= (the sum of concentrations (ppm) of Metabolite M1 and the isomer of Metabolite M1)×1.398

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

(Example)

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

particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from 20 mmol/L acetic acid buffer (pH 4.7)/methanol (9:1, v/v) to (0:10, v/v) in 10 min.

Ionization mode: ESI (+)

Major monitoring ions (*m/z*): Precursor ion 577, product ions 158, 116

Injection volume: 3 μL

Expected retention time: Metabolite M1: 6 min

The isomer of Metabolite M1: 5 min

10. Limit of quantification

0.01 mg/kg (equivalent to tulathromycin).

11. Explanatory note

1) Outline of analytical method

The method consists of extracting tulathromycin and its metabolites from sample with 2 mol/L hydrochloric acid in ethyl acetate, heating the extracts under the condition of hydrochloric acid to convert to Metabolite M1 and the isomer of Metabolite M1, purifying using a sulfonate-modified divinylbenzene-*N*-vinylpyrrolidone copolymer cartridge, quantifying and confirming by LC-MS/MS. In the method, Metabolite M1 and the isomer of Metabolite M1 are quantified, and the sum of concentrations of Metabolite M1 and the isomer of Metabolite M1 is multiplied by a conversion factor to obtain an analytical value equivalent to the concentration of tulathromycin (including Metabolite M1, the isomer of Metabolite M1, and metabolites which are converted into Metabolite M1 or the isomer of Metabolite M1 by hydrolysis).

2) Notes

i) At the time of the development of the analytical method, the reference standard for tulathromycin A was only available with not less than 90% purity. Therefore, "Reference standard: Contains not less than 90%" is mentioned in 4. However, the obtainable reference standard containing not less than 95% is preferably used.

ii) In an aqueous solution, tulathromycin changes into an equilibrated mixture of tulathromycin A and B. When a spike and recovery test is conducted on the reference standard of tulathromycin A, two peaks are detected for Metabolite M1 and the isomer of Metabolite M1.

iii) Tulathromycin A and Metabolite M1 adhere to a glass container depending on the solution. To prevent adherence, a stock standard solution of tulathromycin A may be prepared by dissolving in 20 mmol/L acetic acid buffer (pH 4.7) and methanol (1:1, v/v).

iv) Confirm that hydrolysis reaction is fully undergone by conducting a spike and recovery

test with a reference standard of tulathromycin A.

v) The use of a glass fiber filter is desirable in case filtering with suction takes time.

vi) Filtering with suction is not necessary when suspended materials can be removed with a centrifugation after hydrolysis.

vii) When adjusting the volume, some samples may bubble after mixing vigorously. To avoid bubbling, samples may be gently mixed with inversion.

viii) When the analytical method for Metabolite M1 and the isomer of Metabolite M1 using LC-MS/MS was developed, the following monitoring ions were used:

for quantification (*m/z*): precursor ion 577, product ion 158

for confirmation (*m/z*): precursor ion 577, product ion 116

ix) The relative retention time of the isomer of Metabolite M1 against Metabolite M1 is about 0.8.

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

12. References

None

13. Type

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