Original: Japanese
Provisional translation

Analytical Method for Doxycycline (Animal and Fishery Products)

1. Analyte

Doxycycline

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.

Citrate buffer containing ethylenediaminetetraacetic acid: Dissolve 21.0 g of citric acid in water to make 1,000 mL (Solution I). Dissolve 71.6 g of disodium phosphate in water to make 1,000 mL (Solution II). Dissolve 1.86 g of ethylenediaminetetraacetic acid disodium salt dihydrate in a mixture of 307 mL of Solution I and 193 mL of Solution II.

Reference standard of doxycycline hydrochloride hydrate: Contains not less than 98% of doxycycline hydrochloride hydrate.

5. Procedure

1) Extraction

Add 10 mL of citrate buffer containing ethylenediaminetetraacetic acid and 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, centrifuge as described above, and collect the supernatant. Combine the resulting supernatant and add acetone to make exactly 100 mL.

Take exactly a 5 mL aliquot of the solution, concentrate at below 40°C and remove the solvent. Add 5 mL of *n*-hexane to the residue and extract with shaking twice with 5 mL each of acetonitrile saturated with *n*-hexane. Combine the extracts, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 1 mL of 0.1 vol% formic acid-methanol solution.

2) Clean-up

Inject 10 mL of 0.1 mol/L hydrochloric acid and 20 mL of 0.1 vol% formic acid-methanol solution into an ethylenediamine-*N*-propylsilanized silica gel cartridge (500 mg) sequentially and discard the effluent. Inject 10 mL of 0.1 mol/L hydrochloric acid and 20 mL of 0.1 vol% formic acid-methanol solution into a styrene-divinylbenzene copolymer cartridge (265 mg) sequentially and discard each effluent. Connect the styrene-divinylbenzene copolymer cartridge to the bottom of the ethylenediamine-*N*-propylsilanized

silica gel cartridge. Load the solution obtained in 1) to the cartridge, add 9 mL of 0.1 vol% formic acid-methanol solution, collect the total eluate including the loaded solutions and add 0.1 vol% formic acid-methanol solution to make exactly 10 mL, and use this solution as the test solution.

6. Calibration curve

Dissolve the reference standard of doxycycline hydrochloride hydrate in methanol to make 1,000 mg/L stock standard solution of doxycycline. Dilute the stock standard solution with 0.1 vol% formic acid-methanol solution and prepare standard solutions 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 doxycycline in the test solution corresponding to 0.01 mg/kg in the sample is 0.0005 mg/L.

7. Quantification

Inject the test solution into LC-MS/MS, and calculate the concentration of doxycycline 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, 100 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from a mixed solution of 0.1 vol% formic acid and 0.1 vol% formic acid- methanol solution (19:1) to (1:19) in 8 min and hold for 3 min at (19:1).

Ionization mode: ESI (+)

Major monitoring ions (*m/z*): Precursor ion 445, product ions 428, 154

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 extracting doxycycline from sample with acetone in the presence of ethylenediaminetetraacetic acid, defatting by acetonitrile/hexane partitioning, cleaning up with an ethylenediamine-*N*-propylsilanized silica gel cartridge and a styrene-divinylbenzene copolymer cartridge and quantifying and confirming using LC-MS/MS.

2) Notes

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

for quantitative ions (*m/z*): precursor ion 445, product ion 428

for qualitative ions (*m/z*): precursor ion 445, product ion 154

ii) Since doxycycline adheres easily to glass walls, polypropylene equipment is recommended.

iii) The solvent should be removed under a nitrogen stream as bumping is likely to occur during concentration.

iv) For doxycycline, leading may be observed depending on the octadecylsilanized silica gel cartridge used for measurement.

v) The measurement should be made promptly after preparing the test solution since doxycycline tends to decompose gradually in the test solution. It is also advisable to cool the test solution during measurement.

vi) The stock standard solution (1,000 mg/L) remains stable for about 1 month when stored at −20°C. The solutions for the calibration curves should be prepared just before use.

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

12. Reference

None

13. Type

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