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Method of Test for Veterinary Drug Residues in Foods - Test of Aminoglycoside Antibiotics (1)

1. Scope

This method is applicable to the determination of 7 aminoglycoside antibiotics residues (apramycin etc. listed in the attached **Table 1**) in muscles, visceral and eggs of poultry and livestock products.

2. Method

After extraction, analytes are determined by liquid chromatography/ tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: positive ion electrospray ionization, ESI^+.

2.1.1.2. Column: Agilent ZORBAX eclipse plus C18, 1.7 μm, 2.1 mm i.d. × 50 mm, or an equivalent product.

2.1.2. Centrifuge: temperature control ≤ 10℃.

2.1.3. Shaker.

2.1.4. Homogenizer.

2.1.5. Nitrogen evaporator.

2.1.6. Solid phase extraction vacuum manifolds.

2.1.7. Vortex mixer.

2.2. Chemicals

Formic acid, HPLC grade;
Acetonitrile, HPLC grade;
Ammonia water (25%);
n-Hexane, reagent grade;
Ammonium formate, reagent grade;
Heptafluorobutyric acid, reagent grade;
Potassium dihydrogen phosphate, reagent grade;
Lithium hydroxide, reagent grade;
Hydrogen chloride, reagent grade;
Sodium hydroxide, reagent grade;
Trichloroacetic acid, ragent grade;
Disodium ethylene diamine tetraacetate, EDTA, reagent grade;
Deionized water, resistivity ≥ 18 MΩ·cm (at 25℃);
Apramycin sulfate, dihydrostreptomycin-sesquisulfate, gentamicin

sulfate, kanamycin sulfate, neomycin sulfate, spectinomycin $5H_2O$, and streptomycin sulfate, reference standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.2. Solid phase extraction cartridge: OASIS MCX cartridge, 6 mL, 150 mg, or an equivalent product.

2.3.3. Membrane filter: 0.45 μm, Nylon; 0.22 μm, PVDF.

2.3.4. Volumetric flask: 10 mL.

2.4. Reagents

2.4.1. 5% Trichloroacetic acid buffer solution

Dissolve and dilute 50 g of trichloroacetic acid and 1.36 g of potassium dihydrogen phosphate with deionized water to 1000 mL.

2.4.2. 0.2 M EDTA

Dissolve and dilute 7.5 g of disodium ethylene diamine tetraacetate with deionized water to 100 mL.

2.4.3. Lithium hydroxide solution

Dissolve and dilute 35 g of lithium hydroxide with deionized water to 500 mL. Filter with a 0.45 μm membrane filter assisted with vacuum.

2.4.4. Elute solution

Dissolve and dilute 31.5 g of ammonium formate with 350 mL of deionized water in a 500-mL volumetric flask. Add 100 mL of acetonitrile, and adjust pH to 9.5 with ammonia water. Dilute to volume with deionized water.

2.4.5. 25% Heptafluorobutyric acid

Dilute 2.5 mL of heptafluorobutyric acid with deionized water to 10 mL.

2.4.6. 10% Formic acid

Dilute 10 mL of formic acid with deionized water to 100 mL.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1.3 mL of heptafluorobutyric acid with deionized water to 1000 mL. Filter with a 0.22 μm membrane filter.

2.5.2. Solvent B

Dilute 1.3 mL of heptafluorobutyric acid with acetonitrile to 1000 mL. Filter with a 0.22 μm membrane filter.

2.6. Standard solution preparation

Transfer equivalent 10 mg of reference standards accurately weighed into each 10-mL volumetric flask, dissolve and dilute with deionized water to volume. Transfer to a 15-mL centrifuge tube, and store at -20℃ as standard stock solutions. When to use, mix appropriate volume of each standard stock solution, and dilute with deionized water to 0.05-125 μg/mL as the standard solutions.

Note: aminoglycoside antibiotics normally carry positive charge and may be adsorbed on the surface of glassware. Therefore, PP plastic containers should be used. Otherwise, use the glassware pretreated by the following steps to avoid adsorption problems. Soak the glassware in 1 N hydrochloric acid for 10 min. Take the glassware out, wash away the residual hydrochloric acid with deionized water, and dry.

2.7. Sample solution preparation

2.7.1. Extraction:

Transfer about 5 g of the homogenized sample accurately weighed into a 50-mL centrifuge tube. Add 15 mL of 5% trichloroacetic acid buffer solution and 0.5 mL of 0.2 M EDTA, vortex for 1 min, and shake for 5 min. Centrifuge at 3200 ×g for 5 min at 10℃ and collect the supernatant. Add 15 mL of 5% trichloroacetic acid buffer and 0.5 mL of 0.2 M EDTA to the residue, and repeat the extraction procedure described above. Combine the supernatants, add 10 mL of *n*-hexane, vortex for 1 min, and shake for 5 min. Centrifuge at 3200 ×g for 5 min at 10 ℃, and collect the lower layer (the visceral sample needs to repeat de-fatting procedure once). Adjust pH to 5.5-6.5 with lithium hydroxide solution for further purification.

2.7.2. Purification:

Transfer the solution for purification from section 2.7.1. into an OASIS MCX cartridge prerinsed with 5 mL of acetonitrile, 4 mL of lithium hydroxide solution and 5 mL of deionized water, and discard the eluent. Wash the cartridge with 5 mL of deionized

water and 5 mL of acetonitrile, and discard the eluent. Add 5 mL of the elute solution, and collect the eluent. Add 1 mL of 10% formic acid, and evaporate to about 3 mL with a stream nitrogen in a water bath at 60°C. Add 50 μL of 25% heptafluorobutyric acid, and dilute to 10 mL with deionized water. Filter with a 0.22 μm membrane filter, and take the filtrate as the sample solution.

2.8. Matrix-matched calibration curve

Take a blank sample, and follow the procedure described in section 2.7. to obtain the sample extract after extraction, purification, and evaporation to about 3 mL, and add 50 μL of 25% heptafluorobutyric acid. Separately add 1 mL of the standard solutions at different concentrations, dilute to 10 mL with deionized water, and filter with a 0.22 μm membrane filter. Take the filtrates as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each antibiotic drug by the peak areas of each antibiotic drug vs. the added concentrations.

LC/MS/MS operating conditions(note)

Column: Agilent ZORBAX eclipse plus C18, 1.7 μm, 2.1 mm i.d. × 50 mm.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 0.5	95 → 95	5 → 5
0.5 → 1.0	95 → 80	5 →20
1.0 → 8.5	80 → 70	20 → 30
8.5 → 9.0	70 → 10	30 → 90
9.0 → 10.0	10 → 10	90 → 90
10.0 → 10.1	10 → 95	90 → 5
10.1 → 15.0	95 → 95	5 → 5

Flow rate: 0.3 mL/min.

Injection volume: 5 μL.

Capillary voltage: 5.5 kV.

Desolvation temperature: 550℃.

Curtain gas: 20 psi.

Ion source gas 1: 50 psi.

Ion source gas 2: 55 psi.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in **Table 1**.

Note : All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 5 µL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.8. Identify each antibiotic drug based on the retention time and the relative ion intensities(note). Calculate the amount of each antibiotic drug in the sample by the following formula:

$$\text{The amount of each antibiotic drug in the sample (ppm)} = \frac{C \times V}{M}$$

Where,

C: the concentration of each antibiotic drug in the sample solution calculated by the matrix-matched calibration curve (µg/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤100%). Maximum permitted tolerances of relative ion intensities are as the follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20 ~ 50	± 25
> 10 ~ 20	± 30
≤ 10	± 50

Remark

1. Limits of quantification (LOQs) for 7 antibiotic drugs are listed in **Table 2**.
2. Further validation should be performed when interfering compounds

appear in samples.

Table 1. MRM parameters of 7 antibiotic drugs

No.	Analyte	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
1	Apramycin	540 > 217*	76	35
		540 > 378	76	23
2	Dihydrostreptomycin	584 > 263*	296	43
		584 > 346	296	49
3	Gentamicin			
	Gentamicin C1a	450 > 322*	110	17
		450 > 160	110	29
	Gentamicin C1	478 > 322*	71	19
		478 > 157	71	25
	Gentamicin C2a/b	464 > 322*	121	17
		464 > 160	121	29
4	Kanamycin	485 > 163*	71	31
		485 > 324	71	21
5	Neomycin	615 > 161*	256	39
		615 > 163	256	41
6	Spectinomycin	351 > 333*	81	25
		351 > 207	81	29
7	Streptomycin	582 > 263*	110	43
		582 > 246	110	53

*Quantitative ion pair.

Table 2. LOQs of 7 antibiotic drugs

Analyst	LOQ (ppm)		
	Muscle	Viscera	Egg
Apramycin	0.02	0.2	0.02
Dihydrostreptomycin	0.1	0.1	0.1
Gentamicin	0.05	0.5	0.05
Kanamycin	0.05	0.2	0.05
Neomycin	0.1	0.2	0.1
Spectinomycin	0.1	0.2	0.1
Streptomycin	0.2	0.3	0.2