Multi-residue Method II for Veterinary Drugs by HPLC (Animal and Fishery Products)

1. Analytes

See Table 8.

2. Instruments

High performance liquid chromatograph-photodiode array detector (HPLC-DAD)

High performance liquid chromatograph-electrochemical detector (HPLC-ECD)

Liquid chromatograph-mass spectrometer (LC-MS)

3. Reagents

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

Acetonitrile: Prepared for high-performance liquid chromatography.

Water: Prepared for high-performance liquid chromatography.

Methanol: Prepared for high-performance liquid chromatography.

Phosphate buffer solution (pH 3.0)

Solution 1: Weigh 27.2 g of potassium dihydrogen phosphate, dissolve in water to make 1,000 mL.

Solution 2: Weigh 2.31 g of dipotassium hydrogen phosphate, dissolve in water to make 100 mL.

Add solution 2 to solution 1, mix, and adjust pH to 3.0.

Phosphate buffer solution (pH 5.0)

Solution 1: Weigh 27.2 g of potassium dihydrogen phosphate, dissolve in water to make 1,000 mL.

Solution 2: Weigh 3.48 g of dipotassium hydrogen phosphate, dissolve in water to make 100 mL.

Add solution 2 to solution 1, mix, and adjust pH to 5.0.

Reference standards of veterinary drugs: Reference standards of known purities for each veterinary drug.

4. Procedure

1) Extraction

i) Muscle, liver, kidney, milk and other edible parts

Weigh 5.00 g of sample, add 30 mL of 95% acetonitrile solution, homogenize, centrifuge at 2,500 rpm for 5 minutes, and take the acetonitrile layer. Add 30 mL of 95% acetonitrile solution to the residue, shake vigorously, centrifuge as described above, and combine the obtained acetonitrile layers.

ii) Fat

Weigh 5.00 g of sample, add 30 mL each of 95% acetonitrile solution and *n*-hexane sequentially, homogenize, centrifuge at 2,500 rpm for 5 minutes, and take the acetonitrile layer. Add 30 mL of 95% acetonitrile solution to the residue and *n*-hexane layer shake vigorously, centrifuge as described above, and combine the obtained acetonitrile layers.

2) Clean up

i) Synthetic magnesium silicate column chromatography

Place 8 g of synthetic magnesium silicate for column chromatography suspended in acetonitrile in a chromatographic tube of 15 mm in inside diameter and 300 mm in length, and let flow out acetonitrile to the extent that only a small quantity of acetonitrile remains on the top of the column. Add 100 mL of acetonitrile to the cartridge and discard the effluent. Transfer the solution obtained in 1) to the cartridge, elute with 30 mL of acetonitrile, and collect the total eluate. Add 100 mL of *n*-hexane, shake the separating funnel vigorously for 3 minutes with a shaker, let stand, collect the acetonitrile layer, concentrate at below 40°C and remove the solvent. Dissolve the residue in 4 mL of phosphate buffer solution (pH 5.0) and add 6 mL of water.

ii) Octadecylsilanized silica gel column chromatography

Add 10 mL of methanol, 10 mL of water and 2 mL of phosphate buffer solution (pH 5.0) to an octadecylsilanized silica gel cartridge (360mg) sequentially, and discard the effluents. Transfer the solution obtained in 1) to the cartridge, add 5 mL of phosphate buffer solution (pH 5.0), and discard the effluent. Elute with 10 mL of 40% methanol solution and 10 mL of 70% acetonitrile solution respectively, and collect the eluate respectively, concentrate at below 40°C and remove the solvent. Dissolve the residue obtained from 40% methanol solution in 2 mL of 5% methanol solution, dissolve the residue obtained from 70% acetonitrile solution in 2 mL of 35% methanol solution and use each solution as the test solution.

5. Calibration curve

Prepare standard solutions (methanol) of each veterinary drug, prepare solutions of several concentrations by diluting with 5% methanol solution for the veterinary drug described as A in Fraction C18 of Table 8, and diluting with 35% methanol solution for the veterinary drug described as B in the column of the Table. Inject 200 μL of each standard solution to HPLC, and make calibration curves by peak-height or peak-area method.

6. Quantification

Inject 200 μL of the test solution to HPLC, and calculate the concentration of each veterinary drug from the calibration curves made in **5**.

7. Confirmation

Confirm using LC-MS or LC-MS/MS.

8. Measurement conditions

Detector: See Table 8.

Column: Octadecylsilanized silica gel, 4.6 mm in inside diameter, 250 mm in length and 5 μm in particle diameter

Column temperature: 40°C

Mobile phase:

HPLC-DAD: Linear gradient from acetonitrile/water/phosphate buffer solution (pH 3.0) (1:18:1, v/v/v) to (14:5:1, v/v/v) in 30 min and hold for 10 min.

HPLC-ECD: Acetonitrile / 0.085 mol/L potassium dihydrogen phosphate (2:3, v/v)

Detecting conditions: See Table 8.

9. Limit of quantification

See Table 8.

10. Explanatory note

1) Outline of analytical method

The method consists of extraction of veterinary drugs from sample with 95% acetonitrile solution, clean-up with a synthetic magnesium silicate column chromatography, defatting by acetonitrile/hexane partitioning, clean-up with an octadecylsilanized silica gel chromatography, and quantification and confirmation using HPLC-DAD or HPLC-ECD.

2) Notes

i) Table 8 list the analytes for which this method is applicable in the order they appear in the Japanese syllabary. Note that the maximum residue limits (MRLs) defined for some agricultural chemicals include not only the parent compounds, but also their metabolites or other transformation products, which are inapplicable to this method.

ii) This method does not ensure simultaneous analysis of all of the analytes listed in Table 8. In advance, confirm that degradation or interference does not occur as the result of interaction between the target analytes.

iii) Because some veterinary drugs easily cause the air oxidation and the photolysis, all procedures should be performed under shading promptly.

iv) If a reference standard is difficult to dissolve in methanol, dissolve it in a small amount of *N*,*N*-dimethylformamide and then dilute with methanol.

v) For the preparation of synthetic magnesium silicate column chromatography which is described in 2) Clean up in **4. Procedure** section, the heating process at 130°C for 12 hours or longer described in Section 3 of the General Rules should not be performed.

vi) Concentration and complete removal of the solvent should be performed under a gentle stream of nitrogen.

vii) Depending on the sensitivity of the LC-MS or LC-MS/MS, it may be necessary to dilute

the test solution with HPLC mobile phase.

viii) Table 8 shows expected fractions in agricultural chemicals for two types of test solutions obtained by octadecylsilanized silica gel column chromatography. Because elution behavior may change depending on the lot and storage conditions of the octadecylsilanized silica gel cartridge, check the validity of the elution behavior using reference standards.

ix) Because the limit of quantification differs depending on the instrument used, the concentration rate of the test solution, and the injection volume, it may be necessary to optimize the conditions.

11. Reference

Hisaya Terada, et al., Journal of Nagoya City Public Health Research Institute, 35, 101-105, 1989

12. Type

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Table 8. Multi-residue Method II for Veterinary Drugs by HPLC (Animal and Fishery Products)

Veterinary drugs	Analytes	Monitoring wavelength (nm)	Monitoring ions (m/z)	Fraction C18	Limit of quantification (mg/kg)
2-Acetylamino-5-nitrothiazole	2-Acetylamino-5-nitrothiazole		-186→139	B	0.0001
Aklomide	Aklomide		+201→155	B	0.01
Azaperone	Azaperone		+328→123	A	0.01
Albendazole	5-(Propylsulphonyl)-1H-benzimidazole-2-amine	280	+240→133	A	0.01
Ethopabate	Ethopabate	280	+238→206	A	0.01
Oxacillin	Oxacillin		+402→160	A	0.005
Oxibendazole	Oxibendazole	300	+250→176	A, B	0.01
Ormetoprim	Ormetoprim	280	+175→123	A, B	0.02
Oleandomycin	Oleandomycin		+688→158	B	0.01
Carazolol	Carazolol		+299→116	B	0.0005
Carprofen	Carprofen		+274→228	A, B	0.01
Cloxacillin	Cloxacillin		+437→160	A	0.005
Closantel	Closantel	230		B	0.05
Clopidol	Clopidol	280	+192→101	A	0.01
Ketoprofen	Ketoprofen		+255→105	A	0.005
Melengestrol acetate	Melengestrol acetate	300	+397→337	B	0.001
Diclazuril	Diclazuril	300	-405→334	B	0.01
Dinitolmide	Dinitolmide		+224→181	A	0.03
Sulfaquinoxaline	Sulfaquinoxaline	270	+301→156	A	0.01
Sulfachlorpyridazine	Sulfachlorpyridazine	270	+285→156	A	0.01
Sulfadiazine	Sulfadiazine	270	+251→92	A	0.01
Sulfadimidine	Sulfadimidine	270	+279→92	A	0.01
Sulfadimethoxine	Sulfadimethoxine	270	+311→156	A	0.01
Sulfathiazole	Sulfathiazole	270	+256→92	A	0.01
Sulfadoxine	Sulfadoxine	270	+311→156	A	0.01
Sulfatroxazole	Sulfatroxazole		+268→92	A	0.01
Sulfanitran	Sulfanitran	270	+336→65	A	0.01
Sulfapyridine	Sulfapyridine	270	+250→156	A	0.01
Sulfabromomethazine sodium	Sulfabromomethazine sodium		+357→92	A	0.01
Sulfabenzamide	Sulfabenzamide	270	+277→156	A	0.01
Sulfamethoxazole	Sulfamethoxazole	270	+254→92	A	0.01
Sulfamethoxypyridazine	Sulfamethoxypyridazine	270	+281→92	A	0.01
Sulfamerazine	Sulfamerazine	270	+265→92	A	0.01
Sulfamonomethoxine	Sulfamonomethoxine	270	+281→92	A	0.01

Cefazolin	Cefazolin		+455→323	A	0.01
Cefapirin	Cefapirin		+424→152	A	0.01
Cefoperazone	Cefoperazone		+646→143	A	0.01
Cefuroxime	Cefuroxime		+447→386	A	0.01
Zeranol	Zeranol	*	-321→277	A, B	0.0005
Thiabendazole	Thiabendazole	320	+202→175	A, B	0.01
	5-Hydroxylthiabendazole	320	+218→191	A	0.01
Tiamulin	Tiamulin		+494→192	B	0.05
Thiamphenicol	Thiamphenicol	230	-345→185	A	0.01
Trimethoprim	Trimethoprim	280	+291→230	A	0.02
Tolfenamic acid	Tolfenamic acid		+262→209	B	0.001
Trenbolone acetate	α- Trenbolone (liver)	350	+271→115	B	0.002
	β- Trenbolone (muscle)	350	+271→115	B	0.002
Nicarbazin	*N,N'*-Bis(4-nitrophenyl) urea	300	-301→137	B	0.02
Nafcillin	Nafcillin		+415→199	A	0.01
Nifrustyrenate	Nifrustyrenate		-258→184	A	0.01
Novobiocin	Novobiocin	300	+613→189	B	0.01
Norgestomet	Norgestomet		+373→313	B	0.0001
Bithionol	Bithionol		-355→161	B	0.002
Pyrimethamine	Pyrimethamine		+249→177	B	0.02
Famphur	Famphur		+326→93	A	0.02
Phenoxymethylpenicillin	Phenoxymethylpenicillin		+351→160	A	0.002
Praziquantel	Praziquantel		+313→203	B	0.01
Flubendazole	Flubendazole	300	+314→282	B	0.002
Brotizolam	Brotizolam		+395→314	B	0.0005
Florfenicol	Florfenicol		-358→185	A	0.01
Benzylpenicillin	Benzylpenicillin		+335→91	A	0.005(muscle, fat, organ) 0.001 (milk)
Mebendazole	Mebendazole		+296→264	B	0.0001
Meloxicam	Meloxicam		+352→115	A, B	0.0001
Lasalocid	Lasalocid		+592→337	B	0.005
Lincomycin	Lincomycin		+407→126	A, B	0.05
Levamisole	Levamisole	230	+205→178	A	0.002
Warfarin	Warfarin		+309→163	A, B	0.001

・The compounds are listed in the order of the Japanese syllabary.

・The monitoring wavelengths represent the wavelength measured by a high performance liquid chromatograph equipped with an ultraviolet spectrophotometric detector or a photodiode array detector.

・Ions are monitored with an ESI positive mode (+) and an ESI negative mode (-) in

LC-MS/MS measurement.

- The level of Zeranol should be determined using HPLC-ECD (Eg 850 mV, E1 500 mV, and E2 750 mV).
- In the Fraction C18 column, A represents a 40% methanol-water fraction and B represents a 70% acetonitrile fraction.