Promulgated, April 27, 2023
Implemented, July 01, 2023
MOHWV0052.00

Method of Test for Veterinary Drug Residues in Foods - Multiresidue Analysis of Antiprotozoal Drugs (2)

1. Scope

This method is applicable to the determination of 23 antiprotozoal drug residues (buquinolate etc. listed in the attached table) in muscle, viscera, eggs, milk of poultry and livestock products, and honey.

2. Method

After extraction and purification, 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: electrospray ionization, ESI.

2.1.1.2. Column: Poroshell 120SB-C18, 2.7 μm, 3.0 mm × 15 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Vortex mixer.

2.1.4. Shaker.

2.1.5. Centrifuge: centrifugal force ≥ 5000 ×g.

2.1.6. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, ≥ 1000 rpm, or an equivalent product.

2.1.7. Nitrogen evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Formic acid, reagent grade;
Dimethylsulfoxide, DMSO, reagent grade;
Magnesium sulfate anhydrous, reagent grade;
Sodium acetate, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Buquinolate and other antiprotozoal drugs listed in the attached table, reference standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 50 mL, PP.

2.3.2. Volumetric flask: 50 mL, Pyrex and PP.

2.3.3. Sample vial: 1 mL, PP.

2.3.4. Membrane filter: 0.22 μm, PTFE.

2.3.5. Ceramic homogenizer: Bond Elut QuEChERS P/N 5982-9313,or an equivalent product.

2.3.6. Extraction powder(note): Containing 6 g of magnesium sulfate anhydrous and 1.5 g of sodium acetate anhydrous, or an equivalent product.

Note: Commercial extraction kits can be used as needed.

2.4. Reagents

2.4.1. Acetonitrile: methanol (4:1, v/v).

Mix acetonitrile and methanol at the ratio of 4:1 (v/v).

2.4.2. Acetonitrile containing 10% formic acid

Mix formic acid and acetonitrile at the ratio of 1:9 (v/v).

2.4.3. Acetonitrile: methanol (95:5, v/v)

Mix acetonitrile and methanol at the ratio of 95:5 (v/v).

2.4.4. Extraction solution

Mix acetonitrile: methanol (95:5, v/v) and formic acid at the ratio of 99:1 (v/v).

2.4.5. 50% methanol

Dilute 500 mL of methanol with deionized water to1000 mL.

2.4.6. *n*-Hexane saturated with acetonitrile

Add 100 mL of acetonitrile to 1000 mL of *n*-hexane. Shake and then stand until complete layering. Take the *n*-hexane layer.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 1 mL of formic acid with methanol to 1000 mL, and filter with a membrane filter.

2.6. Standard solution preparation

2.6.1. Standard solution A

Transfer about 5 mg of buquinolate, carnidazole, diaveridine, diclazuril, diminazene(note), halofuginone, HMMNI, isometamidium, ipronidazole-OH, 2-methyl-5-nitroimidazole, nicarbazine, praziquantel, pyrantel, pyrimethamine, robenidine hydrochloride,

tinidazole and zoalene reference standards accurately weighed to each 50-mL volumetric flask, dissolve and dilute with methanol to volume; transfer about 5 mg of imidocarb accurately weighed to a 50-mL volumetric flask, dilute with acetonitrile: methanol (4:1, v/v) to volume; transfer about 5 mg of decoquinate accurately weighed to a 50-mL volumetric flask, dilute with acetonitrile containing 10% formic acid to volume as the standard stock solutions. Store under freezing in the dark. When to use, mix appropriate volume of each standard stock solution, and dilute with methanol to 1 μg/mL as the standard solution.

2.6.2. Standard solution B

Transfer about 5 mg of dimetridazole, metronidazole, metronidazole-OH and ronidazole reference standards accurately weighed to each 50-mL volumetric flask, dissolve and dilute with methanol to volume as the standard stock solutions. Store under freezing in the dark. When to use, mix appropriate volume of each standard stock solution, and dilute with methanol to 1 μg/mL as the standard solution.

Note: As diminazene is a basic drug, its standard stock solution should be prepared in a plastic volumetric flask.

2.7. Sample solution preparation

Transfer about 2 g of the homogenized muscle or visceral sample accurately weighed; remove eggs' shells, and transfer about 2 g of the mixed egg white and yolk sample accurately weighed; transfer about 2 g of the well-mixed honey accurately weighed; accurately transfer 2 mL of the milk sample into a centrifuge tube. Add one ceramic homogenizer and 10 mL of pre-cooled deionized water, and stand for 10 min. Add 10 mL of the extraction solution, and vortex-mix for 1 min. Shake at 1000 rpm for 1 min by the high speed dispersing device or shake vigorously by hands for 1 min. Add the extraction powder, cap the centrifuge tube, shake vigorously several times by hands to prevent coagulation of salts, and then shake at 1000 rpm for 1 min by the high speed dispersing device or shake vigorously by hands for 1 min. Centrifuge at 5000 ×g for 1 min at 10°C, and collect the supernatant. Add 10 mL of the extraction solution to the residue, vortex-mix for 1 min, and shake vigorously several times by hands to prevent coagulation of salts, and then shake at 1000 rpm for 1

min by the high speed dispersing device or shake vigorously by hands for 1 min. Centrifuge at 5000 ×g for 1 min at 10°C, and collect the supernatant. Combine the supernatants, and transfer 5 mL of the supernatant into a centrifuge tube. Add 10 mL of *n*-hexane saturated with acetonitrile, and shake for 1 min. Centrifuge at 5000 ×g for 1 min, and collect the lower layer. Add 10 mL of *n*-hexane saturated with acetonitrile to the lower layer, and repeat the above procedure once. Take 2 mL of the lower layer, add 50 μL of DMSO(note), and then evaporate to near dryness by gently flushing with a stream of nitrogen at 40°C. Dissolve the residue with 1 mL of 50% methanol, and filter with a membrane filter. Take the filtrate as the sample solution.

Note: The purpose of adding a small amount of DMSO is to avoid over dryness by nitrogen causing losses of halofuginone, dimetridzole and metronidazole.

2.8. Calibration standard curve

2.8.1. Muscle, visceral, milk and honey

Take a blank sample, add 10-250 μL of the standard solution A and standard solution B respectively, and follow the procedure described in section 2.7 to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curve of each antiprotozoal drug by the peak areas of each antiprotozoal drug vs. the added concentrations in the range of 1-25 ng/mL.

2.8.2. Egg

Take a blank sample, add 10-250 μL of the standard solution A and 2-250 μL standard solution B respectively, and follow the procedure described in section 2.7 to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curve of each antiprotozoal drug by the peak areas of each antiprotozoal drug vs. the added concentrations in the range of 0.2-25 ng/mL for dimetridazole, metronidazole, metronidazole-OH and ronidazole, and 1-25 ng/mL for other drugs.

LC-MS/MS operating conditions(note)

Column: Poroshell 120SB-C18, 2.7 μm, 3.0 mm × 15 cm.

Column temperature: 40°C.

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

Time (min)	A (%)	B (%)
0 → 1	95 → 95	5 → 5
1 → 15	95 → 0	5 → 100
15 → 21	0 → 0	100 → 100
21 → 22	0 → 95	100 → 5
22 → 24	95 → 95	5 → 5

Flow rate: 0.3 mL/min.

Injection volume: 10 μL.

Capillary voltage: ESI^+, 3.5 kV; ESI^-, 3.0 kv.

Ion source temperature: 150°C.

Desolvation temperature: 500°C.

Cone gas flow rate: 100 L/hr.

Desolvation flow rate: 700 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in the attached 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 10 μL of the sample solution and the calibration standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.8. Identify each antiprotozoal drug based on the retention time and the relative ion intensities(note). Calculate the amount of each antiprotozoal drug in the sample by the following formula:

$$\text{The amount of each antiprotozoal drug in the sample (ppm)} = \frac{C \times V}{M \times 2}$$

Where,

C: the concentration of each antiprotozoal drug in the sample solution calculated by the calibration curve (μg/mL)

V: the volume of the extraction solution for sample extraction (20 mL)

M: the weight of the sample (g) or the volume of the sample (mL)

2: the concentration factor

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

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

Remark

1. Limits of quantification (LOQs) for 23 antiprotozoal drugs are listed in the attached table 2.
2. To avoid adsorption of basic drugs by glass materials to affect the analytical results, the sample solution and standard solutions should be placed in sample vials made of PP material before instrumental analysis and be analyzed promptly.
3. Further validation should be performed when interfering compounds appear in the samples.

Reference

1. Peng, G. J., Huang, C. H., Shen, Y. U., Lin, Y. T., Chang, S. H., Lai, Y. H., Lin, N. C., Liao, C. D., Kao, Y. M., Tseng, S. H. and Wang, D. Y. 2018. Advanced research for multi-residual analysis of pesticides and veterinary drugs in foods. The Research Project of the Food and Drug Administration, Ministry of Health and Welfare, Taiwan.
2. Shao, B., Wu, X., Zhang, J., Duan, H., Chu, X. and Wu, Y. 2009. Development of a rapid LC-MS-MS method for multi-class determination of 14 coccidiostat residues in eggs and chicken. Chromatographia 69: 1083-1088.
3. Yamada, R., Kozono, M., Ohmori, T., Morimatsu, F. and Kitayama, M. 2006. Simultaneous determination of residual veterinary drugs in bovine, porcine, and chicken muscle using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Biosci. Biotechnol. Biochem. 70: 54-65.

Reference chromatogram

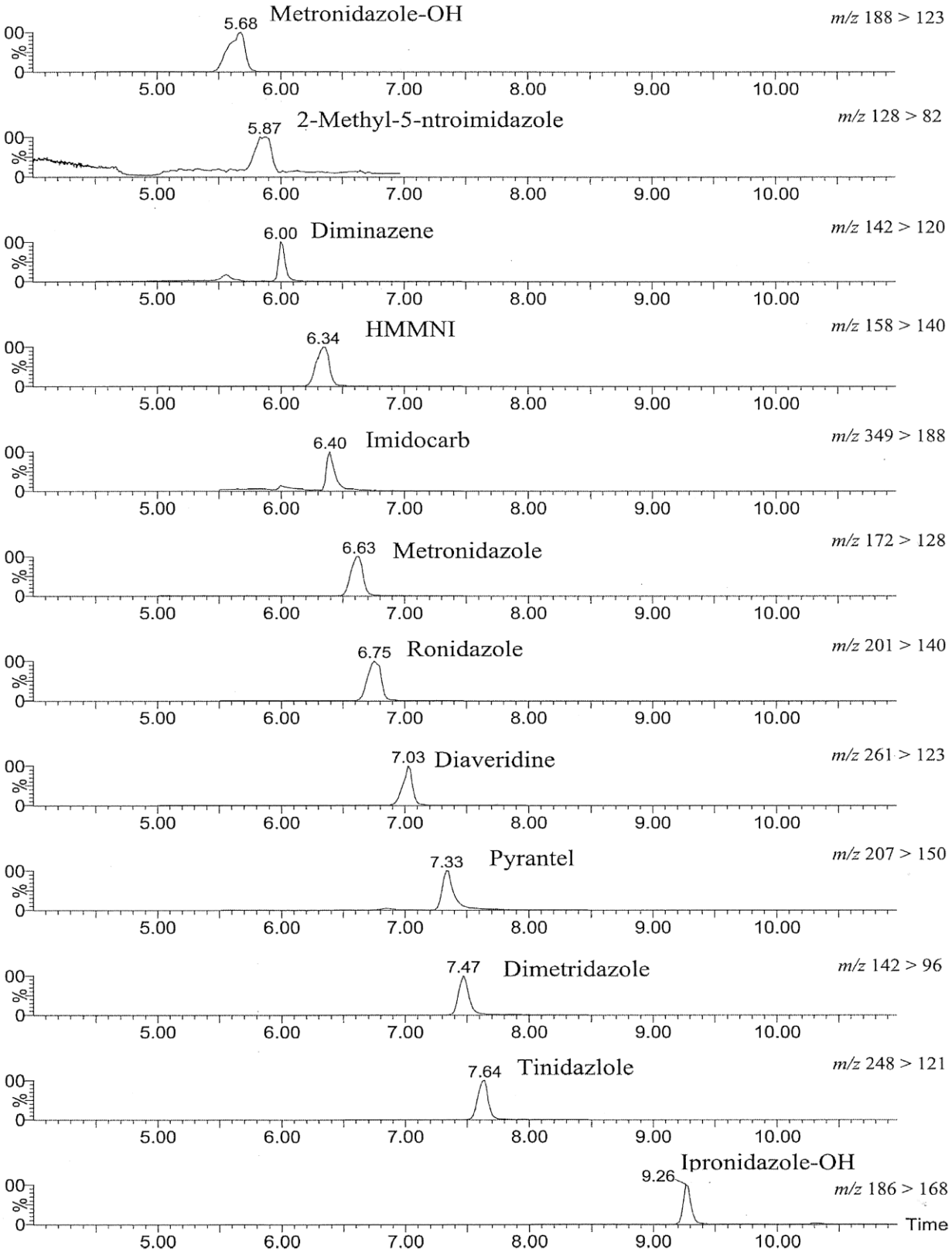

Figure. MRM chromatograms of 23 antiprotozoal drugs analyzed by LC-MS/MS.

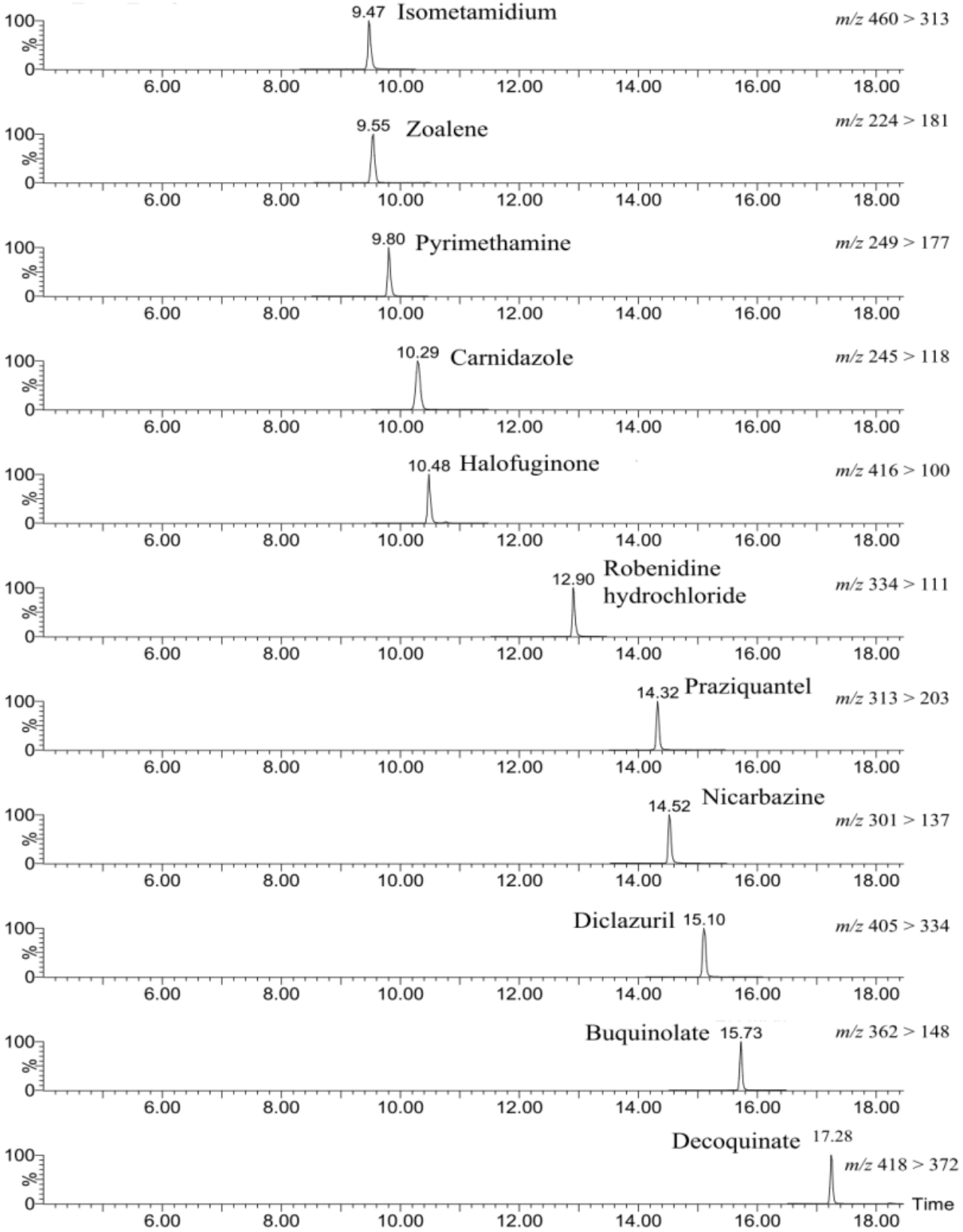

Figure. MRM chromatograms of 23 antiprotozoal drugs analyzed by LC-MS/MS (continued).

Table 1. MRM parameters of 23 antiprotozoal drugs

No	Analyte	Ionization mode	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
1	Buquinolate	ESI^+	362 > 148*	58	50
			362 > 204		40
			362 > 260		22
2	Carnidazole	ESI^+	245 > 118*	10	12
			245 > 75		30
			245 > 60		46
3	Decoquinate	ESI^+	418 > 372*	64	20
			418 > 204		40
			418 > 232		34
4	Diaveridine	ESI^+	261 > 123*	52	22
			261 > 245		26
			261 > 81		42
5	Diclazuril	ESI^-	405 > 334*	20	18
			407 > 336		22
6	Dimetridazole	ESI^+	142 > 96*	12	16
			142 > 81		22
7	Diminazene	ESI^+	142 > 120*	20	5
			142 > 135		5
			282 > 120		5
			282 > 135		5
8	Halofuginone	ESI^+	416 > 100*	24	20
			416 > 120		20
			416 > 138		20
9	HMMNI (2-Hydroxymethyl-1-methyl-5-nitro-1H-imidazole)	ESI^+	158 > 140*	48	10
			158 > 55		16
			158 > 94		22

Table 1. MRM parameters of 23 antiprotozoal drugs (continued)

No.	Analyte	Ionization mode	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
10	Imidocarb	ESI^+	349 > 188*	36	24
			349 > 90		78
			349 > 162		22
11	Ipronidazole-OH	ESI^+	186 > 168*	28	12
			186 > 122		20
			186 > 82		24
12	Isometamidium	ESI^+	460 > 313*	4	18
			460 > 298		26
			460 > 269		46
13	2-Methyl-5-nitroimidazole	ESI^+	128 > 82*	6	14
			128 > 56		12
			128 > 111		14
14	Metronidazole	ESI^+	172 > 128*	20	14
			172 > 82		20
			172 > 111		20
15	Metronidazole-OH	ESI^+	188 > 123*	28	12
			188 > 126		14
			188 > 144		12
16	Nicarbazine	ESI^-	301 > 137*	36	20
			301 > 107		38
			301 > 46		48
17	Praziquantel	ESI^+	313 > 203*	40	14
			313 > 174		26
			313 > 132		44
18	Pyrantel	ESI^+	207 > 150*	24	26
			207 > 136		26
			207 > 97		22

Table 1. MRM parameters of 23 antiprotozoal drugs (continued)

No.	Analyte	Ionization mode	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
19	Pyrimethamine	ESI^+	249 > 177*	20	26
			249 > 198		38
			249 > 233		26
20	Robenidine hydrochloride	ESI^+	334 > 111*	52	42
			334 > 138		24
			334 > 155		18
21	Ronidazole	ESI^+	201 > 140*	24	12
			201 > 55		20
22	Tinidazole	ESI^+	248 > 121*	15	17
			248 > 82		25
23	Zoalene	ESI^-	224 > 181*	10	10
			224 > 77		24
			224 > 151		16

*The quantitative ion. The qualitative ion should be selected at least one ion depending on the matrix.

Table 2. Limits of quantificaion (LOQs) for 23 antiprotozal drugs

No.	Analyte	LOQ (ppm)				
		Muscle	Visceral	Eggs	Milk	Honey
1	Buquinolate	0.005	0.005	0.005	0.005	0.005
2	Carnidazole	0.005	0.005	0.005	0.005	0.005
3	Decoquinate	0.005	0.005	0.005	0.005	0.005
4	Diaveridine	0.005	0.005	0.005	0.005	0.005
5	Diclazuril	0.005	0.005	0.005	0.005	0.005
6	Dimetridazole	0.005	0.005	0.001	0.005	0.005
7	Diminazene	0.005	0.005	0.005	0.005	0.005
8	Halofuginone	0.005	0.005	0.005	0.005	0.005
9	HMMNI	0.01	0.01	0.01	0.01	0.01
10	Imidocarb	0.005	0.005	0.005	0.005	0.025
11	Ipronidazole-OH	0.005	0.005	0.005	0.005	0.005
12	Isometamidium	0.005	0.005	0.005	0.005	0.005
13	2-Methyl-5-nitroimidazole	0.01	0.01	0.01	0.025	0.025
14	Metronidazole	0.005	0.005	0.001	0.005	0.005
15	Metronidazole-OH	0.005	0.005	0.001	0.005	0.005
16	Nicarbazine	0.005	0.005	0.005	0.005	0.005
17	Praziquantel	0.005	0.005	0.005	0.005	0.005
18	Pryrantel	0.005	0.005	0.005	0.005	0.005
19	Pyrimethamine	0.005	0.005	0.005	0.005	0.005
20	Robenidine hydrochloride	0.005	0.005	0.005	0.005	0.005
21	Ronidazole	0.005	0.005	0.001	0.005	0.005
22	Tinidazole	0.005	0.005	0.005	0.005	0.005
23	Zoalene	0.005	0.005	0.01	0.01	0.01