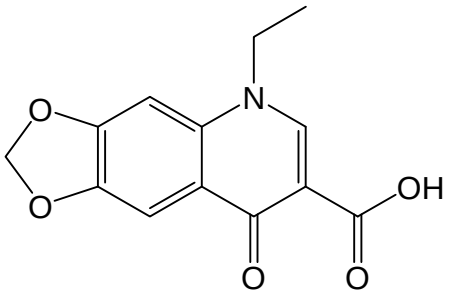
3 Oxolinic acid



5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo(4,5-*g*)quinoline-7-carboxylic acid

$C_{13}H_{11}NO_5$ MW: 261.2 CAS No.: 14698-29-4

【Outline of oxolinic acid】

Oxolinic acid is one of synthetic quinolone antibacterials, being used to pseudotuberculosis, furunculosis, etc. in fish, bacterial diarrhea in calf or piglets, pneumonic pasteurellosis in swine, paratyphoid in chickens. Further, oxolinic acid is used as bactericidal agent for agriculture.

【Methods listed in the Feed Analysis Standards】

1. Simultaneous analysis method of oxolinic acid and flumequin with a liquid chromatograph 〔Feed Analysis Standards Chapter 8, Section 1, 4.1〕

Target chemicals: Oxolinic acid and flumequin (2 components)

A. Reagent preparation

1) oxolinic acid standard stock solution[1]: Place 10 mg of oxolinic acid 〔$C_{13}H_{11}NO_5$〕 exactly measured in a 100 mL volumetric flask, add 1 mL of sodium hydroxide solution (0.1 mol/L) and methanol, ultrasonically treat for dissolving, and further add methanol up to the gauge line to prepare the oxolinic acid standard stock solution (1 mL of this solution contains an amount of oxolinic acid equivalent to 0.1 mg).

2) Flumequin standard stock solution[1]: Place 10 mg of flumequin 〔$C_{14}H_{12}FNO_3$〕 exactly measured in a 100 mL volumetric flask, add methanol for dissolving, and further add the solvent up to the gauge line to prepare the flumequin standard stock solution (1 mL of this solution contains an amount of flumequin equivalent to 0.1 mg).

3) Mixed standard solution: Mix the definite amount of oxolinic acid and flumequin standard stock solutions, dilute exactly with water-methanol (7:3) to prepare several mixed standard solution containing respective amounts of oxolinic acid and flumequin equivalent to 0.01-5 μg/mL.

4) Solvent for elution: Add 2 mL of formic acid to 1,000 mL of acetonitrile-toluene (3:1).

B. Quantification

Extraction: Place 10.0g of the analysis sample measured in a stoppered 200 mL Erlenmeyer flask,

add 20 mL of water and allow still standing for 30 min. Then, add 100 mL of 0.2 % metaphosphoric acid solution-acetonitrile (3:2), and mix them while shaking for 30 min for extraction. Place a 200 mL volumetric flask under a Buchner funnel, and filter the extracted solution with a paper filter (No. 5B) while suctioning. Then, wash that Erlenmeyer flask and the residue sequentially with 50 mL of 0.2 % metaphosphoric acid solution-acetonitrile (3:2), and filter the washings while suctioning in a similar way. Further, add 0.2 % metaphosphoric acid solution-acetonitrile (3:2) up to the gauge line of the volumetric flask. Place 4 mL of this solution exactly in a 100 mL recovery flask, and add 10 mL of water to prepare a sample solution for column treatment.

Column treatment: Wash an octadecylsilylated silica-gel minicolumn (500 mg) Note 1 with 5 mL of acetonitrile and 5 mL of water. Place the sample solution in the minicolumn, and effuse with pressing injection Note 2 until the fluid level reaches the top of the packing material.

Connect a graphite carbon minicolumn (300 mg) Note 3 previously washed with 5 mL of acetonitrile and 5 mL of water to the bottom of that minicolumn. Wash the recovery flask previously containing the sample solution 3 times with respective 5 mL of 0.2 v/v% formic acid solution-acetonitrile (1:1), add the washings sequentially to the column, flow down with pressing injection Note 2 until the fluid level reaches the top of the packing material to transfer oxolinic acid and flumequin to the graphite carbon minicolumn.

Then, remove the octadecylsilylated silica-gel minicolumn, and place a 50 mL recovery flask under the graphite carbon minicolumn. Add 15 mL of elution solvent to the graphite carbon minicolumn to elute oxolinic acid and flumequin.

Concentrate the eluate with a water bath at 40 °C or lower under reduced pressure to approximately 1 mL of volume, and send nitrogen gas to obtain the dry matter. Add exactly 2 mL of water-methanol (7:3) to dissolve the residue, and filter the solution through a membrane filter (pore diameter: 0.45 μm) to obtain a sample solution for liquid chromatography Note 4.

Liquid chromatography: Inject respective 10 mL of the sample solution and each mixed standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Fluorescence detector (excitation wavelength: 325 nm, fluorescence wavelength: 365 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 150 mm, particle diameter: 5 μm) Note 5

Eluent: 0.2 v/v% formic acid solution-methanol (7:3)→15 min→(4:6) (5 min retention)

Flow rate: 1.0 mL/min

Column temperature: 40 °C

Calculation: Obtain the peak area or height from the chromatogram[2] to prepare the calibration curve, and calculate the amounts of the oxolinic acid and flumequin in the sample.

Note 1. InertSep SlimJ C18-B (GL Science) connected with a reservoir with appropriate volume, or an equivalent one.

2 Flow rate is 1 mL/min. A suction manifold is also available.

3 InertSep GC (GL Science, reservoir volume: 6 mL) or an equivalent one.

4 Place the sample solution in a brown vial container.

5 L-column ODS (Chemicals Evaluation and Research Institute) or an equivalent one.

《Summary of analysis method》

This method is intended to determine the amount of oxolinic acid and flumequin in a feed, by extracting with acetonitrile containing metaphosphoric acid solution, and purifying with a C_{18} minicolumn and a graphite carbon minicolumn, and quantifying using a liquid chromatograph with a fluorescence detector.

A flow sheet the analysis method is shown in Fig. 8.2.1-1.

Sample: 10 g
- Water: 20 mL, still standing for 30 min
- 100 mL of 0.2 v/v% metaphosphoric acid—acetonitrile (3:2) : stir for 30 min
- Suction filtration (No. 5B)
- Wash with 50 mL of 0.2 v/v% metaphosphoric acid solution—acetonitrile (3:2)
- Fix the volume of 0.2 v/v% metaphosphoric acid solution—acetonitrile (3:2) at 200 mL

Collect 4 mL of the sample solution (exactly)
- 10 mL of water

InertSep SlimJ C18-B cartridge (previously washed with 5 mL of acetonitrile and 5 mL of water)
- Load the sample solution
- Connect the InertSep GC cartridge (previously washed with 5 mL of acetonitrile and 5 mL of water) to the bottom of InertSep SlimJ C18-B cartridge

  Connect to the bottom of InertSep SlimJ C18-B cartridge
- Wash with 5 mL of 0.2 v/v% formic acid solution—acetonitrile (1:1) (3 times)
- Remove the InertSep SlimJ C18-B cartridg
- Elute with 15 mL of acetonitrile-toluene (3:1) (containing 0.2 v/v% formic acid)
- Concentrate with reduced pressure (40 °C or lower), and dry with nitrogen gas
- 2 mL of Water—methanol (7:3)
- Membrane filter (pore diameter: 0.45 μm)

LC-FL (Ex: 325 nm, Em: 365 nm)

Fig. 8.2.1-1 A flow sheet of simultaneous analysis method of oxolinic acid and flumequin

Reference: Yasutoshi Sugimoto, Masayo Nomura, Kazuya Washio: Research Report of Animal Feed, 34, 28 (2009)

History in the Feed Analysis Standards: 【33】new

《Validation of analysis method》

・Recovery rate and repeat accuracy

Added compound	Type of sample	Concentration (mg/kg)	Repeat	Mean recovery rate (%)	Repeat accuracy RSD (% or less)
Oxolinic acid	Formula feed for adult chicken	0.5~5	3	72.6~94.4	7.3
	Formula feed for red sea bream	1~5	3	71.1~74.5	11
	Fish meal 1	0.5~5	3	76.3~90.8	12
	Fish meal 2	1~5	3	71.8~83.3	5.7
	Fish meal 3	1~5	3	78.2~80.3	3.6
Flumequin	Formula feed for adult chicken	0.3~5	3	78.9~90.5	12
	Formula feed for red sea bream	1~5	3	74.2~74.7	12
	Fish meal 1	0.3~5	3	71.5~86.7	11
	Fish meal 2	1~5	3	73.3~85.3	16
	Fish meal 3	1~5	3	80.5~82.0	5.2

・Cooperative testing

Added component	Type of sample	No of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Oxolinic acid	Fish meal	8	3	83.0	6.1	9.5	0.68
	Formula feed for adult chicken	8	3	88.7	5.9	6.6	0.48
Flumequin	Fish meal	8	3	82.0	4.1	8.3	0.59
	Formula feed for adult chicken	8	3	87.7	5.5	6.9	0.50

・Lower detection limit: 0.5 mg/kg for oxolinic acid, 0.3 mg/kg for flumequin

《Notes and precautions》

[1] Available from Kanto Chemical and others

[2] An example of chromatogram is shown in Fig. 8.2.1-2.

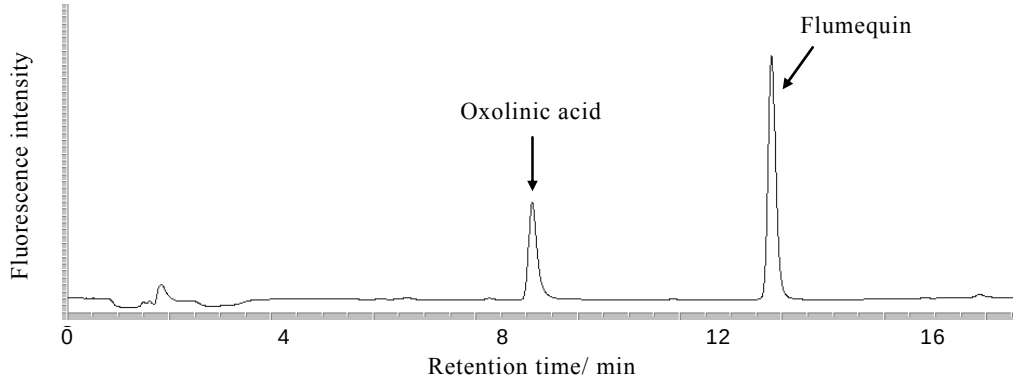


Fig. 8.2.1-2 A chromatogram of oxolinic acid and flumequin added at respective amounts equivalent to 5 mg/kg to formula feed