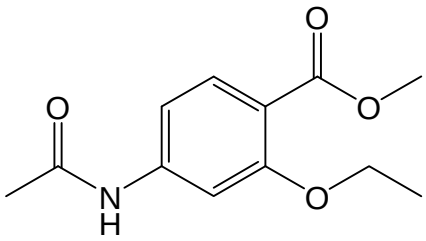
2 Ethopabate



Methyl 4-acetamido-2-ethoxybenzoate

$C_{12}H_{15}NO_4$ MW: 237.25 CAS No.: 59-06-3

【Outline of ethopabate】

Ethopabate is almost odorless white to pale reddish white powder. It is freely soluble in chloroform, soluble in methanol or ethanol, very slightly soluble in ether, and practically insoluble in water.

Ethopabate is designated as a feed additive to promote the effective use of nutrient components contained in feeds, and the ordinance of MAFF allows the use of it in the range of 2.56-16 g/t in feeds for starting chicks, growing chicks, prior stage or later stage broiler chickens (up to 7 days before killing for edible use). Further, use of ethopabate at a concentration of 5 g/t is approved for three-combination with amprolium and sulfaquinoxaline in formula feeds for chickens.

【Methods listed in the Feed Analysis Standards】

1 Quantitative test methods

1.1 Liquid chromatography

1.1.1 Premix

〔Feed Analysis Standards Chapter 8, Section 1, 2.1.1-(1)〕

A. Reagent preparation

Ethopabate standard solution: Place 20 mg of ethopabate〔C12H15NO4〕exactly measured in a 100 mL brown volumetric flask, add methanol for dissolving, further add the solvent up to the gauge line to prepare the ethopabate standard stock solution（1 mL of this solution contains an amount of ethopabate equivalent to 0.2 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with methanol to prepare several ethopabate standard solutions containing amounts of ethopabate equivalent to 0.25-2 μg per mL.

B. Quantification

Extraction: Place 1-2 g of analysis sample [2] exactly measured in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of methanol, and stir it for 30 min for extraction. Place the extracted

solution in a stoppered brown centrifuging tube to centrifuge at 1,000×g for 5 min, and exactly dilute a definite amount of the supernatant with methanol. Filter this solution with a membrane filter [2] (pore diameter: 0.5 μm or less) to obtain a sample solution for liquid chromatography.

Liquid chromatography: Inject respective 20 μL of the sample solution and each ethopabate standard solution into a liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 270 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 6 mm, length: 150 mm, particle diameter: 5 μm) Note1[3]

Eluent: Water-acetonitrile (7:3)

Flow rate: 1.0mL/min

Calculation: Obtain the peak height or area from the chromatogram [4] to prepare the calibration curve, and calculate the ethopabate amount in the sample.

Note 1. Use YMC-Pack ODS-A (YMC) or an equivalent one.

《Summary of analysis method》

This method is intended to determine the amount of ethopabate in premixes by extracting with methanol, diluting with the solvent, and quantifying using a liquid chromatograph with an ultraviolet spectrophotometer. This is an improved method of one listed in Feed Analysis Standards in 1980, based on the method of Ishiguro.

References: Eiichi Ishiguro：Research Report of Animal Feed, 7, 82 (1981)

Norio Hikichi：Research Report of Animal Feed, 20, 67 (1995)

History in the Feed Analysis Standards: 【2】new,【15】revision,【17】revision,【22】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Premix for chicken 1	0.5-7.5	3	99.0-101.7	4.8
Premix for chicken 2	0.5-7.5	3	99.0-100.7	8.0
Premix for chicken 3	0.5-7.5	3	99.7-102.0	1.5

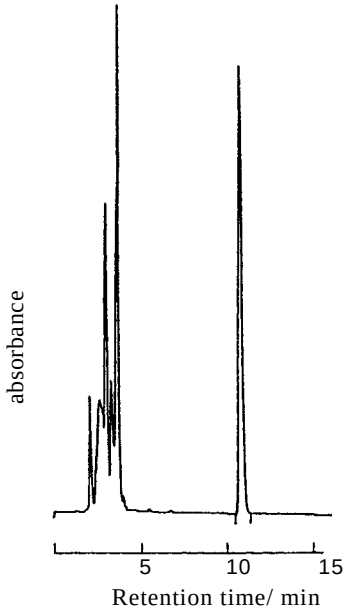
《Notes and precautions》

[1] As for a premix, exactly measure 1 to 2 g, and dilute the supernatant obtained by centrifuging up to 10 to 100-fold.

[2] Hydrophilic one such as made of PTFE (Toyo Roshi) is available.

[3] Any column with an equivalent end-capped packing material is applicable.

[4] An example of chromatogram of ethopabate is shown in Fig. 8.1.2-1.



Measurement conditions

Detector：Wavelength：270 nm

Column：YMC-Pack ODS-A-312

Eluent：Water-acetonitrile（7:3）

Flow rate：1.0 mL/min

Fig. 8.1.2-1 A chromatogram of ethopabate added to a premix for chickens
（The arrow indicates the peak of ethopabate）

1.1.2 Formula feed 〔Feed Analysis Standards Chapter 8, Section 1, 2.1.1-(2)〕

A. Reagent preparation

1) Ethopabate standard solution: Place 20 mg of ethopabate〔$C_{12}H_{15}NO_4$〕 exactly measured in a 100 mL brown volumetric flask, add methanol for dissolving, further add the solvent up to the gauge line to prepare the ethopabate standard stock solution（1 mL of this solution contains an amount of ethopabate equivalent to 0.2 mg）.

At the time of use, exactly dilute a definite amount of the standard stock solution with methanol-water (7:3) to prepare several ethopabate standard solutions containing amounts of ethopabate equivalent to 0.25-2 μg per mL.

2) Neutral alumina: Dry the neutral alumina（particle diameter: 63-200 μm（230-70 mesh））[Note1] for column chromatograph at 120 °C for 2 hr.

B. Quantification

Extraction: Measure 10.0 g of analysis sample, place it in a 200 mL stoppered brown Erlenmeyer flask, add 100 mL of methanol-water (7:3), stir it for 30 min for extraction. Place the extracted solution in a stoppered brown centrifuging tube, centrifuge at 1,000×g for 5 min to obtain a sample solution for column treatment.

Column treatment: Pack 5 g of neutral alumina in a column tube (internal diameter: 10 mm) by dry processing to prepare the column.

Place the sample solution in the column, discard the first 3 mL of the outflow, and filter the second 5 mL of outflow with a membrane filter (0.5 μm or less pore diameter) to obtain a sample solution for the liquid chromatography.

Liquid chromatography: Inject respective 20 μL of the sample solution and each ethopabate standard solution into the liquid chromatograph to obtain the chromatogram.

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (excitation wavelength: 306 nm, fluorescence wavelength: 350 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note2[1]

Eluent: Water-acetonitrile (7:3)

Flow rate: 1.0mL/min

Column temperature: 40℃

Calculation: Obtain the peak height or area from the chromatogram [2] to prepare the calibration curve, and calculate the ethopabate amount in the sample.

Note 1. Use Aluminiumoxid 90 aktiv neutral Art. 1077 (Merck) or an equivalent one.

2. Use Mightsil RP-18 GP (Kanto Chemical) or an equivalent one

《Summary of analysis method》

This method is intended to determine the amount of ethopabate in formula feeds by extracting with methanol-water (7:3), purifying with a neutral alumina column, and quantifying using a liquid chromatograph with a fluorescence detector.

A flowsheet of analysis method is shown in Fig. 8.1.2-2.

Sample10.0 g
↓
Stoppered 200 mL Erlenmeyer flask
├ Methanol-water (7+3) 100 mL
├ Stir for 30 min
Stoppered brown centrifuging sedimentation tube
├ Centrifugation (1,000×*g* for 5min)
Column for cleanup (column [internal diameter: 10 mm] packed with 5 g of neutral alumina with dry processi
├ Discard the fiest 3 mL of effluent
├ Collect 5 mL of subsequent effluent
Filter through a membrane filter (pore diameter: 0.5 μm or less)
↓
LC-FL (Ex: 306 nm, Em: 350 nm)

Fig. 8.1.2-2. A flowsheet of quantification method of ethopabate in a formula feed

References Hiroshi Hibino, Takeshi Utiyama, Akihito Ikezawa, Kyoko Akimoto, Eiichi Ishiguro: Research Report of Animal Feed, 25, 21 (2000)

History in the Feed Analysis Standards: 【2】new【15】revision【17】revision【22】revision

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Formula feed for starting chick	3 to 15	3	95.0 to 98.6	6.0
Formula feed for growing chick	3 to 15	3	94.4 to 97.1	6.2
Formula feed for prior stage broiler	3 to 15	3	99.7 to 100.0	7.3

・Cooperative testing

Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for starting chicken	6	5	88.1	1.1	1.5	0.12

《Notes and precautions》

[1] Any column with an equivalent end-capped packing material is applicable. **The column used at the time of discussing about development of this analysis method was** Mightysil RP-18GP**.**

[2] An example of chromatogram of ethopabate is shown in Fig. 8.1.2-3.

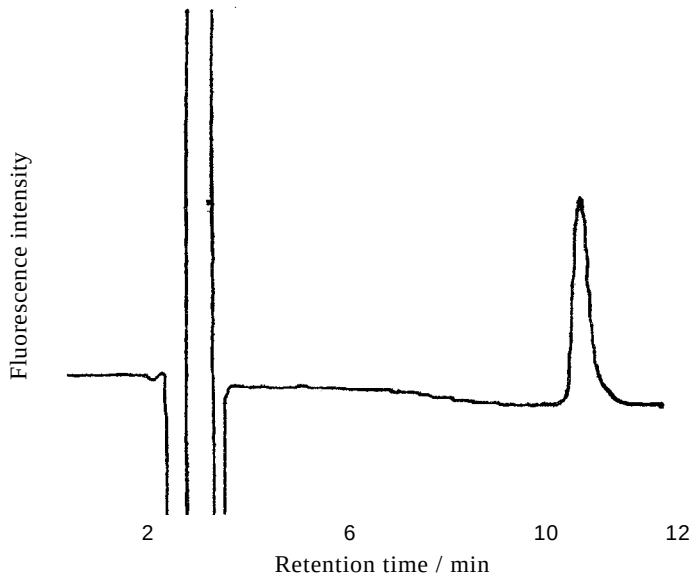


Fig. 8.1.2-3. A chromatogram of ethopabate (5 g/t) added to a formula feed for growing chick (The arrow indicates the peak of ethopabate)

2 Microquantitative test method

2.1 Liquid chromatography 〔Feed Analysis Standards Chapter 8, Section 1, 2.2.1〕

Scope of application: Formula feed

A. Reagent preparation

1) Ethopabate standard solution: Place 20 mg of ethopabate〔$C_{12}H_{15}NO_4$〕exactly measured in a100 mL brown volumetric flask, add methanol for dissolving, further add the solvent up to the gauge line to

prepare the ethopabate standard stock solution. (1 mL of this solution contains an amount of ethopabate equivalent to 0.2 mg).

At the time of use, exactly dilute a definite amount of the standard stock solution with acetonitrile-water (1:1) to prepare several ethopabate standard solutions containing an amount of ethopabate equivalent to 0.2-1 μg per mL.

2) Neutral alumina: Dry the neutral alumina（particle diameter: 63-200 μm（230-70 mesh)) Note1 for the column chromatograph at 120 °C for 2 hr.

B. Quantification

Extraction: Measure 10.0 g of analysis sample [1], place it in a stoppered 200 mL brown Erlenmeyer flask, add 100 mL of methanol-water (4:1), and stir it for 30 min for extraction. Place the extracted solution in a stoppered brown centrifuging tube, and centrifuge at 1,000×g for 5 min to obtain the supernatant as a sample solution for column treatment I.

Column treatment I: Pack 10 mg of neutral alumina in a column tube（internal diameter: 10 mm）with dry processing to prepare the column.

Place the sample solution in the column, and discard 3 mL of the first effluent. Place exactly 10 mL Note 2 of subsequent effluent in a 50 mL recovery flask, concentrate it under reduced pressure in water bath at 40 °C or lower until almost dry out, and then send nitrogen gas to obtain the dry matter [2]. Dissolve the residues by adding 5 mL of chloroform to obtain the sample solution for column treatment II.

Column treatment II: Wash a synthetic magnesium silicate minicolumn (910 mg) with 5 mL of chloroform. Place the sample solution in a minicolumn, and flow out with pressure injection Note 3. Wash the recovery flask which previously contained sample solution with 10 mL of chloroform, and process in a same manner. Then, add 10 mL of diethyl ether-hexane (4:1) to the minicolumn for washing. Place a 50 mL recovery flask under the minicolumn, to which add 5 mL of ethyl acetate to elute ethopabate with pressure injection Note3. Concentrate the eluate under reduced pressure in a water bath at 40 °C or lower to almost dry out, and then send nitrogen gas to obtain the dry matter. Dissolve the residues by exactly adding 1 mL of acetonitrile-water（1:1）, and filter this solution through a membrane filter (pore diameter: 0.5 μm or less) to obtain the sample solution for liquid chromatography.

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

Measurement conditions (example)

Detector: Ultraviolet spectrophotometer (measurement wavelength: 270 nm)

Column: Octadecylsilylated silica-gel column (internal diameter: 4.6 mm, length: 250 mm, particle diameter: 5 μm) Note 4[3]

Eluent: Phosphate buffer solution Note 5—methanol (11:9)[4]

Flow rate: 0.8 mL/min

Column temperature: 40°C

Calculation: Obtain the peak height from the chromatogram [5], and calculate the ethopabate amount in the sample.

Note 1. Aluminiumoxid 90 activ neutral Art. 1077 (Merck) or an equivalent one.

2. Collect from subsequent 13 mL of effluent.

3. Flow rate is approximately 2 mL/min.

4. Wakosil II $5C_{18}$HG (Wako Pure Chemical) or an equivalent one.

5. Dilute 9.0 g of sodium dihydrogen phosphate-12 water and 3.4 g of potassium dihydrogen phosphate with water up to a total amount of 1 L.

《Summary of analysis method》

This method has been developed to quantify a minute amount of ethopabate remaining in formula feeds caused by carrying over, etc. and is intended to determine the amount of ethopabate in feeds by extracting with methanol-water (4:1), purifying with a neutral alumina minicolumn and a Florisil minicolumn and by quantifying using a liquid chromatograph with an ultraviolet spectrophotometer.

References: Haruyoshi Harada, Norio Hikichi: Research Report of Animal Feed, 18, 55 (1993)

History in the Feed Analysis Standards: 【15】new

《Validation of analysis method》

・Recovery rate and repeat accuracy

Type of sample	Concentration (mg/kg)	Repeat	Recovery rate (%)	Repeat accuracy RSD (% or less)
Formula feed for adult chicken	0.2-0.8	3	92.7-109.0	11.5
Formula feed for developing boar	0.2-0.8	3	95.3-113.3	6.4
Formula feed for growing beef cattle	0.2-0.8	3	92.0-104.0	10.8

・Cooperative testing

Type of sample	No. of labs	Concentration (mg/kg)	Recovery rate (%)	Repeat accuracy in room RSD_r (%)	Reproducibility RSD_R (%)	HorRat
Formula feed for growing pig	6	0.5	95.7	4.5	6.0	0.47

《Notes and precautions》

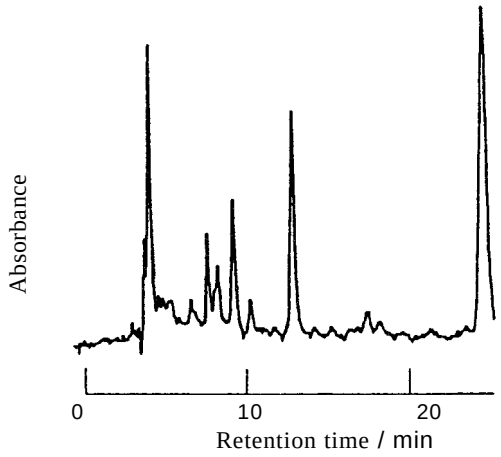
[1] Since a large amount of sample causes inadequate purification for column chromatography, the amount of sample should be 10 g or less.

[2] Otherwise, interference peaks appear; be careful to sufficiently dry so that no water remains.

[3] Any column with an equivalent end-capped packing material is applicable.

[4] Since the eluent contains buffer solution, LC apparatus and columns used should be washed enough, and the eluent should be replaced entirely with methanol, acetonitrile or others before storing. At the time of use, give eluent after replacing with water.

[5] An example of chromatogram of ethopabate is shown in Fig. 8.1.1-3.



Measurement conditions

Detector: Measurement wavelength270 nm

Column: Wakosil II $5C_{18}$HG

Eluent: Phosphate buffer solution — methanol

（11:9）

Flow rate: 0.8 mL/min

Column temperature: 40 °C

Fig. 8.1.2-4. A chromatogram of ethopabate added to a formula feed for adult chickens
（The arrow indicates the peak of ethopabate）