Chapter IV

Determination of Drug and Chemical Residue by HPLC

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OXOLINIC ACID

1. Principle

The Quinolone oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g] quino-line-7-carboxylic acid) is a commonly used antibiotic for treatment of infectious, diseases in fish farming. Tissue is extracted by homogenizing with dry ethyl acetate the organic phase is evaporated, the residue partitioned between aqueous oxalic acid and hexane. The aqueous phase is chromatographed using fluorometric detection. The LC is based on the method described by Ikai et. al.(1989).

2. Apparatus

2.1 Liquid Chromatography: Isocratic pump system, injector, fluorometric detector capable of monitoring emission at 369 nm and excitation at 327 nm.

2.2 Chromatographic column: Reverse phase, Nova-Pak C18 (150 mm x 3.9 mm). Operating condition: set flow rate 1.0 ml/min. As a part of the system shut-down at the end of the day, the mobile phase is pumped through the column for a minimum of fifteen minutes followed by a fifteen-minute rinse with methanol at 1 ml/min.

2.3 Homogenizer

2.4 Centrifuge

2.5 Rotary Evaporator

2.6 Filter Unit: Hyperclean Syringe Filters, 0.45μm Nylon, Non-Sterile, 13mm id. (Shandon Cat. No. 66982331)

2.7 Centrifuge tube: Oak Ridge Centrifuge Tube, PA (Nalgene Cat. No. 3119-0010)

3. Reagents

3.1 Anhydrous Na_2SO_4

3.2 Ethyl acetate AR-grade

3.3 n-Hexane AR-grade

3.4 Methanol HPLC-grade

3.5 Acetonitril HPLC-grade

3.6 Oxolinic acid (Sigma Chemical Company): Store all standard solutions below 10°C. Stock solution is stable for at least 3 months, but diluted solution should be kept no longer than 2 weeks.

Primary Standard Solution (100 μg/ml): Weigh Oxolinic acid 0.01g into a 100ml volumetric flask. Add ca. 10ml of dimethyl sulfoxide and swirl until completely dissolved. Dilute to 100ml with acetonitril and mix.

Secondary Standard Solution (10 μg/ml) : Pipette 10ml of Primary Standard Solution into 100ml volumetric flask and dilute to volume with acetonitril.

Working Standard Solution (1 μg/ml): Pipette 10ml of Secondary Standard Solution into 100ml volumetric flask and dilute to volume with acetonitril.

3.7 Oxalic acid 0.01M: Weigh 1.26g Oxalic acid dihydrate to 1 litre volumetric flask and dilute to volume with water, using 3N NaOH to adjust pH of 0.01M Oxalic acid to pH3.3.

3.8 Mobile Phase : Methanol:Acetonitril:0.01M Oxalic acid (pH3.3) = 1:3:6, filtered through Nylon-66 membrane. Mobile phase should be prepared daily and stored in a glass container.

4. Procedure

4.1 Weigh homogeneous sample 5.0g into centrifuge tube and add 10.0 of anh. Na_2SO_4 and 30ml of Ethyl acetate.

4.2 Homogenize with Polytron Homogenizer at 5,000rpm for 1 minute. Rinse probe with 5ml Ethyl acetate.

4.3 Centrifuge at 5,000rpm for 5 minutes. Filter the supernatant through filter paper Whatman No. 541 to 250ml Round Bottom Flask (RBF).

4.4 Rehomogenize the sediments with another 30ml Ethyl acetate and rinse probe well with 5ml of Ethyl acetate. Centrifuge at 5,000rpm for 5 minutes and combine the supernatant with the supernatant from the first extraction.

4.5 Evaporate the solvent in 250ml RBF to dryness by using Rotary Evaporator at 40°C until an oily liquid residue remains.

4.6 Redissolve residue with 5ml of mobile phase and 1ml of n-Hexane. Tube is capped and liquid is swirled, making sure that walls of the tube is thoroughly rinsed.

4.7 Centrifuge at 5000rpm for 5 minutes. Discard upper layer (n-Hexane) and transfer only clear aqueous liquid into a HPLC Autosampler vial. Sample preparations are stable in a refrigerator for 2-3 days.

4.8 Inject 200μl aliquots using a flow rate of 1 ml/min. with detector set at 327 nm excitation and 369 nm emission.

4.9 At the end of each day, rinse the system for 15 - 30 minutes with Methanol at 1 ml/min.

5. Calculation

$$\text{Oxolinic acid } (\mu g/g) = \frac{\text{Peak Area sample}}{\text{eak Area Std.}} \times \text{Conc. Std}$$

6. Method Validation

The recovery of Oxolinic acid from shrimp tissue was determined by spiking shrimp muscle tissue with 50, 80, 120, 160, 200 and 240 ng/g of Oxolinic acid, and submitting the samples to the described procedure. Each amount was added to five replicate portions. The recoveries ranged from 79 - 98%. The limit of quantification was 20 ng/g. Precision, expressed as percentage relative standard deviation, was below 4.82%.

The linearity of the method was checked by spiking the tissue with Oxolinic acid in the range 50 - 240 ng/g. Least-squares linear regression analysis of the data gave the equation: $y = 13.93x + 0.0087$, where y was the peak area and x was the amount of Oxolinic acid added. The correlation coefficient (r) was 0.9997.

REFERENCES:

1. Larocque, L., M. Sciinurr, S. Sved and A. Weninger (1991) Determination of Oxolinic Acid Residues in Salmon Tissue by Liquid Chromatography with Fluorescence Detection. J. Assoc. Off. Anal. Chem. vol. 74, No. 4 .

2. Ikai, Y., II. Oka, N. Kawamura, M. Yamada, K. Harada, M. Suzuki and H. Nakazawa (1989) J.Chromatography, 477, 397-406.

OXYTETRACYCLINE

1. Scope and Application

1.1 The method is applicable to fish and fish products.

2. Principle

This method permits the detection and identification of Oxytetracycline in animal tissues. The tetracyclines are extracted from the tissues with McIlvaine Buffer (pH4) and the filtered extract is then passed through a conditioned C-18 minicolumn (Sep-Pak C18). The tetracyclines are removed from the Sep-Pak C18 with methanol, the solvent evaporated and the residues dissolved in the HPLC mobile phase. The solution is chromatographed using fluorometric detection based on Japan Frozen Food Inspection Corporation.

3. Interferences

3.1 There are no known significant interferences.

4. Sampling Procedure And Storage

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. Sample Preparation

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.2 For raw fish, fresh or thawed: pass the sample through a grinder a sufficient number of times to obtain a homogeneous blend.

5.3 For processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix.

5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. Apparatus

6.1 50ml disposable polypropylene centrifuge tubes.

6.2 Polytron Homogenizer: Ika - Labortechnik Ultra - Turrax T25.

6.3 Centrifuge (capable of 5,000 rpm): Tomy Seiko Co., Ltd. with Rotor No. 9N.

6.4 Pasteur pipette, disposable.

6.5 Bond Elute C-18 disposable columns (Sep-Pak Plus C18), Part No. 20515.

6.6 Sample filter, Hyperclean Syringe Filter, 0.45 μm, Nylon membrane 13 mm.

6.7 Liquid Chromatography: Waters system consisting of 510 pump, fluorometric detector 470 capable of monitoring emission at 380 nm and excitation at 520 nm.

6.8 Chromatographic column: Reverse phase, Nova-Pak C18 (150 mm x 3.9 mm). Operating condition: set flow rate 0.8 ml /min. As a part of the system shut-down at the end of the day, the mobile phase is pumped through the column for a minimum of fifteen minutes, followed by a fifteen-minute rinse with methanol at 1 ml/min.

7. Reagents

7.1 Methanol HPLC-grade.

7.2 Imidazole AR-grade.

7.3 Disodium Ethylene Diamine Tetra-acetic acid AR-grade (Na_2EDTA).

7.4 Disodium hydrogen phosphate AR-grade (Na_2HPO_4).

7.5 McIlvaine Buffer/EDTA solution (pH = 4): Dissolve 28.41g of Na_2HPO_4 in distilled water in a 1 litre flask. Dilute to volume and mix. Dissolve 21.01g of citric acid monohydrate in distilled water in a 1 litre flask. Dilute to volume and mix. Combine 1 litre of the citric acid solution with 625ml of the Na_2HPO_4 solution in a 2 litres flask. Check the pH of this mixture; it should be 4.0 + 0.05.

Make the McIlvaine Buffer to 0.1 M EDTA by adding the appropriate weight and dissolving. For example, 37.224g EDTA/L x 1.625 L = 60.49 g.

7.6 Mobile Phase: 1 M Imidazole-Methanol. Dissolve Imidazole 68.08g, Magnesium acetate 10.72g and Na_2EDTA 0.37g with 800ml of deionized water, then adjust pH to 7.2 with Glacial Acetic acid. Adjust volume to 1 litre with deionized water. Mix the solution with Methanol at ratio 77:23.

7.7 Oxytetracycline (Sigma Chemical Company): Store the standard solutions below 10°C. Stock solution is stable for at least 3 months, but diluted solution should be kept no longer than 2 weeks.

Primary Standard Solution (100μg/ml): Weigh 0.01g of Oxytetracycline and into a 100ml volumetric flask. Add 0.01 N HCl a little and swirl until completely dissolved, dilute to volume with 0.1 N HCl.

Secondary Standard Solution (10μg/ml): Pipette 10ml of Primary Standard Solution into 100ml volumetric flask and dilute to volume with 0.1 N HCl.

Working Standard Solution (1μg/ml): Pipette 10ml of Secondary Standard Solution into 100ml volumetric flask and dilute to volume with 0.1 N HCl.

8. Procedure

8.1 Weigh 5.0g of whole or freshly homogenized tissues (see Note 1) into a 50ml polypropylene centrifuge tube.

8.2 Add 25ml of McIlvaine Buffer/EDTA solution (pH4) and homogenize with Polytron Homogenizer at 5,000rpm for 1 minute.

8.3 Centrifuge at 3,500rpm for 10 minutes. Filter the supernatant through filter paper Whatman No. 1 to 100ml beaker.

8.4 Rehomogenize the sediments with another 25ml McIlvaine Buffer / EDTA solution (pH = 4) and rinse probe well with 5ml of McIlvaine Buffer / EDTA solution. Centrifuge at 3.500rpm for 10 minutes and combine the supernatant with the one from the first extraction.

8.5 Load the extract on to the Sep-Pak C18 which had previously been deactivated by passing 10ml of Methanol, followed by 10ml of water dropwise through column. Regulate flow rate at ca 1 to 2 drops/sec. using valve to intriduce air leak into system.

8.6 Rinse the beaker with 10ml water and add to the reservoir when the extract is loaded on the column. Allow the column to run dry when the water rinse is conpleted and continue to draw air through the column for 5 minutes with vacuum at maximum.

8.8 Elute oxytetrscycline with 10ml of methanol to 125ml RBF. Evaporate the methanol by using Rotary Evaporator. Redissolve the residues with 5ml of mobile phase.

8.9 Filter the samples and standards through the Hyperclean Syringe Filter 0.45 um Nylon Membrane into HPLC vials. Inject 50μl to LC system at flow rate of 0.8ml/min. with detector set at 380 nm excitation and 520 nm emission.

8.10 At the end of each day, rinse the system for 15-30 min. with methanol at 1 ml/min.

9. Calculation

$$\text{Tetracyclines } (\mu g/g) = \frac{\text{Peak Area sample}}{\text{Peak Area Std.}} \times \text{Conc. Std.} \times \text{DF}$$

where: DF = Dilution Factor

10. Appendix

10.1 Note 1

It is preferable to use whole or freshly homogenized tissue as pre-homogenized tissue tends to cause the minicolumns to become plugged. This may be due to altered proteins in the homogenate. If pre-homogenized tissue is used, split the extract into two equal volumes and filter. changing filters between volumes.

11. Method Validation

The recovery of Oxytetracycline from shrimp tissue was determined by spiking shrimp muscle tissue with 50, 100, 200, 400, 800 and 1,600 ng/g of OTC, and submitting the samples to the described procedure. Each amount was added to five replicate portions. The recoveries ranged from 78 to 120%. The limit of quantification was 10 ng/g. Precision, expressed as percentage relative standard deviation, was below 15.1%.

The linearity of the method was checked by spiking the tissue with OTC in the range 50 - 1,600 ng/g. Least-squares linear regression analysis of the data gave the equation: $y = 8.4957 x + 0.1220$, where y was the peak area and x was the amount of OTC added. The correlation coefficient (r) was 0.9948.

REFERENCES

1. Japan Frozen Foods Inspection Institute Authorized by Ministry of Health and Welfare of the Japanese Government.

2. Oka, H.; Matsumoto, H. and Uno, K. (1985) Determination of Tetracycline, Oxytetracycline and Chlortetracycline in animal tissues and whole egg using HPLC, J. Chromatography 325, 265-274.

Determination of Oxytetracycline by HPLC

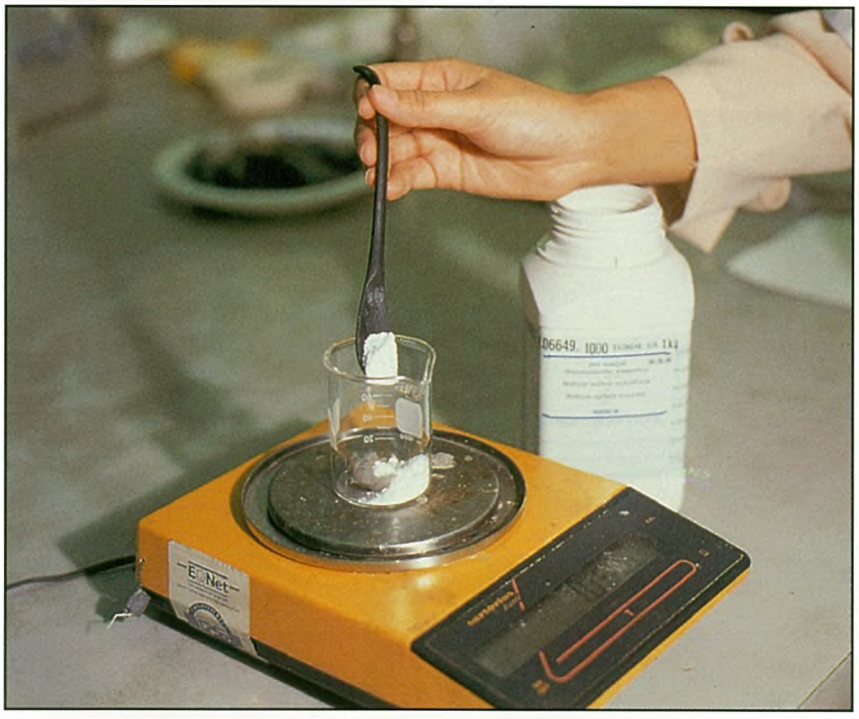
Weigh sample 5.0 g. + McIlvaine Buffer/EDTA solution (pH 4) 20 ml.

↓

Homogenize 5,000 rpm for 1 min.

↓

Centrifuge at 3,500 rpm. for 10 mins.



Re-homogenize the sediments with another 20 ml. McIlvaine Buffer/EDTA solution (pH 4)

↓

Centrifuge at 3,500 rpm. for 10 mins.

↓

Combine the supernatant with the supernatant from the first extraction.

Load the extract on to the Sep-Pak C18 which had previously been deactivated by padding 10 ml. of Methanol,

↓

followed by 10 ml. of water dropwise through column. Regulate Flow Rate at ca 1-2 drops/sec.

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Elute Oxytetracycline with 10 ml. of methanol to 250 ml. RBF.

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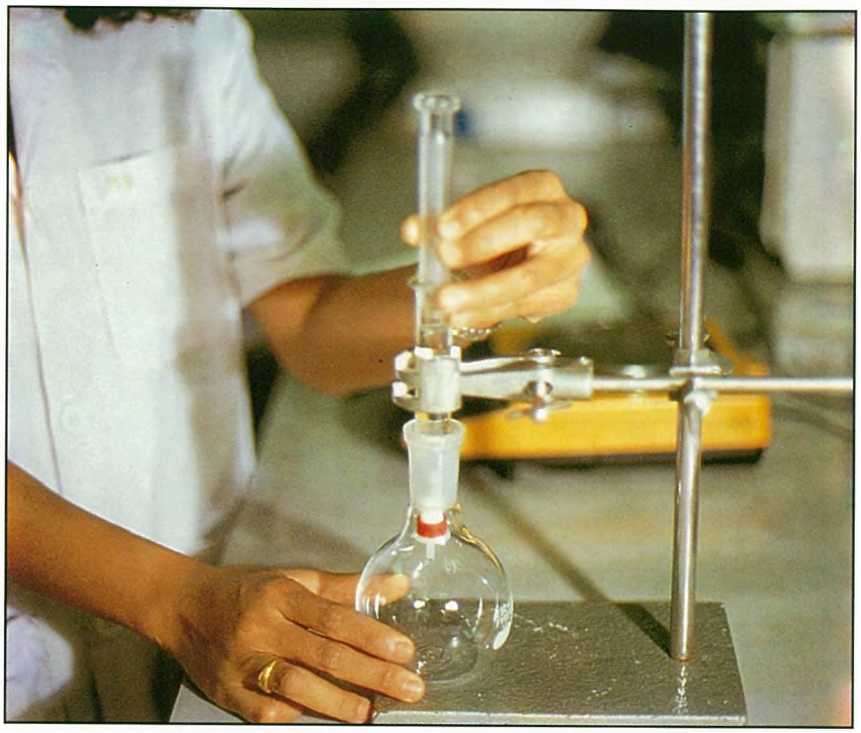
Evaporate the methanol by using Rotary Evaporator at 40°C.



Re dissolve residue with 5.0 ml. of mobile phase.

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Filter the samples through the Hyperclean Syringe Filter 0.45 μm Nylon menbrane into HPLC vials.

↓

Inject 50 μl to LC system at flow rate of 0.8 ml./min. with detector set at 380 nm. excitation and 520 nm. emission.

Determination of Oxolinic Acid by HPLC

Weigh sample 5.0 g. +
Na_2SO_4 10 g. +
Ethyl acetate 30 ml

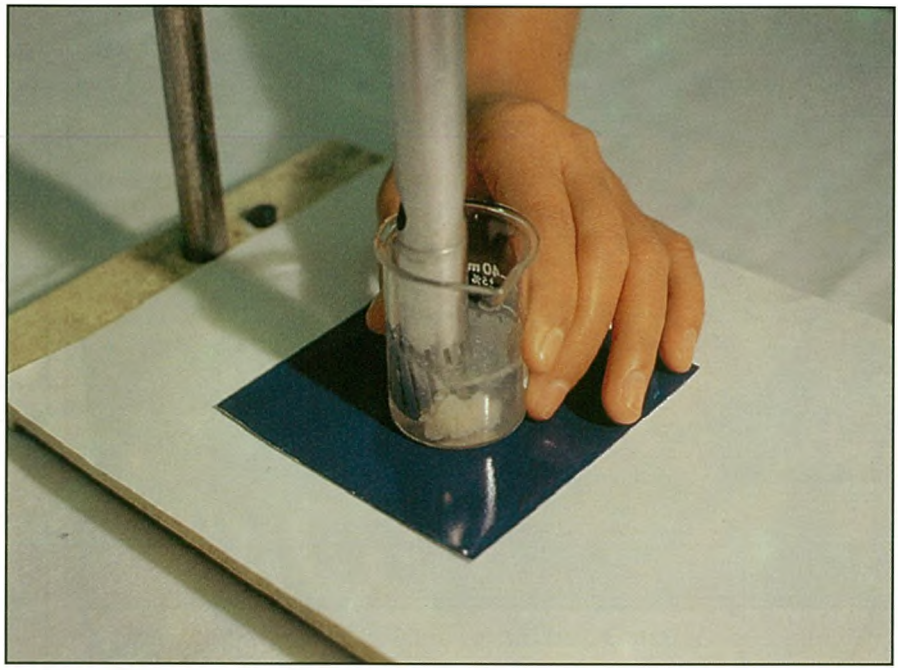
↓

Homogenize 5,000 rpm
for 1 min.

↓

Filter the supernatant
through filter paper
Whatman No.1

↓



Rehomogenize the sediments with
another 30 ml. Ethyl acetate.

→

Combine the solvent in 250 ml. RBF.
to dryness by using Rotary Evaporator at
40°C until an liquid remains.

Add solvent

Redissolve residue with
5 ml. of mobile phase and
1 ml. of n-Hexane.

↓

Centrifuge at 5,000 rpm
for 5 mins.

↓

Discard upper layer
(n-Hexane) and transfer only
aqueous liquid into
a HPLC Autosample vial.

↓

Inject 200μl aliquots
using a flow rate
of 1 ml./min. with
detector set at
327 nm. excitation and
369 nm. emission.

Prepare for reading