Original: Japanese
Provisional translation

Analytical Method for Trenbolone Acetate

1. **Analyte**
   α-trenbolone
   β-trenbolone
2. **Instrument**
   Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)
3. **Reagents**
   Use the reagents listed in Section C ***Reagent/Test Solution, Etc.***, Part II ***Food Additives***, except the following. Reagents designated as “special grade” in this section must meet the requirements for “special grade” specified in the Japan Industrial Standards for the reagents.
   Acetonitrile: Use a reagent not containing any substances that may interfere with the analysis of the target compounds.
   Formic Acid: Use a reagent not containing any substances that may interfere with the analysis of the target compounds.
   Ethyl acetate: Use a reagent not containing any substances that may interfere with th e analysis of the target compounds.
   Silica gel cartridge (500mg): A polyethylene tube of 8-9 mm in inside diameter packed with 500 mg of silica gel, or an equivalent cartridge in separation capability.
   Triethylamine: Triethylamine (special grade)
   2-fluoro-1-methylpyridinium *p*-toluenesulfonate: Use a reagent with a purity of 98% or higher.
   n-hexane: Use a reagent not containing any substances that may interfere with the analysis of the target compounds.
   Water: Use water suitable for chemical analysis, including distilled water, purified water, or pure water. If it contains any substances that may interfere with the analysis of the target compounds, wash with a solvent such as n-hexane before use.
   Anhydrous sodium sulfate: Use a reagent not containing any substances that may interfere with the analysis of the target compounds.
4. **Reference standard**
   Reference standard of α-trenbolone: Contains not less than 95% of α-trenbolone.
   Reference standard of β-trenbolone: Contains not less than 95% of β-trenbolone.
5. **Procedure**
   1) Extraction
      Add 50 mL of acetonitrile saturated with *n*-hexane and 50 mL of *n*-hexane to 10.0 g of sample, and homogenize. Add 20 g of anhydrous sodium sulphate thereto, and homogenize again. Centrifuge at 3,000 rpm for 5 minutes, discard the *n*-hexane layer, and collect an acetonitrile layer. Add 50 mL of acetonitrile to the residue, homogenize, and centrifuge as described above. Collect an acetonitrile layer again, combine the previously

obtained acetonitrile layer, and add another portion of acetonitrile to make exactly 100 mL. Take an exact 10 mL aliquot of the solution, concentrate it at below 40ºC, and remove the solvent. Add 20 mL of water to the residue, and extract with shaking twice with 20 mL each of ethyl acetate/*n*-hexane (1:4, v/v). Collect the ethyl acetate/*n*-hexane layer, concentrate at below 40°C, and remove the solvent. Dissolve the residue in 2 mL of ethyl acetate/*n*-hexane (1:4, v/v).

2) Clean-up

Add 5 mL of ethyl acetate/*n*-hexane (1:4, v/v) to a silica gel cartridge (500 mg), and discard the effluent. Transfer the solution obtained in "1) Extraction" to the cartridge, add 10 mL of ethyl acetate/*n*-hexane (1:4, v/v), and discard the effluent. Then, add 10 mL of ethyl acetate/*n*-hexane (1:1, v/v) to the cartridge, collect the eluate, concentrate it at below 40ºC, and remove the solvent.

3) Derivatization

Add 1 mL of 20 mg/mL 2-fluoro-1-methylpyridinium *p*-toluenesulfonate・acetonitrile and 0.05 mL of acetonitrile/triethylamine (9:1, v/v) to the residue obtained in "2) Clean-up", stir, and let stand at room temperature for 90 minutes. Concentrate the solution after derivatization reaction at below 40°C, and remove the solvent. Dissolve the residue in 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (3:1, v/v) to make exactly 1 mL, and use this solution as the test solution.

6. Calibration curve

Dissolved the reference standards of α-trenbolone and β-trenbolone respectively in acetonitrile to prepare standard stock solutions. Mix these stock standard solutions appropriately, dilute with acetonitrile, and prepare standard solutions of concentrations.

Take an appropriate aliquot of the solutions, concentrate at below 40°C, and remove the solvent. Add 1 mL of 20 mg/mL 2-fluoro-1-methylpyridinium *p*-toluenesulfonate・acetonitrile and 0.05 mL of acetonitrile/triethylamine (9:1, v/v) to the residue, stir, and let stand at room temperature for 90 minutes. Concentrate the solution after derivatization reaction at below 40℃, and remove the solvent. Dissolve the residue in 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (3:1, v/v), dilute with 0.1 vol% formic acid and 0.1 vol% formic acid/acetonitrile (3:1, v/v), and prepare standard solutions of several concentrations. Inject each standard solution to LC-MS/MS, and make calibration curves by peak-height or peak-area method. When the test solution is prepared following the above procedure, the sample containing 0.001 mg/kg of each analyte gives the test solution of 0.001 mg/L in concentration.

7. Quantification

Inject the test solution to LC-MS/MS and calculate the concentration of α-trenbolone and β-trenbolone from the calibration curve made in **6**.

8. Confirmation

Confirm using LC-MS/MS.

9. Measurement conditions

(Example)

Column: Silica gel with adamantyl group (2.1 mm in inside diameter, 150 mm in length, 3

µm in particle diameter)

Column temperature: 40ºC

Mobile phase: Initially 0.1 vol% formic acid/0.1 vol% formic acid/acetonitrile (3:1, v/v) for 5 min, followed by a linear gradient from 0.1 vol% formic acid/0.1 vol% formic acid/acetonitrile (3:1, v/v) to (11:9, v/v) in 5 min.

Ionization mode: Electrospray ionization (positive ion mode)

Major monitoring ion (*m/z*)

α-trenbolone-1-methylpyridinium derivatives: Precursor ion 362, product ions 253, 197

β-trenbolone-1-methylpyridinium derivatives: Precursor ion 362, product ions 253, 197

Injection volume: 5 µL

Expected retention time

α-trenbolone-1-methylpyridinium derivatives: 8 min.

β-trenbolone-1-methylpyridinium derivatives: 9 min.