Analytical Method for Brotizolam
(Targeted to animal products)

The target compound to be determined is brotizolam.

1. Instrument

Liquid chromatograph-tandem mass spectrometer (LC-MS/MS)

2. Reagents

Use the reagents listed in Section C *Reagents/Test Solutions*, Etc., Part II *Food additives*, except the following.

Acetone: Use a reagent not containing any substance that may interfere with the analysis of the target compound.

Acetonitrile: Use a reagent not containing any substance that may interfere with the analysis of the target compound.

Octadecylsilanized silica gel cartridge (1,000 mg): A polyethylene tube of 12–13 mm in inside diameter packed with 1,000 mg of octadecylsilanized silica gel, or a cartridge equivalent to the specified one in separation capability. Trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500mg): A polyethylene tube of 12–13 mm in inside diameter packed with 500 mg of trimethylaminopropylsilanized silica gel in the upper layer and 500 mg of ethylenediamine-*N*-propylsilanized silica gel in the lower layer, or a cartridge equivalent to the specified one in separation capability.

n-Hexane: Use a reagent not containing any substance that may interfere with analysis of the target compound.

Sodium chloride: Use a reagent not containing any substance that may interfere with analysis of the target compound.

Anhydrous sodium sulfate: Use a reagent not containing any substance that may interfere with analysis of the target compound.

Water: Use water suitable for chemical analysis, including distilled water, purified water, or pure water. If it contains any substance that may interfere with analysis of the target compound, wash with a solvent such as *n*-hexane before use.

3. Reference standard

Reference standard of brotizolam: Contains not less than 98% of brotizolam.

4. Procedure

a. Extraction

(i) Muscle, fat, liver, kidney and fish/shellfish

For fat, weigh 5.00 g of sample. For muscle, liver, kidney and fish/shellfish, weigh 10.0 g of sample.

Add 50 mL of acetone/*n*-hexane (1:1, v/v) to the sample, homogenize, and filter with suction. Add 25 mL of acetone/*n*-hexane (1:1, v/v) to the residue on the filter paper, homogenize, and filter as described above. Combine the resulting filtrates, and concentrate to about 15 mL at below 40°C. Add 100 mL of saturated sodium chloride solution, and extract with shaking twice with ethyl acetate (100 mL and then 50 mL). Combine the resulting extracts, dehydrate with anhydrous sodium sulfate, filter out the anhydrous sodium sulfate, concentrate the filtrate at below 40°C, and remove the solvent. Add 30 mL of *n*-hexane to the residue, and extract with shaking twice with 30 mL of acetonitrile saturated with *n*-hexane. Combine the resulting extracts, and concentrate to about 5 mL at below 40°C.

(ii) Milk, egg and honey

For milk and egg, weigh 5.00 g of sample. For honey, weigh 5.00 g of sample and dissolve with 5 mL of water.

Add 30 mL of acetonitrile to the sample, homogenize, centrifuge at 2,500 rpm for 5 minutes, and collect the acetonitrile layer. Add 20 mL of acetonitrile to the residue (the residue and the water layer for honey), homogenize, and centrifuge as described above. Combine the resulting acetonitrile layer, dehydrate with anhydrous sodium sulfate, filter out the anhydrous sodium sulfate, and concentrate the filtrate to about 5 mL at below 40°C.

b. Clean-up

Connect a trimethylaminopropylsilanized silica gel/ethylenediamine-*N*-propylsilanized silica gel layered cartridge (500 mg/500 mg) to the lower part of an octadecylsilanized silica gel cartridge (1,000 mg). Add 10 mL of acetonitrile to the cartridge and discard the effluent. Transfer the solution obtained in a. “Extraction” to the cartridge, elute with 10 mL of acetonitrile, collect the total eluate, concentrate at below 40°C and remove the solvent. Dissolve the residue in acetonitrile/water (1:1, v/v) to make exactly 5 mL (2.5 mL for fat, milk, egg and honey), and use this solution as the test solution.

5. Measurement

a. Calibration curve

Prepare brotizolam standard solutions (acetonitrile/water (1:1, v/v)) of several concentrations. Inject each standard solution to LC-MS/MS, and make a calibration curve by peak-height or peak-area method. When the test solution is prepared following the above procedure, the sample containing 0.0005 mg/kg of brotizolam gives the test solution of 0.001 mg/L in concentration.

b. Quantification

Inject the test solutions to LC-MS/MS and calculate the concentration of brotizolam from the calibration curve made in a. “Calibration curve”.

c. Confirmation

Confirm using LC-MS/MS.

d. Measurement conditions

Column: Octadecylsilanized silica gel, 2.1 mm in inside diameter, 150 mm in length and 3 μm in particle diameter

Column temperature: 40°C

Mobile phase: Linear gradient from mobile phase acetonitrile/mobile phase 0.1 vol% formic acid (3:7, v/v) to (7:3, v/v) in 15 minutes.

Ionization mode: Electrospray ionization method (positive ion)

Major monitoring ions (*m/z*): Precursor ion 380, product ion 344, 314

Injection volume: 5 μL

Expected retention time: 13 minutes

6. Limit of Quantification

0.0005 mg/kg