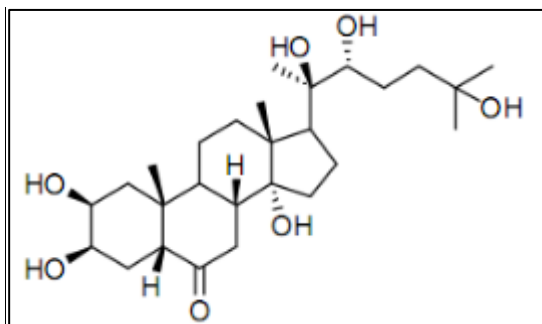


14-HYDROXYLATED BRASSINOSTEROID



Common name	14-Hydroxylated brassinosteroid
Chemical name	(2β,3β,5β,22R)-2,3,14,20,22,25-hexahydroxy-Cholestan-6-one
Empirical formula	C ₂₇ H ₄₆ O ₇
RMM	482.7
m.p.	149-150 °C
v.p.	2.01E-21 mmHg at 25°C
Solubility	In water 13 g/l, methanol and acetonitrile > 100 g/l(all at 20°C)
Description	Off-White Solid
Stability	Stable in weak acidic and low temperature condition
Formulation	Soluble liquid

14-HYDROXYLATED BRASSINOSTEROID TECHNICAL CONCENTRATE

XXX/TK/M/-

1. **Sampling.** Take at least 100 g.

2. Identity tests

2.1 **HPLC.** Use the HPLC method below. The relative retention time of 14-hydroxylated brassinosteroid in the sample solution should not deviate by more than 1.5% from that of calibration solution.

2.2 **UV spectrometry.** Record the UV spectrum during the HPLC determination from 190 to 400 nm using a diode array detector. The spectrum obtained from the sample should not differ significantly from that of the standard.

3. 14-Hydroxylated brassinosteroid

OUTLINE OF METHOD

14-Hydroxylated brassinosteroid is determined by high performance liquid chromatography on a reversed phase column with UV detection and external standardization.

REAGENTS

Acetonitrile: HPLC grade

Methanol: HPLC grade

Water: Ultra-pure

Phenylboronic acid: AR grade

14-hydroxylated brassinosteroid standard: Known purity

APPARATUS

Balance

Ultrasonic water bath

High performance liquid chromatography equipped with a detector suitable for operation at 222nm

Column stainless steel, Eclipse Plus 100mm × 4.6mm (i.d) columns, C18 packed with octadecyl silane filler (3.5µm), or equivalent.

Filter pore diameter: 0.45 µm

Water bath

PROCEDURES

(a) LIQUID CHROMATOGRAPHIC CONDITIONS

Mobile phase: Acetonitrile / Water= 45 / 55 (v/v)

Flow rate: 1.0 ml/min

Detector wavelength: 222 nm

Injection volume: 10 µl

Column temperature: 30 °C

Retention time: approximately 9.1 min

(b) Equilibration of the chromatographic system. Inject the calibration solution and repeat the injections until retention times and the response factors calculated from the peak areas vary by less than 1.5 % for successive injections.

(c) Preparation of solvent: 10 mg/ml Phenylboronic Acid solution was prepared with methanol.

(d) Preparation of standard solution: Weigh 0.02 g (to the nearest 0.02 mg) 14-hydroxylated brassinosteroid standard into a 10 ml volumetric flask and filled to volume with methanol to prepare the stock standard solution. Then 1 ml of above solution is transferred to a 25 ml volumetric flask, 3 ml of Phenylboronic Acid solution is added to the flask and filled to 20 ml with methanol. The solution is mixed well and placed in water bath at $70\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for reaction for 0.5 hour. After the reaction is done and the solution cools down to room temperature, the volumetric flask is filled to line with methanol. Mix thoroughly and place the flask in an ultrasonic bath for 5-10 min, then filter the solution through a $0.45\text{ }\mu\text{m}$ filter membrane prior to analysis (solution C).

(e) Preparation of sample solution: Prepare solutions in duplicate for each sample. Weigh sufficient sample (to the nearest 0.02 mg) to contain about 0.02 g 14-hydroxylated brassinosteroid into a 10 ml volumetric flask and filled to volume with methanol. Then 1ml of above solution is transferred to a 25 ml volumetric flask, 3 ml of Phenylboronic Acid solution is added to the flask and filled to 20 ml with methanol. The solution is mixed well and placed in water bath at $70\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for reaction for 0.5 hour. After the reaction is done and the solution cools down to room temperature, the volumetric flask is filled to line with methanol. Mix thoroughly and place the flask in an ultrasonic bath for 5-10 min, then filter the solution through a $0.45\text{ }\mu\text{m}$ filter membrane prior to analysis (solution S).

(f) Determination: Inject in duplicate 10 μl portions of each sample solution bracketing them by injections of the calibration solutions as follows:

$C_A, S_1, S_1, C_B, S_2, S_2, C_A$, etc

(g) Calculation

$$f_i = \frac{S \times P}{H_s}$$

$$\text{Content of} = \frac{H_w \times f}{w} \quad \text{g/kg}$$

Where:

f_i = individual response factor

f = mean response factor

H_s = peak areas of 14-hydroxylated brassinosteroid in the calibration solution

Hw =peak areas of 14-hydroxylated brassinosteroid in the sample solution

s =mass of 14-hydroxylated brassinosteroid standard (mg)

w =mass of sample taken (mg)

P =purity of 14-hydroxylated brassinosteroid standard (g/kg)

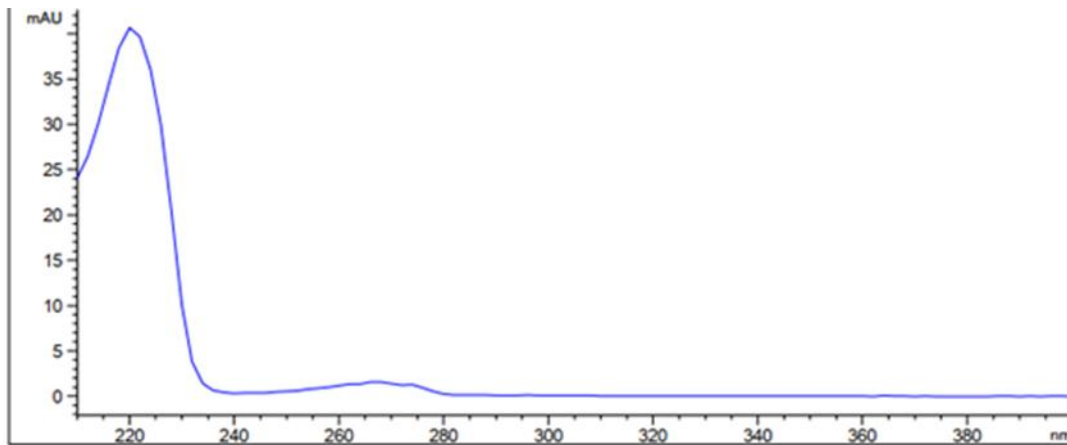


Fig 1 UV spectra of 14-hydroxylated brassinosteroid

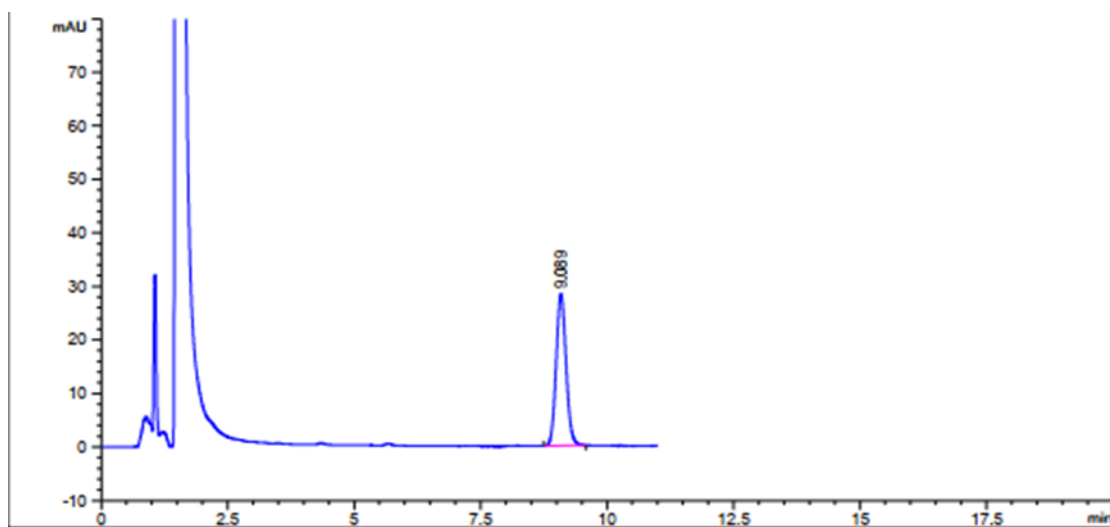


Fig. 2 Chromatogram of 14-hydroxylated brassinosteroid standard

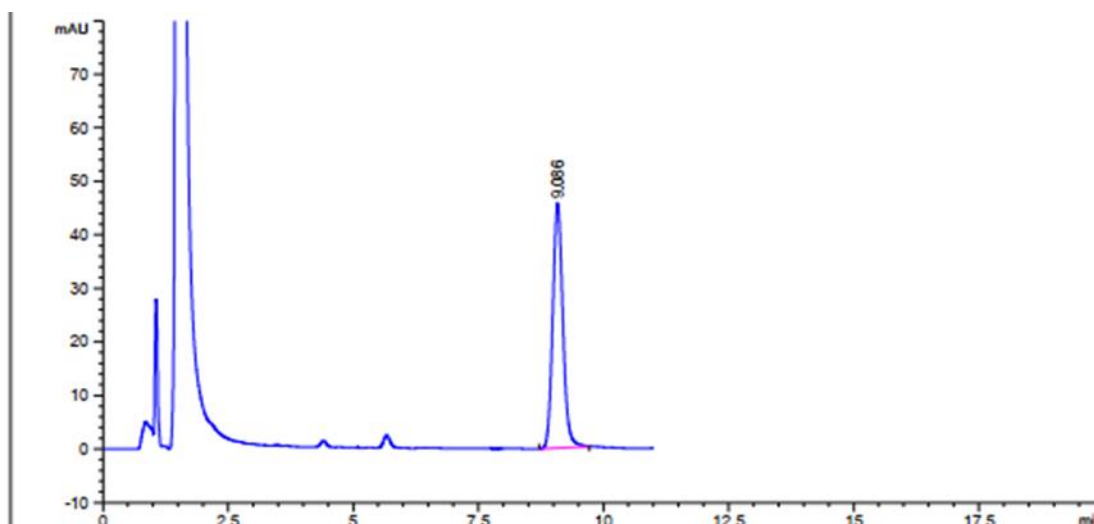


Fig. 3 Chromatogram of 14-hydroxylated brassinosteroid 80% TK sample

14-HYDROXYLATED BRASSINOSTEROID SOLUBLE LIQUID
XXX/SL/M/-

1. **Sampling.** Take at least 1l.
2. **Identity tests.** As for 14-Hydroxylated brassinosteroid XXX/TK/M/2
3. **14-Hydroxylated brassinosteroid.** As for 14-Hydroxylated brassinosteroid XXX/TK/M/3 except:

PROCEDURES

(a) LIQUID CHROMATOGRAPHIC CONDITIONS

Mobile phase:

Time [min]	Acetonitrile[% v/v]	Water[% v/v]
0	45	55
11	45	55
12	85	15
16	85	15
17	45	55
19	45	55

(d)Preparation of standard solution: Weigh 0.01 g (to the nearest 0.02 mg) 14-hydroxylated brassinosteroid standard into a 10 ml volumetric flask and filled to volume with methanol to prepare the stock standard solution. Then 1 ml of above solution is transferred to a 25 ml volumetric flask, 3 ml of Phenylboronic Acid solution is added to the flask and filled to 20 ml with methanol. The solution is mixed well and placed in water bath at 70 °C ±2 °C for reaction for 0.5 hour. After the reaction is done and the solution cools down to room temperature, the volumetric flask is filled to line with methanol. Mix thoroughly and filter the solution through a 0.45 µm filter membrane prior to analysis(solution C).

(e) Preparation of sample solution: Prepare solutions in duplicate for each sample. Weigh sufficient sample (to the nearest 0.2 mg) to contain about 0.001 g of 14-hydroxylated brassinosteroid into a 250 ml round bottom flask. Use rotary evaporator at 85°C~90°C for 4 min at least to concentrate to nearby dry. The concentrate is transfer into a 25 ml volumetric flask and the flask is washed 3 times with methanol, then the solution is transferred to above 25 ml volumetric flask. 3.0 ml of Phenylboronic acid Solution is added to the flask and filled to 20 ml with methanol. The solution is mixed well and placed in water bath at 70°C±2°C for reaction for 0.5 hour. After the reaction is done and the solution cools down to room temperature, the volumetric flask is filled to line with methanol. Mix thoroughly and filter the solution through a 0.45 µm filter membrane prior to analysis (solution S).

(g) Calculation

$$f_i = \frac{S \times P}{H_s}$$

$$\text{Content of} = \frac{H_w \times f}{10 \times W} \quad \text{g/kg}$$

Where:

f_i =individual response factor

f = mean response factor

H_s =peak areas of 14-hydroxylated brassinosteroid in the calibration solution

H_w =peak areas of 14-hydroxylated brassinosteroid in the sample solution

s =mass of 14-hydroxylated brassinosteroid standard (mg)

w =mass of sample taken (mg)

P =purity of 14-hydroxylated brassinosteroid standard (g/kg)

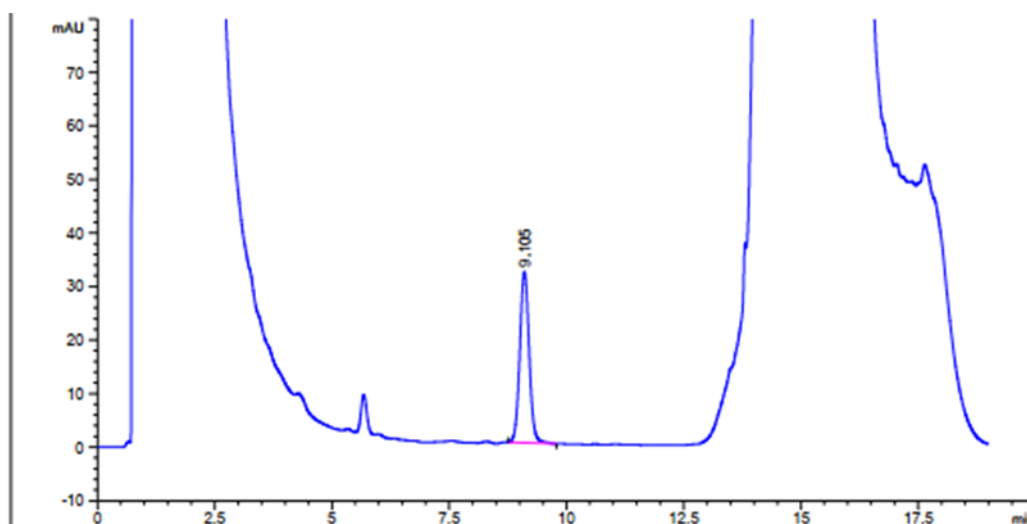


Fig. 4 Chromatogram of 14-Hydroxylated brassinosteroid 0.0075% SL sample