Bufonis Venenum

Figure 1 (i) A photograph of dried secretion of *Bufo bufo gargarizans* Cantor

A. Lumps of dried secretion B. Magnified image of fracture of lump
C. Magnified image of fracture of lump (after water has been dripped on it)

Figure 1 (ii) A photograph of dried secretion of *Bufo melanostictus* Schneider

A. Lumps of dried secretion B. Magnified image of fracture of lump
C. Magnified image of fracture of lump (after water has been dripped on it)

1. NAMES

Official Name: Bufonis Venenum

Chinese Name: 蟾酥

Chinese Phonetic Name: Chansu

2. SOURCE

Bufonis Venenum is the dried secretion of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider (Bufonidae). Usually the toad is collected in summer and autumn, washed clean. The white serous fluid of the parotid glands and skin glands is squeezed out, filtered, then dried to obtain Bufonis Venenum.

3. DESCRIPTION

***Bufo bufo gargarizans* Cantor:** Flattened and rounded lumps, brown or reddish-brown, 65-117 mm in diameter, 2.5-20 mm thick. Texture hard, uneasily broken. Fracture brown, corneous, slightly lustrous. Odour slightly stinky, smelling the powder causes sneezing. A creamy white bump is produced on the fracture when water is dripped on it [Fig. 1 (i)].

***Bufo melanostictus* Schneider:** 65-112 mm in diameter, 3-16 mm thick [Fig. 1 (ii)].

4.2 Thin-Layer Chromatographic Identification *[Appendix IV(A)]*

Standard solutions

Cinobufagin standard solution

Weigh 1.0 mg of cinobufagin CRS (Fig. 2) and dissolve in 5 mL of methanol.

Resibufogenin standard solution

Weigh 1.0 mg of resibufogenin CRS (Fig. 2) and dissolve in 5 mL of methanol.

Developing solvent system

Prepare a mixture of cyclohexane, acetone and ethyl acetate (4:3:2, v/v).

Spray reagent

Add slowly 10 mL of sulphuric acid to 90 mL of ethanol.

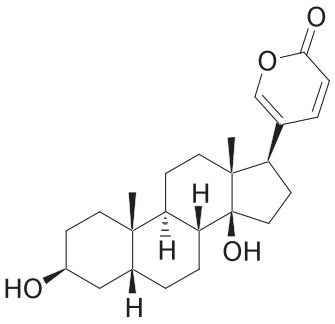
Test solution

Weigh 0.1 g of the powdered sample and place it in a 50-mL round-bottomed flask, then add 25 mL of methanol. Reflux the mixture for 30 min. Cool down to room temperature. Filter the mixture.

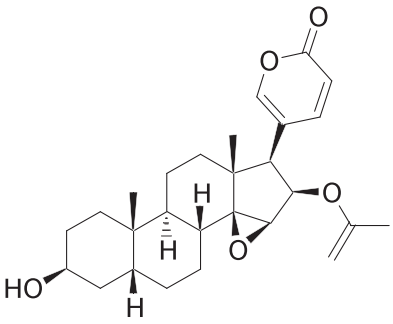
Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate and a freshly prepared developing solvent system as described above. Apply separately cinobufagin standard solution, resibufogenin standard solution and the test solution (10 µL each) to the plate. Develop over a path of about 8 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Spray the plate evenly with the spray reagent and heat at about 105ºC (about 10 min). Examine the plate under UV light (366 nm). Calculate the R_f values by using the equation as indicated in Appendix IV (A).

(i)



(ii)



(iii)

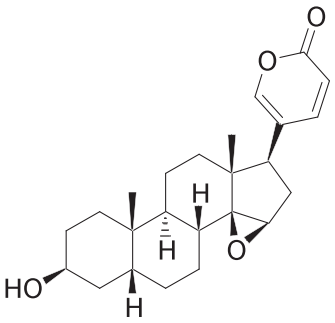


Figure 2 Chemical structures of (i) bufalin (ii) cinobufagin and (iii) resibufogenin

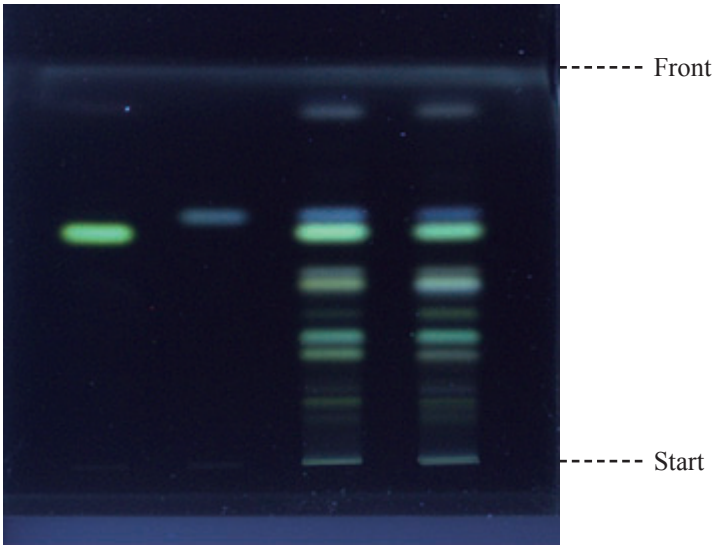


Figure 3 A reference HPTLC chromatogram of Bufonis Venenum extract observed under UV light (366 nm) after staining

1. Cinobufagin standard solution 2. Resibufogenin standard solution
3. Test solution of
(i) dried secretion of *Bufo bufo gargarizans* Cantor
(ii) dried secretion of *Bufo melanostictus* Schneider

For positive identification, the sample must give spots or bands with chromatographic characteristics, including the colour and the R_f values, corresponding to those of cinobufagin and resibufogenin (Fig. 3).

4.3 High-Performance Liquid Chromatographic Fingerprinting *(Appendix XII)*

Standard solutions

Bufalin standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 2.0 mg of bufalin CRS (Fig. 2) and dissolve in 10 mL of methanol.

Cinobufagin standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 2.0 mg of cinobufagin CRS and dissolve in 10 mL of methanol.

Resibufogenin standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 2.0 mg of resibufogenin CRS and dissolve in 10 mL of methanol.

Test solution

Weigh 0.1 g of the powdered sample and place it in a 50-mL conical flask, then add 20 mL of methanol. Sonicate (250 W) the mixture for 30 min. Filter and transfer the filtrate to a 25-mL

volumetric flask. Wash the residue with methanol. Combine the solutions and make up to the mark with methanol. Filter through a 0.45-µm RC filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (296 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 µm particle size, 120 Å pore size and 18% carbon loading). The flow rate is about 0.7 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	0.1% Formic acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 15	70 → 55	30 → 45	linear gradient
15 – 40	55	45	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of bufalin Std-FP, cinobufagin Std-FP and resibufogenin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of bufalin, cinobufagin and resibufogenin should not be more than 5.0%; the RSD of the retention times of bufalin, cinobufagin and resibufogenin peaks should not be more than 2.0%; the column efficiencies determined from bufalin, cinobufagin and resibufogenin peaks should not be less than 20000 theoretical plates.

The *R* value between peak 4 and the closest peak; the *R* value between peak 5 and the closest peak; and the *R* value between peak 6 and the closest peak in the chromatogram of the test solution should not be less than 1.5 [Fig. 4 (i) or (ii)].

Procedure

Separately inject bufalin Std-FP, cinobufagin Std-FP, resibufogenin Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of bufalin, cinobufagin and resibufogenin peaks in the chromatograms of bufalin Std-FP, cinobufagin Std-FP, resibufogenin Std-FP and the retention times of the six characteristic peaks [Fig. 4 (i) or (ii)] in the chromatogram of the test solution. Identify bufalin, cinobufagin and resibufogenin peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of bufalin Std-FP, cinobufagin Std-FP and resibufogenin Std-FP. The retention times of bufalin, cinobufagin and resibufogenin peaks in the chromatograms of the test solution and the corresponding Std-FP should not differ by more than 2.0%. Calculate the RRTs of the characteristic peaks by using the equation as indicated in Appendix XII.

The RRTs and acceptable ranges of the six characteristic peaks of Bufonis Venenum extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the six characteristic peaks of Bufonis Venenum extract

Peak No.	RRT	Acceptable Range
1 (arenobufagin)	0.36	± 0.03
2 (telocinobufagin)	0.52	± 0.03
3 (bufotalin)	0.56	± 0.03
4 (bufalin)	0.75	± 0.03
5 (marker, cinobufagin)	1.00	-
6 (resibufogenin)	1.05	± 0.03

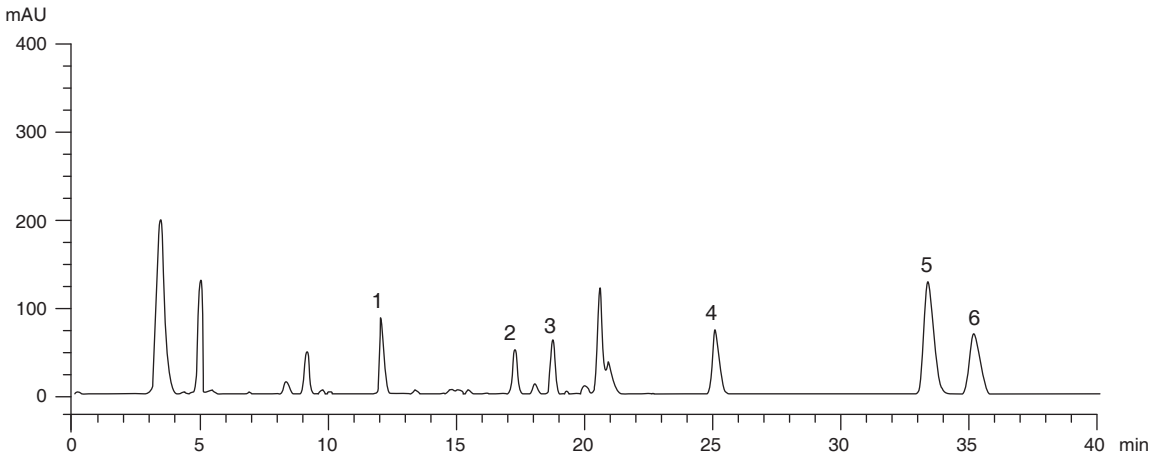


Figure 4 (i) A reference fingerprint chromatogram of dried secretion of *Bufo bufo gargarizans* Cantor extract

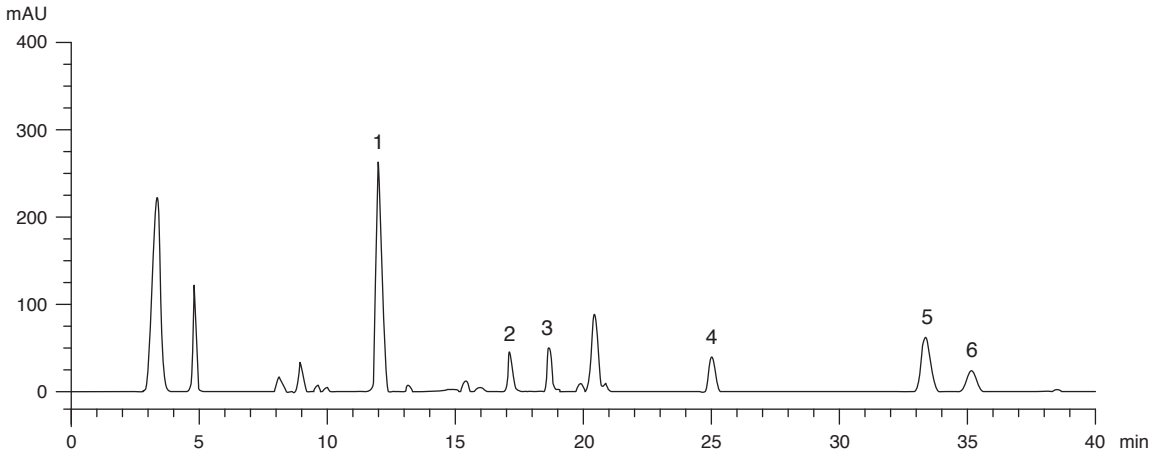


Figure 4 (ii) A reference fingerprint chromatogram of dried secretion of *Bufo melanostictus* Schneider extract

For positive identification, the sample must give the above six characteristic peaks with RRTs falling within the acceptable range of the corresponding peaks in the respective reference fingerprint chromatograms [Fig. 4 (i) or (ii)].

5. TESTS

5.1 Heavy Metals *(Appendix V)*: meet the requirements.

5.2 Pesticide Residues *(Appendix VI)*: meet the requirements.

5.3 Mycotoxins *(Appendix VII)*: meet the requirements.

5.4 Sulphur Dioxide Residues *(Appendix XVII)*: meet the requirements.

5.5 Ash *(Appendix IX)*

Total ash: not more than 4.5%.
Acid-insoluble ash: not more than 2.0%.

5.6 Water Content *(Appendix X)*

Oven dried method: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 28.0%.
Ethanol-soluble extractives (hot extraction method): not less than 31.0%.

7. ASSAY

Carry out the method as directed in Appendix IV (B).

Standard solution

Mixed bufalin, cinobufagin and resibufogenin standard stock solution, Std-Stock (500 mg/L each)
Weigh accurately 12.5 mg of bufalin CRS, 12.5 mg of cinobufagin CRS and 12.5 mg of resibufogenin CRS, and dissolve in 25 mL of methanol.
Mixed bufalin, cinobufagin and resibufogenin standard solution for assay, Std-AS
Measure accurately the volume of the mixed bufalin, cinobufagin and resibufogenin Std-Stock, dilute with methanol to produce a series of solutions of 6, 40, 80, 100, 200 mg/L for bufalin, 6, 100, 200, 300, 500 mg/L for cinobufagin and 6, 40, 80, 100, 200 mg/L for resibufogenin.

Test solution

Weigh accurately 0.1 g of the powdered sample and place it in a 50-mL conical flask, then add 20 mL of methanol. Sonicate (250 W) the mixture for 30 min. Filter and transfer the filtrate to a 25-mL volumetric flask. Wash the residue with methanol. Combine the solutions and make up to the mark with methanol. Filter through a 0.45-μm RC filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (296 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size, 120 Å pore size and 18% carbon loading). The flow rate is about 0.7 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	0.1% Formic acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 15	70 → 55	30 → 45	linear gradient
15 – 40	55	45	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 μL of the mixed bufalin, cinobufagin and resibufogenin Std-AS (80 mg/L for bufalin, 200 mg/L for cinobufagin and 80 mg/L for resibufogenin). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of bufalin, cinobufagin and resibufogenin should not be more than 5.0%; the RSD of the retention times of bufalin, cinobufagin and resibufogenin peaks should not be more than 2.0%; the column efficiencies determined from bufalin, cinobufagin and resibufogenin peaks should not be less than 20000 theoretical plates.

The *R* value between bufalin peak and the closest peak; the *R* value between cinobufagin peak and the closest peak; and the *R* value between resibufogenin peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curves

Inject a series of the mixed bufalin, cinobufagin and resibufogenin Std-AS (10 μL each) into the HPLC system and record the chromatograms. Plot the peak areas of bufalin, cinobufagin and resibufogenin against the corresponding concentrations of the mixed bufalin, cinobufagin and resibufogenin Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify bufalin, cinobufagin and resibufogenin peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed bufalin, cinobufagin and resibufogenin Std-AS. The retention times of bufalin, cinobufagin and resibufogenin peaks in the chromatograms of the test solution and the Std-AS should not differ by more than 5.0%. Measure the peak areas and calculate the concentrations (in milligram per litre) of bufalin, cinobufagin and resibufogenin in the test solution, and calculate the percentage contents of bufalin, cinobufagin and resibufogenin in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 5.8% of the total content of bufalin ($C_{24}H_{34}O_4$), cinobufagin ($C_{26}H_{34}O_6$) and resibufogenin ($C_{24}H_{32}O_4$), calculated with reference to the dried substance.

8. CAUTION

This CMM is potent/toxic and should be prescribed by registered Chinese medicine practitioner.