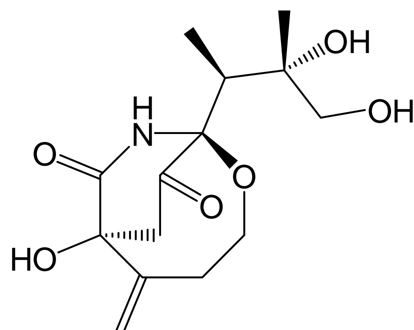
22 Bicozamycin

(Bicyclomycin)



$C_{12}H_{18}N_2O_7$ MW: 302.3 CAS No.: 38129-37-2

[Summary of bicozamycin]

Bicozamycin (BZM) is an antibiotic obtained from a fermented culture of *Streptomyces sapporonensis*.

For physicochemical properties, BZM technical occurs as white to brownish white crystals or powder, and has no odor or slightly has a characteristic odor. It is freely soluble in water, soluble in methanol, slightly soluble in acetone and in ethanol, and practically insoluble in ether and in chloroform.

BZM has an antibacterial effect on Gram-negative bacteria and a growth promoting effect on chickens (including broilers) and pigs.

«Standards and specifications in the Act on Safety Assurance and Quality Improvement of Feeds»

BZM is a pure-grade antibiotic that was designated as a feed additive as of July 6, 1983. The specifications for feeds containing this ingredient are specified in Appended Table No.1, 1-(1)–C of the Standards and Specifications in the Act on Safety Assurance and Quality Improvement of Feeds.

(in g(potency)/t)

Feed of interest	For chickens (except for broilers)	For broilers	For pigs	
	Starting chiks Growing chiks	Starting period broilers Finishing period broilers	Suckling piglets	Piglets
Added amount	5~20	5~20	10	10

The amount of BZM added to a commercial premix is roughly 2 to 15 g (potency)/kg.

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

1.1 Premix

[Feed Analysis Standards, Chapter 9, Section 2, 22.1.1]

A. Reagent preparation

1) Buffer solution: Buffer No.3
2) Diution solvent. Add about 250 mL of chloroform per 1,000 mL of Buffer No.3, shake, and use the buffer solution layer (upper phase) as the dilution solvent.
3) Bicozamycin standard solution. Weigh accurately not less than 40 mg of bicozamycin working standard[1], accurately add Buffer No.3 and dissolve to prepare a bicozamycin standard stock solution with a concentration of 1 mg (potency)/mL[2].

   At the time of use, accurately dilute a quantity of the standard stock solution with the dilution solvent to prepare hith- and low-concentration standard solutions with concentrations of 0.1 and 0.025 mg (potency)/mL, respectively[3].
4) Culture medium: Medium F-111[4]
5) Bacterial suspension and amount of addition. Use *Escherichia coli* ATCC 27166[5] as the test organism. Add about 0.5 mL of a 10-fold diluted suspension of the test organism per 100 mL of the culture medium.
6) Agar plate. Proceed by the cylinder plate method.
7) Extracting solvent: A mixture of chloroform and methanol (1:1)

B. Preparation of sample solution

Weigh accurately a quantity of the analysis sample (equivalent to 0.01 to 0.1 g (potency) as BZM), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer the extract to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Accurately transfer a quantity of the filtrate (equivalent to 1 mg (potency) as BZM) to a 50-mL recovery flask, evaporate under reduced pressure into dryness in a water bath at 50°C, and add 5 mL of chloroform to dissolve the residue[6]. To this solution add accurately 10 mL of Buffer No.3, shake[7], place in a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 10 minutes, and use the water phase (upper phase) as a high-concentration sample solution with a concentration of 0.1 mg (potency)/mL. Further, accurately dilute this solution with the dilution solvent to prepare a low-concentration sample solution with a concentration of 25 μg (potency)/mL.

C. Quantification[8]

Proceed by the 2-2 dose method[9].

«Summary of analysis method»

This method is intended to determine the amount of BZM in a premix by microbiological assay using a sample solution prepared by extracting with a mixture of chloroform and methanol (1:1) and purifying by liquid-liquid partition with a mixture of Buffer No.3 and chloroform.

The flow sheet of this method is shown in Figure 9.2.22-1.

Sample (equivalent to 0.01-0.1 g (potency))

↓

Extract with 100 mL of chloroform-methanol (1:1)
(with a magnetic stirrer for 20 min).

↓

Centrifuge (at 1,500×*g* for 5 min).

↓

Filter (through filtre paper No.5A).

↓

Collect a quantity of the filtrate (into a 50-mL recovery flask).

↓

Evaporate into dryness under reduced pressure (in a water bath at 50°C).

↓

Dissolve the residue with 5 mL of chloroform (with ultrasonic waves for 2-3 min), and add 10 mL of Buffer No.3.

↓

Transfer to a stoppered centrifuge tube and centrifuge (at 1,500×*g* for 10 min).

↓

Collect the upper phase and use as the high-concentration sample solution (100 µg (potenc)/mL).

↓

Dilute a quantity of the high-concentration sample solution with chloroform-saturated Buffer No.3 to prepare a low-concentration sample solution (25 µg (potency)/mL).

↓

Dispense to agar plates (allow to stand at 10-20°C for 2 hr).

↓

Incubate (at 35-37°C for 16-24 hr).

↓

Measure the inhibition zone diameter.

↓

Calculate the potency by the 2-2 dose method.

Figure 9.2.22-1 Quantitative test method for bicozamycin (premix)

References: Kiyoshi Kanno, Masanobu Kajiyama: Research Report of Animal Feed, 12, 85 (1987)

History in the Feed Analysis Standards [9] New

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Premix 1	0.5~20	3	92.6~98.7	3.9
Premix 2	0.5~20	3	98.8~101.9	4.9
Premix 3	0.5~20	3	96.0~99.9	5.1

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Pig premix	8	2	95.9	2.1	2.8

«Notes and precautions»

[1] The definition etc. of bicozamycin working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.

[2] For the method of preparation for the standard stock solution, refer to «Notes and precautions» [10] in Section 1, 1 of this Chapter.

Method of preparation: Example (When the weighed amount is 50 mg)

When the labeled potency of the working standard is 987 µg (potency)/mg, 50 mg of the working standard contains 49,350 µg (potency) (i.e., 50 mg × 987 µg (potency)/mg). To prepare a standard stock solution with a concentration of 1,000 µg (potency)/mL, the required amount of solvent is thus calculated to be 49.35 mL (i.e., 49,350 µg (potency) / 1,000 µg (potency)/mL). Therefore, completely transfer 50 mL of the working standard to an Erlenmeyer flask containing 49.35 mL of Buffer No.3 and dissolve to prepare the standard stock solution with a concentration of 1,000 µg (potency)/mL.

[3] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for bicozamycin standard solution is shown in Table 9.2.22-1.

Table 9.2.22-1 Method of preparation for bicozamycin standard solution (premix, example)

Test tube No.	1	2
Amount (mL) of standard solution	②	5
Amount (mL) of chloroform-saturated Buffer No.	18	15
Concentration (µg(potency)/mL)	100	25

Note"②mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

[4] Use Antibiotic Medium 11 (Difco) or an equivalent.

[5] For the number of bacteria, refer to «Notes and precautions» [33] in Section 1, 1 of this Chapter.

[6] It is recommended to apply ultrasonic waves for 2 to 3 minutes.

[7] Shake for 2 to 3 minutes.

[8] An example standard response line for BZM is shown in Figure 9.2.22-2.

[9] Refer to «Notes and precautions» [53] to [60] in Section 1, 1 of this Chapter.

When using an automatic apparatus for measuring the inhibition zone diameter, it is recommended to achieve a clear-cut zone of inhibition by addyin 2 to 3 mL of hydrochloric aicd (1:5) to the agar plates after incubation and allowing to stand for 1 to 2 minutes.

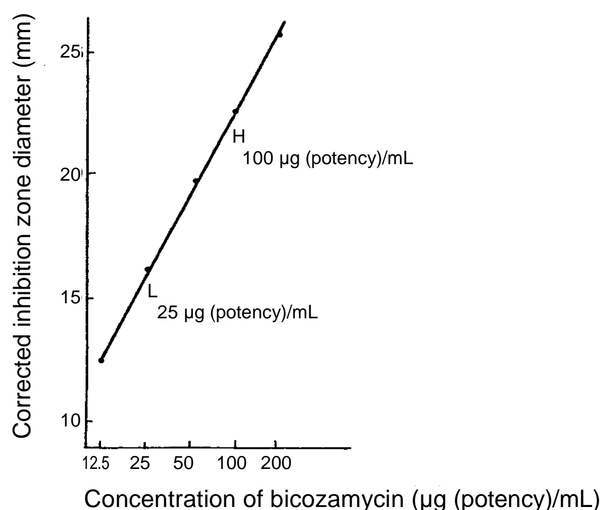


Figure 9.2.22-2 Standard response line for bicozamycin (premix, example)

(*Escherichia coli* ATCC 27166, Medium F-111, Cylinder plate method)

1.2 Feed

[Feed Analysis Standards, Chapter 9, Section 2, 22.2.1]

A. Reagent preparation

1) Buffer solution: Buffer No.3

2) Diution solvent. Add about 250 mL of chloroform per 1,000 mL of Buffer No.3, shake, and use the buffer solution phase (upper phase) as the dilution solvent.

3) Bicozamycin standard solution. Weigh accurately not less than 40 mg of bicozamycin working standard, accurately add Buffer No.3 and dissolve to prepare a bicozamycin standard stock solution with a concentration of 1 mg (potency)/mL.

At the time of use, accurately dilute a quantity of the standard stock solution with the dilution solvent to prepare standard solutions with concentrations of 12, 6, 3, 1.5 and 0.75 µg (potency)/mL[1].

4) Culture medium: Medium F-23

5) Bacterial suspension and amount of addition. Use *Escherichia coli* BS-10 as the test organism. Add about 1 mL of the suspension of the test organism per 100 mL of the culture medium.

6) Agar plate. Proceed by the cylinder plate method.

7) Extracting solvent

i) When the analysis sample is not such a heat-processed feed as pellets, use a mixture of chloroform and methanol (1:1)

ii) When the analysis sample is such a heat-processed feed as pellets, use a mixture of chloroform, methanol and water (9:9:2)

B. Preparation of sample solution

1) When the BZM content is not less than 10 g (potency)/t

Weigh accurately a quantity of analysis sample (equivalent to 0.2 mg (potency) as BZM), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 15 mL filtrate (equivalent to 30 µg (potency) as BZM) to a 50-mL recovery flask, evaporate into dryness under reduced pressure in a water bath at 50°C, and add 15 mL of chloroform to dissolve the residue [2]. To this solution add accurately 10 mL of Buffer No.3, shake, transfer to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 10 minutes, and use the water phase (upper phase) as a sample solution with a concentration of 3 µg (potency)/mL[3].

2) When the BZM content is less than 10 g (potency)/t

Weigh accurately a quantity of the analysis sample (equivalent to 0.1 mg (potency) as BZM), place in a 200-mL stoppered Erlenmeyer flask, add 100 mL of the extracting solvent, and extract with stirring for 20 minutes. Transfer 50 mL of the extract to a stoppered centrifuge tube, centrifuge at 1,500×*g* for 5 minutes, and filter the supernatant liquid through filter paper (No.5A).

Transfer accurately 30 mL of the filtrate (equivalent to 30 µg (potency) as BZM) to a 50-mL recovery flask, evaporate into dryness under reduced pressure in a water bath at 50°C, add 15 mL of chloroform to dissolve the residue[2]. To this solution add accurately 10 mL of Buffer No.3, shake, transfer to a 50-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 10 minutes, and use the water phase (upper phase) as a sample solution with a concentration of 3 µg (potency)/mL [3].

C. Quantification[4]

Proceed by the standard response line method[5].

«Summary of analysis method»

This method is intended to determine the amount of BZM in a feed by microbiological assay using a sample solution prepared by extracting with a chloroform-methanol solvent and purifying by liquid-liquid partition with a mixture of Buffer No.3 and chloroform. The type of extracting solvent, the amount of analysis sample, and the amount of sample solution subject to purification differ depending on the manufacturing conditions of the feed of interest (heat-processed feeds, such as pellets, and others) and the concentration of BZM. Refer to the following table.

Table 9.2.22-2 Quantification procedures for different conditions of manufacture and BZM concentrations

Feed manufacturing conditions		Concentration of BZM in feed (g(potency)/t)	
	Item	Not less than 10	Less than 10
Not heat-processed	Extracting solvent	Chloroform-methanol (1:1)	Chloroform-methanol (1:1)
	Sampled amount	Equivalent to 0.2 mg (potency) as BZM	Equivalent to 0.1 mg (potency) as BZM
	Amount of filtrate for purification	15 mL	30 mL
Heat-processed	Extracting solvent	Chloroform-methanol-water (9:9:2)	Chloroform-methanol-water (9:9:2)
	Sampled amount	Equivalent to 0.2 mg (potency) as BZM	Equivalent to 0.1 mg (potency) as BZM
	Amount of filtrate for purification	15 mL	30 mL

The flow sheet of this method is shown in Figure 9.2.22-3.

Sample (equivalent to 0.2 or 0.1 mg (potency) as BZM)

Extract with 100 mL of chloroform-methanol (1:1) (non-heat-processed feed) or chloroform-methanol-water (9:9:2) (heat-processed feed)
(with a magnetic stirrer for 20 min).

Centrifuge (at 1,500×*g* for 5 min).

Filter (through filter paper No.5A).

Collect a quantity of the filtrate (15 mL for a feed with BZM content not less than 10 g (potency/t) or 30 mL for a feed with BZM content 5-10 g (potency)/t) (into a 50-mL recovery flask).

Evaporate into dryness under reduced pressure (in a water bath at 50°C).

Dissolve the residue with 15 mL of chloroform (with ultrasonic waves for 2-3 min), and add 10 mL of Buffer No.3.

Transfer to a stoppered centrifuge tube and centrifuge (at 1,500×*g* for 10 min).

Use the upper phase as a sample solution with a concentration of 3 µg (potency)/mL.

Dispense to agar plates (allow to stand at 10-20°C for 2 hr).

Incubate (at 35-37°C for 16-24 hr).

Measure the inhibition zone diameter.

Calculate the potency by the standard response line method.

Figure 9.2.22-3 Quantitative test method for bicozamycin (feed)

References: Kiyoshi Kanno: Research Report of Animal Feed, 9, 53 (1984)
Seiji Araki: Research Report of Animal Feed, 19, 149 (1994)

History in the Feed Analysis Standards [5] New, [16] Revision

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Starting chick formula feed (non-heat-processed)	5~10	6	98.9~99.9	4.4
Starting period broiler formula feed	5~10	6	98.6~102.9	4.9
Suckling piglet formula feed (non-heat-processed)	5~10	6	99.8~106.0	4.3
Suckling calf formula feed (non-heat-processed)	5~10	6	99.4~106.5	4.0

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Starting chick formula feed (non-heat-processed)	5	10	103.6	3.6	5.0
Starting chick formula feed (heat-processed)	7	10	105.3	2.9	5.7

«Notes and precautions»

[1] For the method of preparation for the standard solution, refer to «Notes and precautions» [8] in Section 1, 1 of this Chapter.

An example method of preparation for bicozamycin standard solution is shown in Table 9.2.22-3.

Table 9.2.22-3 Method of preparation for bicozamycin standard solution (feed, example)

Test tube No.	1	2	3	4	5	6
Amount (mL) of standard solution	②	3	10	10	10	5
Amount (mL) of chloroform-saturated Buffer No.3	18	22	10	10	10	5
Concentration (μg(potency)/mL)	100	12	6	<3>	1.5	0.75

Note:"② mL" means "2 mL of standard stock solution (1 mg(potency)/mL)".

[2] While dissolving the residue, scrape off the fatty material on the wall of the recovery flask with a medicine spoon etc.

Further apply ultrasonic waves for 2 to 3 minutes to dissolve the residue completely.

[3] When the obtained supernatant lisuid is not clear, further filter through filter paper (No.5A or No.6).

[4] An example standard response line for BZM is shown in Figure 9.2.22-4.

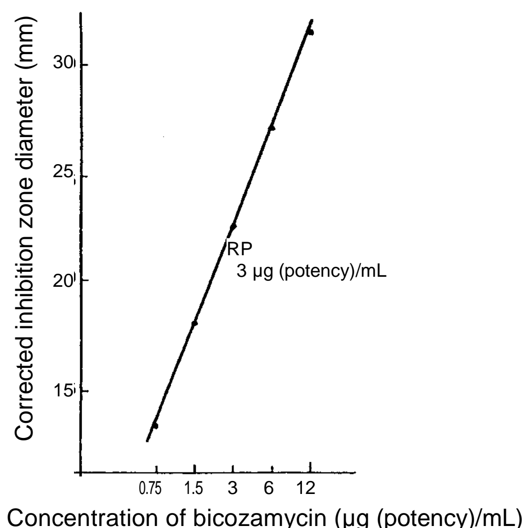


Figure 9.2.22-4 Standard response line for bicozamycin (feed, example)
(*Escherichia coli* BS-10, Medium F-23, Cylinder plate method)

[5] Refer to «Notes and precautions» [53] to [57] and [61] in Section 1, 1 of this Chapter.

When using an automatic apparatus for measuring the inhibition zone diameter, it is recommended to achieve clear-cut zone of inhibition by adding 2 to 3 mL of hydrochloric aicd (1:5) to the agar plates after incubation and allowing to stand for 1 to 2 minutes.