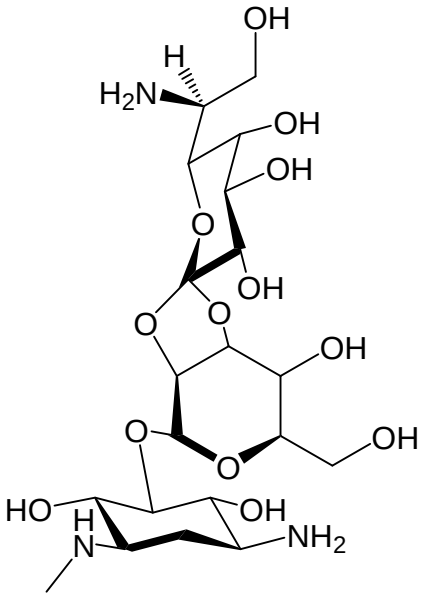
17 Destomycin A



$C_{20}H_{37}N_3O_{13}$ MW: 527.5 CAS No.: 14918-35-5

[Summary of destomycin A]

Destomycin A (DM-A) is a aminoglycosid antibiotic obtained by the incubation of *Streptomyces rimofaciens*.

For physicochemical properties, DM-A occurs as a basic white powder. It is freely soluble in water, in methanol and in ethanol, and practically insoluble in ether, in chloroform and in benzene. DM-A technical for a feed additive is a dark brown liquid.

DM-A has an antibacterial effect on Gram-positive bacteria, Gram-negative bacteria and fungi. It prevents reduction in productivity due to Ascaris suum (the mechanism of the anthelmintic activity involves inhibition of vitelline coat formation of parasite eggs), and promotes the growth of pigs. Analogous (diastereomeric) antibiotics include hygromycin B, which was once designated as a feed additive but cancelled as of October 12, 2004.

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

DM-A is a pure-grade antibiotic that was designated as a feed additive as of July 24, 1976. The specifications for feeds containing this ingredient are specified in Appended Table No.1, 1-(1)-C of the Act on Safety Assurance and Quality Improvement of Feeds.

(in g(potency)/t)

Feed of interest	For pigs	
	Suckling piglets	Piglets
Added amount	5~10	5~10

[Methods listed in the Feed Analysis Standards]

1 Quantitative test method - Plate method

[Feed Analysis Standards, Chapter 9, Section 2, 17.1.1]

Scope of application: Feed not containing OTC

A. Reagent preparation

1) Buffer solution: Buffer No.4

2) Diution solvent. Disolve 1 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate[1] in Buffer No.4 to make 1,000 mL.

3) Destomycin A[2] standard solution. Dry a suitable amount of destomycin A working standard under reduced pressure (not exceeding 0.67 kPa) at 60°C for 3 hours, weigh accurately not less than 40 mg, accurately add Buffer No.4 and dissolve to prepare a destomycin A standard stock solution with a concentration of 1 mg (potency)/mL [3].

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 80, 40, 20, 10 and 5 μg (potency)/mL [4].

4) Culture medium: Medium F-3

5) Spore suspension and amount of addition. Use *Bacillus subtilis* ATCC 6633 as the test organism. Add about 0.2 mL of the spore suspension with a concentration of 1×10^7 spores/mL per 100 mL of the culture medium.

6) Agar plate[5]. Proceed by the agar well method.

7) Ion-exchange resin. Weigh 100 g of an ion-exchange resin (weakly acidic cation-exchange resin)[Note 1], place in a 1-L beaker, wash with 500 mL of ammonia solution (1 mol/L), discard the washings, further add 500 mL of ammonia solution (1 mol/L), and allow to stand over night. Then wash the resin thoroughly with water[6] to prepate an NH_4^+ resin.

Separately, weigh 100 g of an ion-exchange resin, place in a 1-L beaker, wash with 500 mL of hydrochloric acid (1 mol/L), discard the washings, further add 500 mL of hydrochloric acid (1 mol/L), and allow to stand over night. Then wash the resin thoroughly with water[7] to prepare H^+ resin.

Thoroughly stir 70 mL of the NH_4^+ resin and 30 mL of the H^+ resin, and store in water[8].

B. Preparation of sample solution

Extraction. Weigh accurately a quantity of the analysis sample (equivalent to 0.2 mg (potency) as DM-A)[9], place in a 300-mL stoppered Erlenmeyer flask, add 200 mL of potassium hydrogen sulfate solution (1 w/v%), and extract with stirring for 20 minutes. Transfer the extract to a 200-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 10 minutes, transfer the whole amount of of the supernatant liquid to a

200-mL beaker, and adjust the pH to 7.9 to 8.1 with ammonia solution[10]. Transfer this liquid to a 200-mL stoppered centrifuge tube, centrifuge at 1,500×*g* for 10 minutes, filter the supernatant liquid through filter paper (No.5A), and use as a sample solution subject to column treatment[11].

Column treatment. Suspend the ion-exchange resin in water, pour into a column tube (10 mm in internal diameter)[12] to a height of 6 cm, loosely stuff the upper part of the column with glass wool[13], add 20 mL of water, allow to flow out until the liquid surface is 3 mm below the top of the column packing material to prepare the column.

Load 100 mL of the sample solution onto the column and allow to flow out at a rate of 2 mL/min[14] until the liquid surface is 3 mm above the column packing material. Further, load 40 mL of water onto the column and allow to flow out in the same manner[15].

Place a 200-mL recovery flask under the column, add 40 mL of a mixture of ammonia and water (1:49)[16] to the column to elute DM-A[17], evaporate the eluate into dryness under reduced pressure in a water bath at 70°C[18], and add accurately 5 mL of the dilution solvent to dissolve the residue[19] to prepare a sample solution with a concentration of 20 µg (potency)/mL.

C. Quantification[20]

Proceed by the standard response line method[21].

Note 1. Use Amberlite CG-50 type-1 (Rohm and Haas) or an equivalent

«Summary of analysis method»

This method is inteded to determine the amount of DM-A in a feed by microbiological assay using a sample solution prepared by extracting with 1% potassium hydrogen sulfate solution and purifying through a cation-exchange resin (Amberlite CG-50) column. None of the antibacterial substances approved for combined use with DM-A interfere with the quantification of DM-A.

As the blank solution can produce inhibition zones in agar plates prepared by the cylinder plate method, the plates shall be prepared by the agar well method.

The flow sheet of this method is shown in Figure 9.2.17-1.

Sample (in an amount equevalent to 0.2 mg (potency) as DM-A)
↓
Extract with 200 mL of 1% potassium hydrogensulfate solution
(with a magnetic stirrer for 20 min).
↓
Centrifuge (at 1,500×*g* for 10 min).
↓
Collect the whole amount of the supernatant liquid（into a 200-mL beaker).
↓
Adjust the pH to 7.9-8.1 (with ammonia solution).
↓
Centrifuge (at 1,500×*g* for 10 min).
↓
Filter the supernatant liquid (through filter perper No.5A).
↓
Load 100 mL of the filtrate onto an ion-exchange column (Amberlite CG-50) to adsorb DM-A (at a flow rate of 2 mL/min).
↓
Wash with 40 mL of water (at a flow rate of 4 mL/min).
↓
Elute DM-A with 40 mL of ammonia-water (1:49)
(into a 200-mL recovery flask at a flow rate of 2 mL/min).
↓
Evaporate into dryness under reduced pressure (in a water bath at 70℃).
↓
Dissolve the residue with 5 mL of dilution solvent (with ultrasonic waves for 2-3 min).
↓
Dispense to agar plates (allow to stand at 10-20℃ for 2 hr).
↓
Incubate (at 35-37℃ for 16-24 hr).
↓
Measure the inhibition zone diameter.
↓
Calculate the potency by the standard response line method.

Figure 9.2.17-1 quantitative test method for destomycin A (feed)

References: Toyoko Kusama, Shoichi Yamatani: Test of Feed, 275, 1 (1986)

Takashi Sasaki: Research Report of Animal Feed, 27, 249 (2002)

History in the Feed Analysis Standards [8] New, [21] Revision

«Validation of analysis method»

・Spike recovery and repeatability

Sample type	Spike concentration (g(potency)/t)	Repeat	Spike recovery (%)	Repeatability RSD(% or less)
Suckling piglet formyla feed (2 types) and piglet formula feed	5~10	3	99.3	6.6

・Collaborative study

Sample type	No. of labs	Spike concentration (g(potency)/t)	Spike recovery (%)	Intra-lab repeatability RSDr(%)	Inter-lab reproducibility RSDR(%)
Piglet formula feed	6	8	97.0	5.6	6.6

«Notes and precautions»

[1] Although it is a chelating agent, disodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA) has the effect of enhancing the sensitivity of DM-A to the test organism, and therefore used by being added to the buffer solution in this method. Care should be taken that the test organism becomes susceptible to EDTA when its concentration in the buffer solution is not less than 0.4%.

[2] For the definition etc. of destomycin A working standard, refer to «Notes and precautions» [9] in Section 1, 1 of this Chapter.

[3] 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 996 μg (potency)/mg, 50 mg of the working standard contains 49,800 μg (potency) (i.e., 50 mg × 996 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 49.8 mL (i.e., 49,800 μg (potency) / 1,000 μg (potency)/mL. Therefore, completely transfer 50 mg of the working standard to an Erlenmeyer flask containing 49.8 mL of Buffer No.4 and dissolve to prepare the standard stock solution with a concentration of 1,000 μg (potency)/mL.

[4] 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 destomycin Astandard solution is shown in Table 9.2.17-1.

Table 9.2.17-1 Method of preparation for destomycin A standard solution (feed, example)

Test tube No.	1	2	3	4	5
Amount (mL) of standard solution	②	10	10	10	5
Amount (mL) of dilution solvent	23	10	10	10	5
Concentration (μg(potency)/mL)	80	40	<20>	10	5

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

[5] When the inhibition zone produced by the lowest-concentration standard solution (5 μg (potency)/mL) is too small to measure, proceed as directed below to improve the size of the inhibition zone.

A. Prolong the period of allowing to stand before incubation from 2 hours to about 3 hours.

B. Decrease the amount of the culture medium dispensed to each Petri dish from 20 mL to about 18 mL.

C. Decrease the amount of the spore suspension added to the culture medium from 0.2 mL to 0.1 mL.

[6] Suspend the resin in a beaker of water, repeat decantation, and perform this procedure once or twice per day to wash the resin until the pH of the supernatant liquid is 9.0 to 9.5 (it requires 2 weeks to 1 month). For a long-term storage, it is recommended to wash the resin with sterile water.

[7] Perform the same procedure as described in [6], and wash the resin until the pH of the supernatant liquid is 6.0 to 7.0 (it requires 2 weeks to 1 month). For a long-term storage, it is recommended to

wash the resin with sterile water.

[8] They are stable for 1 month when stored at room temperature and for 3 months when stored under refrigeration.

[9] Usually, about 20 to 40 g.

[10] Requires approximately 1.5 mL.

[11] The sample solution prepared by the following procedure is equivalent to the one prepared as described in this method, and applicable to column chromatography.

After centrifuging the extract, transfer 100 mL of the supernatant liquid to a 200-mL beaker, adjust the pH to 7.9 to 8.1, centrifuge, and collect the whole amount of the supernatant liquid. Separately, to the remaining sediment add 5 mL of water, centrifuge at 1,500×*g* for 10 minutes, and collect the supernatant liquid. Bring these supernanat liquids together and use the whole amount as the sample stock solution to load onto the column.

[12] Use a column tube with a fluid reservoir.

[13] Absorbent cotton is also applicable.

[14] Receive the filtrate into a volumetric cylinder etc. to control the flow rate.

[15] Flow out at a rate of approximately 4 mL per 1 minute. It is recommended to do this with the cock of the column almost full open.

[16] In the resin of the column tube can be seen a yellow pigment, derived from the feed of interest, other than DM-A (which is unobservable with the naked eye). Under the conditions of this method, it has been comfirmed that this yellow pigment elutes out immediately after DM-A. It is therefore recommended to use this yellow pigment as an index of whther DM-A has eluted out or not: when no yellow pigment has not eluted out with the specified amount of the eluting solvent (40 mL), increase that amount until the yellow pigment elutes out to make sure that the DM-A has eluted out.

[17] Elute at a rate of 2 mL per 1 minute.

[18] Care should be taken that the sample solution is likely to bump. To prevent this bumping, it is recommended to 1) lower the water bath temperature from 70°C to 50°C or 2) add an equial amount of methanol when the amount of the sample solution in the recovery flask has been reduced by half, etc.

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

[20] An example standard response line for DM-A is shown in Figure 9.2.17-2.

Linearity is observed in the quantification range for DM-A.

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

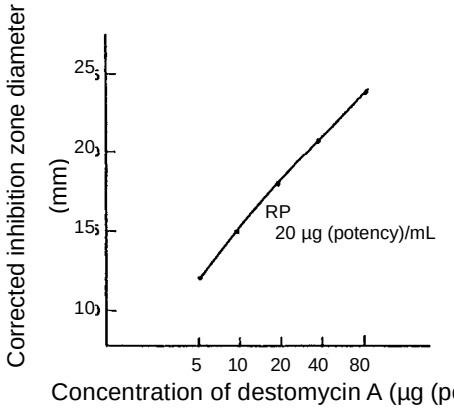


Figure 9.2.17-2 Standard response line for DM-A (feed, example)

(*Bacillus subtilis* ATCC 6633, Medium F-3, Agar well method)