Outline of Details for Microbiological Assays of Antibiotics: Second Revision

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Abstract □ Pertinent data are presented for the performance of 83 different microbiological assays for antibiotics. These include the official methods for all antibiotics approved for human use in the United States plus methods for 10 other antibiotics. For analysts already familiar with basic procedures, these data will serve as a ready guide for the performance of assays.

Keyphrases □ Microbiological assays—guide to antibiotics □ Antibiotics—outline of 83 microbiological assays

An outline of details of microbiological assays for antibiotics was published by Kirshbaum and Arret (1) in 1959. A second outline, revising and updating the original version, was published by the same authors (2) in 1967. Since new antibiotics continue to be discovered, requiring new assay methods, and since existing methods have been revised and modified, a new outline reflecting the latest available microbiological assays is now needed.

The 1967 outline gave details for 57 different official microbiological assays; the present outline gives details for 83 different assays, including 10 that are nonofficial. As in the previous two publications, pertinent information is tabulated in a condensed form for ready reference by the analyst who performs microbiological assays of antibiotics. Detailed directions for performing the assays are not given, since the tabulations are intended for analysts already familiar with the basic assay techniques.

Since the last outline appeared (2), the Federal Register published condensations of microbiological assays in which the outline approach to details of assays is followed to some extent. These were codified in the Code of Federal Regulations (3). For sample preparation methods for individual drugs, one must refer to the specific monograph (4).

In 1955, Grove and Randall (5) published their laboratory manual which received worldwide acceptance as an authoritative guide for antibiotic assay and analysis. They used a sequential numbering system to identify assay culture media. Since commercial manufacturers of culture media now use these numbers to identify their media, we, as well as the Code of Federal Regulations, have uniformly continued this numbering system to avoid confusion in specifying the proper culture media.

The test organisms used for the various assays and their methods of preparation are listed in Table I. The media and buffers are listed in Tables II and III, respectively. Pertinent information for cylinder plate assays and for turbidimetric assays is given in Tables IV and V, respectively.

Table I—Test Organisms for Various Assays

Test Organism		Method of Preparation
A	*Staphylococcus aureus* (ATCC 6538P)	1
B	*Sarcina subflava* (ATCC 7468)	1
C	*Sarcina lutea* (ATCC 9341)	1
D	*Staphylococcus epidermidis* (ATCC 12228)	1
E	*Saccharomyces cerevisiae* (ATCC 9763)	6[a]
F	*Bordetella bronchiseptica* (ATCC 4617)	1
G	*Bacillus cereus* var. *mycoides* (ATCC 11778)	3
H	*Bacillus subtilis* (ATCC 6633)	2
I	*Klebsiella pneumoniae* (ATCC 10031)	1
J	*Escherichia coli* (ATCC 10536)	1
K	*Streptococcus faecalis* (ATCC 10541)	5
L	*Micrococcus flavus* (ATCC 10240)	1
M	*Microsporum gypseum* (ATCC 14683)	4
N	*S. lutea*, resistant to dihydrostreptomycin (ATCC 9341a)	1
O	*S. aureus*, resistant to novobiocin (ATCC 12692)	1
P	*S. aureus*, re istant to dihydrostreptomycin (ATCC 6538-DR)	1
R	*S. subflava*, resistant to dihydrostreptomycin (ATCC 7468/d)	1
T	*S. cerevisiae* (ATCC 2601)	6
U	*M. flavus*, resistant to neomycin (ATCC 14452)	1
V	*M. flavus*, resistant to dihydrostreptomycin (ATCC 10240A)	1
W	*Pseudomonas pyocyanea* (ATCC 25619)	1

[a] For candicidin assay, use Method 1.

PREPARATION OF MICROBIAL SUSPENSIONS

Several different methods are used for the preparation of microbial suspensions.

Method 1—Maintain the test organism on slants of Medium 1 and transfer to a fresh slant once a week. Using 3 ml. of saline T.S. USP, wash the organism from the agar slant (which has been incubated for 24 hr. at 32–37°) onto a large agar surface of Medium 1[1], such as that provided by a Roux bottle containing 250 ml. of agar. Incubate for 24 hr. at 32–37°. Wash the resulting growth from the nutrient surface, using 50 ml. of sterile saline T.S. USP Store the test organism under refrigeration.

[1] For the preparation and maintenance of the antibiotic-resistant organisms listed in Table I, prepare Medium 1 to contain the appropriate antibiotic as described in Sections 25, 27, 28, and 33 of Table II.

Table II—Media

Medium	Ingredients	Grams
1	Peptone	6.0
	Pancreatic digest of casein	4.0
	Yeast extract	3.0
	Beef extract	1.5
	Dextrose	1.0
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 6.55 ± 0.05 after sterilization	
2	Same as Medium 1, except omit pancreatic digest of casein and dextrose	
3	Peptone	5.0
	Yeast extract	1.5
	Beef extract	1.5
	Sodium chloride	3.5
	Dextrose	1.0
	Monobasic potassium phosphate	1.32
	Dibasic potassium phosphate	3.68
	Distilled water to make 1000 ml.	
	pH 7.0 ± 0.05 after sterilization	
5	Same as Medium 2, except adjust pH so that it is 7.9 ± 0.1 after sterilization	
8	Same as Medium 2, except adjust pH so that it is 5.90 ± 0.05 after sterilization	
9	Pancreatic digest of casein	17.0
	Papaic digest of soybean	3.0
	Sodium chloride	5.0
	Dibasic potassium phosphate	2.5
	Dextrose	2.5
	Agar	20.0
	Distilled water to make 1000 ml.	
	pH 7.25 ± 0.05 after sterilization	
10	Same as Medium 9, except use 12 g. instead of 20 g. of agar. Boil to dissolve ingredients; then add 10 ml. of polysorbate 80	
11	Same as Medium 1, except adjust pH so that it is 7.9 ± 0.1 after sterilization	
13	Peptone	10.0
	Dextrose	20.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.05 after sterilization	
18	Same as Medium 11, except boil to dissolve ingredients; then add 20 ml. of polysorbate 80	
19	Peptone	9.4
	Yeast extract	4.7
	Beef extract	2.4
	Sodium chloride	10.0
	Dextrose	10.0
	Agar	23.5
	Distilled water to make 1000 ml.	
	pH 6.1 ± 0.1 after sterilization	
20	Dextrose	40.0
	Peptone	10.0
	Chloramphenicol (activity)	0.05
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.05 after sterilization	
21	Dissolve 10 mg. cycloheximide/ml. of distilled water and sterilize by filtration through a membrane filter having a porosity of 0.22 μ. Aseptically, add 20 ml. sterile solution/l. Medium 20 which has been autoclaved and cooled to 50°	
22	Dextrose	40.0
	Peptone	10.0
	Distilled water to make 1000 ml.	
	pH 5.65 ± 0.5 after sterilization	
25	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile dihydrostreptomycin sulfate solution to give a final concentration of 1000 mcg. dihydrostreptomycin activity/ml. of solution. Sterile dihydrostreptomycin sulfate solution is prepared by filtering a solution containing 10 mg. dihydrostreptomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
27	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile neomycin sulfate solution to give a final concentration of 100 mcg. neomycin activity/ml. medium. Sterile neomycin sulfate solution is prepared by filtering a solution containing 10 mg. neomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
28	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile dihydrostreptomycin sulfate solution to give a final concentration of 500 mcg. dihydrostreptomycin activity/ml. medium. Sterile dihydrostreptomycin sulfate solution is prepared by filtering a solution containing 10 mg. dihydrostreptomycin/ml. distilled water through a membrane filter of 0.22-μ porosity	
29	Beef extract	6.0
	Peptone	10.0
	Agar	15.0
	Distilled water to make 1000 ml.	
	pH 7.9 ± 0.1 after sterilization	
32	Same as Medium 1, except add 300 mg. hydrated manganese sulfate ($MnSO_4H_2O$)/l. of medium	
33	Use Medium 1, sterilized and cooled to 50°. Aseptically, add sufficient sterile sodium novobiocin solution to give a final concentration of 10 mcg. novobiocin activity/ml. medium. Sterile sodium novobiocin solution is prepared by filtering a solution containing 2.5 mg. novobiocin/ml. distilled water through a membrane filter of 0.22-μ porosity	

Method 2—Proceed as described in Method 1, but standardize the suspension as follows. Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50–70 ml. of sterile saline USP T.S., and heat the suspension for 30 min. at 70°. Store the spore suspension under refrigeration.

Table III—Buffers

	Buffer	Ingredients	Amount
I	1% phosphate buffer, pH 6.0 ± 0.05	Dibasic potassium phosphate	2 g.
		Monobasic potassium phosphate	8 g.
		Distilled water to make	1000 ml.
III	0.1 *M* phosphate buffer, pH 7.9 ± 0.1	Dibasic potassium phosphate	16.73 g.
		Monobasic potassium phosphate	0.523 g.
		Distilled water to make	1000 ml.
IV	0.1 *M* monopotassium phosphate buffer, pH 4.5 ± 0.05	Monobasic potassium phosphate	13.6 g.
		Distilled water to make	1000 ml.
VI	10% phosphate buffer, pH 6.0 ± 0.05	Same as I, except use 10 times as much of each salt	
X	0.2 *M* phosphate buffer, pH 10.5 ± 0.1	Dibasic potassium phosphate	35 g.
		10 *N* potassium hydroxide	2 ml.
		Distilled water to make	1000 ml.
XIII	0.01 *N* methanolic hydrochloric acid	1.0 *N* hydrochloric acid	10 ml.
		Methyl alcohol to make	1000 ml.
XV	80% isopropyl alcohol solution	Isopropyl alcohol	800 ml.
		Distilled water to make	1000 ml.
XVI	1% sodium carbonate	Sodium carbonate	10 g.
		Distilled water to make	1000 ml.

Table IV—Plate Diffusion Assays

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Optimal Zone size (mm.) Produced by Reference Concentration[a]	Medium—Base	Medium—Seed	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature
Amphomycin	U	0.5	12–14	2	1	100 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Amphotericin B	E	0.2	16–18	None	19[b]	1000 mcg./ml. in dimethyl sulfoxide	X	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Ampicillin	C	1.0	18–20	11	11	100 mcg./ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35°
Bacitracin	B or R[c]	0.2	16–18	2	1	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Bacitracin	L or V[d]	0.2	17–19	2	1	100 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Carbenicillin	W	0.05	16–18	9	10	1000 mcg./ml. in I	I	64, 80, *100*, 125, 156	37°
Cephalexin	A	0.05	18–20	2	1	1000 mcg./ml. in I	I	12.8, 16.0, *20.0*, 25.0, 31.2	37°
Cephaloglycin	A	0.2	18–20	2	1	100 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Cephaloridine	A	0.3	19–21	2	1	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Cephalothin	A	0.05	14–16	2	1	1000 mcg./ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	32–35°
Chloramphenicol	C	2.0	17–19	1	1	10,000 mcg./ml. in ethanol	I	32, 40, *50*, 62.5, 78.1	32–35°
Chlortetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.01 *N* HCl	IV	0.064, 0.08, *0.1*, 0.125, 0.156	30°
Clindamycin	C	1.5	18–20	11	11	1000 mcg./ml. in distilled water	III	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Cloxacillin	A	0.2	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Sodium colistimethate	F	0.04	13–15	9	10	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Colistin	F	0.04	15–17	9	10	10,000 mcg./ml. in distilled water	VI	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Cycloserine	A	0.04	14–16	2[e]	1	1000 mcg./ml. in distilled water	I	32, 40, *50*, 62.5, 78.1	30°
Dactinomycin	H	0.02	16–18	5[e]	5	10,000 mcg./ml. in methanol	III	0.5, 0.71, *1.0*, 1.41, 2.0	37°
Demeclocycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Dicloxacillin	A	0.2	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Dihydrostreptomycin	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Doxycycline	G	0.4	18–20	8	8	1000 mcg./ml. in 0.1 *N* HCl	III	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Erythromycin	C	1.0	17–19	11	11	10,000 mcg./ml. in methanol	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Gentamicin	D	0.03	15–17	11	11	1000 mcg./ml. in III	III	0.064, 0.08, *0.1*, 0.125, 0.156	37°
Griseofulvin	M	1.0	16–18	20	21	1000 mcg./ml. in dimethylformamide[f]	III	3.2, 4.0, *5.0*, 6.25, 7.81 (48 hr.)	30°
Hetacillin	C	0.5	15–17	11	11	1000 mcg./ml. in III	III	0.064, 0.080, *0.10*, 0.125, 0.156	32°
Kanamycin	A	0.01	16–18	11	11	1000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Kanamycin B	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Lincomycin	C	1.0	16–18	11	11	1000 mcg./ml. in distilled water	III	1.28, 1.6, *2.0*, 2.5, 3.12	37°
Leucomycin[g]	C	1.0	18–20	None	19[b]	10,000 mcg./ml. in ethanol, 1000 mcg./ml. in I	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Methacycline[g]	G	0.4	17–19	8	8	1000 mcg./ml. in XIII	IV	0.064, 0.080, *0.10*, 0.125, 0.156	30°
Methicillin	A	0.2	14–16	2	1	1000 mcg./ml. in I	I	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Mithromycin	A	0.1	15–17	8[e]	8	100 mcg./ml. in distilled water	I	0.5, 0.71, *1.0*, 1.41, 2.0	32°

(*Continued*)

Table IV—(*Continued*)

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Optimal Zone size (mm.) Produced by Reference Concentration[a]	—Medium— Base	Seed	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature
Nafcillin	A	0.2	14–16	2	1	1000 mcg./ml. in I	I	1.28, 1.6, *2.0*, 2.5, 3.12	32–35°
Neomycin	A	0.04	16–18	11	11	1000 mcg./ml. in III	III	6.4, 8.0, *10.0*, 12.5, 15.6	32–35°
Neomycin	D	0.2	16–18	11	11	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Novobiocin	D	2.0	16–18	2	1	10,000 mcg./ml. in ethanol, 1000 mcg./ml. in III	VI	0.32, 0.4, *0.5*, 0.625, 0.781	35°
Nystatin[h]	T	1.0	16–18	None	19[b]	1000 mcg./ml. in dimethylformamide[f]	VI	12.8, 16, *20*, 25, 31.2	30°
Oleandomycin	D	0.2	16–18	11	11	10,000 mcg./ml. in ethanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	37°
Oxacillin	A	0.1	18–20	2	1	1000 mcg./ml. in I	I	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Oxytetracycline	G	0.4	18–20	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Paromomycin	D	0.2	19–21	11	11	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Paromomycin	H	0.05	16–18	29	29	1000 mcg./ml. in III	III	1.28, 1.56, *2.00*, 2.50, 3.12	37°
Penicillin G	A	0.2	17–19	2	1	1000 units/ml. in I	I	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Phenethicillin	C	0.4	17–19	11	11	1000 units/ml. in distilled water	III	0.064, 0.08, *0.1*, 0.125, 0.156	32–35°
Phenoxymethylpenicillin	A	0.2	17–19	2	1	1000 units/ml. in methanol	I	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Polymyxin	F	0.04	15–17	9	10	20,000 units/ml. in distilled water	VI	6.4, 8.0, *10.0*, 12.5, 15.6	37°
Puromycin[g]	C	0.1	16–18	2	1	1000 mcg./ml. in III	III	0.64, 0.80, 1.0, 1.25, 1.56	30°
Rifampin	H	0.1	15–17	2	2	1000 mcg./ml. in methanol	I	3.2, 4.0, *5.0*, *6.25*, *7.81*	30°
Ristocetin[g]	G	0.4	15–17	8	8	1000 mcg./ml. in I	I[i]	6.4, 8.0, *10.0*, 12.5, 15.6	30°
Rolitetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in methanol	IV	0.64, 0.80, *1.0*, 1.25, 1.56	30°
Streptomycin	H	0.05	14–16	5	5	1000 mcg./ml. in III	III	0.64, 0.8, *1.0*, 1.25, 1.56	37°
Tennecitin[g]	E	0.3	17–19	None	19[b]	4000 mcg./ml. in dimethyl sulfoxide	I	0.64, 0.80, *1.0*, 1.25, 1.56	37°
Tetracycline	G	0.4	17–19	8	8	1000 mcg./ml. in 0.1 *N* HCl	IV	0.64, 0.8, *1.0*, 1.25, 1.56	30°
Thiostrepton[g]	A	0.1	14–16	None	19[b]	1000 mcg./ml. in newly adjusted dimethylformamide (pH 6.5–7.0)	III	0.64, 0.80, *1.0*, 1.25, 1.56	32–35°
Troleandomycin	D	0.2	15–17	18	18	1000 mcg./ml. in XV	X	9.6, 12, *15*, 18.8, 23.4	37°
Tylosin[g]	C	0.5	14–16	11	11	10,000 mcg./ml. in methanol	III	3.2, 4.0, *5.0*, 6.25, 7.81	32°
Tylosin[g]	N	1.0	14–16	11	11	10,000 mcg./ml. in III	III	3.2, 4.0, *5.0*, 6.25, 7.81	32–35°
Vancomycin	G	0.2	16–18	8[e]	8	400 mcg./ml. in distilled water	IV	6.4, 8.0, *10.0*, 12.5, 15.6	30°
Viomycin	H	0.05	17–19	5[e]	5	1000 mcg./ml. in distilled water	III	32, 40, *50*, 62.5, 78.1	37°

[a] Zone sizes listed here should be construed as guides only rather than as absolute values. [b] No base layer is used. Add 8 ml. of inoculated seed layer only. [c] Test Organism R is used for products containing bacitracin and dihydrostreptomycin. [d] Test Organism V is used for products containing bacitracin and dihydrostreptomycin. [e] Use 10 ml. instead of 21 ml. [f] Solution is further diluted in solvent to 20 times the final concentration of each concentration of the standard curve so that each final dilution is 1 + 19 with indicated buffer. [g] Not official methods. [h] Use low actinic glassware. [i] Containing 0.1% $NaNO_2$.

Method 3—Use Method 2, but heat shock for 30 min. at 70° prior to centrifugation and wash the spore suspension three times with 25–50 ml. of sterile distilled water each time. Reconstitute with 50–70 ml. of sterile distilled water. Store the spore suspension under refrigeration.

Method 4—Grow the organisms for 6–8 weeks at 25° in several 3-l., wide-mouth conical flasks, each containing 200 ml. of Medium 22. Check the growth for sporulation. When sporulation is 80% or greater, harvest the spores on the mycelial layer with a sterile spatula or other convenient instrument. The spores are on top of the

floating mat. Place harvested spore material in 50 ml. of saline. Store the spore suspension under refrigeration.

Method 5—Maintain the test organisms in 100-ml. portions of nutrient broth—Medium 3. For the test, prepare a fresh subculture by transferring a loopful of the stock culture to 100 ml. of the same nutrient broth and incubate for 16–18 hr. at 37°. Store this broth culture under refrigeration.

Method 6—Use Method 1, but incubate the slants at 30° for 24 hr. and incubate the Roux bottles at 30° for 48 hr.

DETERMINATION OF INOCULUM TO BE USED

For many years, official methods in the Federal Regulations gave instructions for standardizing microbial suspensions by spectrophotometric measurement of light transmission or absorbance. A uniform method for this measurement, described by Kirshbaum *et al.* (6), was incorporated into the Regulations. However, the great number and variety of microbial assays make such an approach to standardization confusing; the point of diminishing returns in giving details probably has been reached. If suspensions are prepared as described in Methods 1–6, growth characteristics are sufficiently uniform so that the inoculum for assays can be adequately determined by test trials without any other adjunctive standardization steps.

For Plate Assays—After the suspension is prepared, add a different volume of it (*e.g.*, 1.0, 2.0, and 3.0 ml., respectively) to each of several different flasks containing 100 ml. of the agar specified for the seed layer in Table IV. Use the volume listed under "Suggested Milliliters of Suspension . . ." in Table IV as a guide. Using the

Table V—Turbidimetric Assays

Antibiotic	Test Organism	Suggested Milliliters of Suspension per 100 ml. of Media	Medium	Initial Solution of Standard	Diluent for Further Dilutions	Final Concentration for Standard Response Line, units or mcg./ml.	Incubation Temperature[a]
Candicidin	E	0.2	13	1000 mcg./ml. in dimethyl sulfoxide	Distilled water	0.03, 0.042, *0.06*, 0.084, 0.12	25°
Capreomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37°
Chloramphenicol	J	0.1	3	10,000 mcg./ml. in ethanol	I	2.0, 2.24, *2.5*, 2.79, 3.12	37°
Chlortetracycline	A	0.1	3	1000 mcg./ml. in 0.01 *N* HCl	IV	0.038, 0.048, *0.060*, 0.075, 0.094	37°
Cycloserine	A	0.1	3	1000 mcg./ml. in distilled water	Distilled water	32.0, 40.0, *50.0*, 62.5, 78.1	37°
Demeclocycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.080, *0.10*, 0.125, 0.156	37°
Dihydrostreptomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	24, 26.8, *30*, 33.5, 37.5	37°
Doxycycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.064, 0.08, *0.10*, 0.125, 0.156	37°
Sodium fusidate[b]	A	0.25	3	1000 mcg./ml. in 1% sodium carbonate XVI	Distilled water	80, 89, *100*, 112, 125	37°
Gramicidin	K	1.0	3	1000 mcg./ml. in 95% ethanol USP	95% ethanol USP	0.028, 0.034, *0.040*,[c] 0.048, 0.057	37°
Methacycline	A	0.1	3	1000 mcg./ml. in XIII	IV	0.032, 0.040, *0.050*, 0.062, 0.078	37°
Minocycline	A	0.2	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.032, 0.040, *0.050*, 0.062, 0.078	37°
Oleandomycin	A	0.1	3	1000 mcg./ml. in III	III	2.00, 2.24, *2.5*, 2.80, 3.12	37°
Oxytetracycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Ristocetin[b]	A	0.3	3	1000 mcg./ml. in I	I	9.0, 11.6, *15.0*, 19.4, 25.0	37°
Rolitetracycline	A	0.1	3	1000 mcg./ml. in methanol	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Soframycin[b]	A	0.2	3	500 mcg./ml. in distilled water	Distilled water	1.92, 2.40, *3.0*, 3.75, 4.65	37°
Spectinomycin	J	0.02	3	1000 mcg./ml. in III	III	12.8, 16.0, *20.0*, 25.0, 31.2	37°
Streptomycin	I	0.1	3	1000 mcg./ml. in distilled water	Distilled water	24, 26.8, *30*, 33.5, 37.5	37°
Tetracycline	A	0.1	3	1000 mcg./ml. in 0.1 *N* HCl	IV	0.160, 0.200, *0.250*, 0.312, 0.390	37°
Troleandomycin	I	0.05	3	1000 mcg./ml. in XV	I	16.0, 20.0, *25*, 31.2, 39.0	37°
Tyrothricin	K	1.0	3	1000 mcg./ml. in 95% ethanol USP	95% ethanol USP	0.14, 0.17, *0.20*, 0.24, 0.285[c]	37°
Viomycin	I	0.05	3	5000 mcg./ml. in distilled water	Distilled water	64, 80, *100*, 125, 156	37°

[a] Overnight incubation is recommended for the candicidin assay. An incubation period of 3 hr. is recommended for assays using Test Organisms I and K. An incubation period of 4 hr. is recommended for assays using Test Organisms A. [b] Not official methods. [c] One-tenth milliliter of each solution is added to each tube.

several inocula prepared in this manner, prepare inoculated plates as described for the specific antibiotic assay. Fill each cylinder with the reference concentration of the antibiotic and then incubate the plates. After incubation, examine and measure the zones of inhibition produced on the plates. The volume of suspension that produces the optimum zones of inhibition with respect to both clarity and diameter determines the inoculum to be used for the assay. Table IV gives optimal zone sizes that might be expected for each assay.

For Turbidimetric Assays—Proceed as described for plate assays, but instead of agar, add varying amounts of the suspension to 100 ml. of the broth described for the assay in Table V, using the volume described under "Suggested Milliliters of Suspension . . ." as a guide. Using the several inocula prepared in this manner, proceed as described for the specific antibiotic assay, but run only the high and low concentrations of the standard response line. After incubation, read the absorbances of the appropriate tubes. Determine which inoculum produces the best response between the low and high antibiotic concentrations, and use this inoculum for the assay.

DETAILS OF ASSAY PROCEDURES

For those assays to which the uniform procedure does not apply, reference should be made to the appropriate section of the Code of Federal Regulations (3).

In Table IV, under the column headed "Medium," there are the subheadings "Base" and "Seed." Unless otherwise specified, 21 ml. of base agar and 4 ml. of seed agar should be used for each plate. For turbidimetric assays in Table V, 9 ml. of inoculated broth should be added to each tube containing 1 ml. of sample, unless otherwise specified.

The column marked "Initial Solution of Standard" indicates the method for dissolving the reference standard to be used for the assay. Unless a concentration of 1000 mcg. or units per milliliter is indicated, the solution should immediately be further diluted with the indicated buffer to obtain a stock solution of convenient concentration.

The entries under "Final Concentration for Standard Response Line" are self-explanatory. The italicized concentration in each series is the reference concentration. Official FDA working standards are supplied to laboratories receiving antibiotic certification services. For other laboratories, reference standards are supplied by the USP or NF for some antibiotics listed in those compendia

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Determination of Sodium *p*-Aminosalicylate in the Presence of *m*-Aminophenol

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Abstract □ The *p*-aminosalicylic acid content in mixtures containing *m*-aminophenol is determined by a modification of the official assay procedure. The *m*-aminophenol is removed by passing a solution of the mixture in dimethylformamide through a column containing a strong cation-exchange resin. The eluate is then treated according to the official method.

Keyphrases □ *p*-Aminosalicylic acid—determination in *m*-aminophenol mixtures, ion-exchange chromatography □ Ion-exchange chromatography—determination of *p*-aminosalicylic acid in *m*-aminophenol mixtures

The official procedure for determining *p*-aminosalicylic acid, its salts and dosage forms, is based on the diazotization reaction involving aromatic amines. In USP XVII, an external indicator was used; while in USP XVIII, the end-point is detected potentiometrically. *m*-Aminophenol, the major breakdown product of *p*-aminosalicylic acid, if present, is also diazotized and constitutes an interference in the official assay procedure. However, the *m*-aminophenol content is determined by spectrophotometric analysis.

A variety of techniques have been proposed for the determination of *p*-aminosalicylic acid and its salts. These were reviewed by Lach and Cohen (1) and were referred to in an earlier paper (2). In the latter paper, a nonaqueous differentiating titration procedure was proposed for analyzing mixtures of *p*-aminosalicylic acid and *m*-aminophenol. Titrations were conducted potentiometrically, with sodium methoxide as the titrant and dimethylformamide as the titration solvent. Titrations were also performed visually, using thymol blue as the indicator. The presence of *m*-aminophenol does not interfere with the end-point detection for *p*-aminosalicylic acid. In a subsequent paper (3), a procedure was described for the separation and determination of mixtures containing *p*-aminosalicylic acid and *m*-aminophenol. Separation was effected by use of a strong cation-exchange resin. *m*-Aminophenol was