3.1 Microbiological assay of antibiotics

The potency (activity) of an antibiotic product is expressed as the ratio of the dose that inhibits the growth of a suitable susceptible microorganism to the dose of an International Biological Standard, an International Biological Reference Preparation, or an International Chemical Reference Substance of that antibiotic that produces similar inhibition. Properly validated secondary reference materials may also be utilized in the assay. To carry out the assay a comparison is made between the inhibition of the growth of microorganisms produced by known concentrations of the reference material and that produced by measured dilutions of the test substance. This response can be measured by the diffusion method, as described below, or in a liquid medium by the turbidimetric method.

An International Unit is the specific activity contained in such an amount (weight) of the relevant International Biological Standard or International Biological Reference Preparation as the WHO Expert Committee on Biological Standardization may from time to indicate as the quantity exactly equivalent to the unit accepted for international use. In some cases, when owing to the properties of the material, difficulties are experienced in weighing with adequate accuracy small amounts of the relevant International Biological Standard or International Biological Reference Preparation, International Units are defined on the basis of the total contents of the material in an ampoule or a vial. A defined number of International Units is then assigned to the total contents of an ampoule or a vial; this material has to be carefully removed with an appropriate solvent and the final volume of the solution has to be accurately adjusted.

International Chemical Reference Substances do not have defined units of biological activity. The potency of those products for which biological assays are required are in such cases expressed in terms of an equivalent weight of the pure substance.

Recommended procedure

Use Petri dishes or rectangular trays filled to a depth of 3-4 mm, unless otherwise indicated in the monograph, with a culture medium that has previously been inoculated with a suitable inoculum of a susceptible test organism prepared as described below. The nutrient agar may be composed of two separate layers of which only the upper one may be inoculated. The concentration of the inoculum should be so selected that the sharpest zones of inhibition and suitable dose response at different concentrations of the standard are obtained. When using the inoculum prepared as described below, an inoculated medium containing 1 mL of inoculum per 100 mL of the culture medium is usually suitable. When the inoculum consists of a suspension of vegetative organisms, the temperature of the molten agar medium must not exceed 48-50 °C at the time of inoculation. The dishes or trays should be specially selected with flat bottoms. During the filling they should be placed on a flat, horizontal surface so as to ensure that the layer of the medium will be of a uniform thickness. With some test organisms, the procedure may be improved if the inoculated plates are allowed to dry for 30 minutes at room temperature before use, or refrigerated at 4 °C for several hours.

For the application of the test solution, small sterile cylinders of uniform size, approximately 10 mm high and having an internal diameter of approximately 5 mm, made of suitable material such as glass, porcelain, or stainless steel, are placed on the surface of the inoculated medium. Instead of cylinders, holes 8-10 mm in diameter may be bored in the medium with a previously sterilized borer. Other methods of application of the test solution may also be used. The arrangement on the plate should be such that overlapping of zones is avoided.

Solutions of the reference material of known concentration and corresponding dilutions of the test substance, presumed to be of approximately the same concentration, are prepared in a sterile buffer of a suitable pH value. To assess the validity of the assay at least 3 different doses of the reference material should be used together with an equal number of doses of the test substance having the same presumed activity as the solutions of the reference material. The dose levels used should be in geometric progression, for example, by preparing a series of dilutions in the ratio 2:1. Once the relationship between the logarithm of concentration of the antibiotic and the diameter of the zone of inhibition has been shown to be approximately rectilinear for the system used, routine assays may be carried out using only 2 concentrations of the reference material and 2 dilutions of the test substance. Where a monograph gives directions for the initial preparation of a solution of the substance, this solution is then diluted as necessary with the appropriate sterile buffer.

The solutions of the reference material and the test substance are preferably arranged in the form of a Latin square when rectangular trays are employed. When Petri dishes are used, the solutions are arranged on each dish so that the solutions of the reference material and those of the test substance alternate around the dish and are placed in such a manner that the highest concentrations of the reference material and of the test substance are not adjacent. The solutions are placed in the cylinders or holes by means of a pipette that delivers a uniform volume of liquid. When the holes are used the delivered volume should be sufficient to fill them almost completely.

The plates are incubated at a suitable temperature, the selected temperature being controlled at ±0.5 °C, for approximately 16 hours, and the diameters or areas of the zones of inhibition produced by the varied concentrations of the standard and of the test substance are measured accurately, preferably to the nearest 0.1 mm of the actual zone size, by using a suitable measuring device. From the results, the potency of the tested substance is calculated. Suitable publications on the statistics of bioassays are listed below.

Conditions for the assay of individual antibiotics and suitable test organisms are given in the monographs. The choice of an

appropriate strain of test organism may be critical for the assay. For easy reference, examples of suitable test organisms for a number of antibiotics are shown in Table 4. The designations of the test strains are as follows:

NCTC - National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, England

NCYC - National Collection of Yeast Cultures, AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA, England

ATCC - American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA

Other suitable strains of test organisms can be used. Additional information regarding sources of suitable strains may be obtained from Quality Assurance and Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

Precision of the assay

In order to determine whether or not a substance satisfies the requirements for potency specified in the monograph, the assay should, if necessary, be repeated until the required precision has been attained. This precision is such that the fiducial limits (P = 0.95) of the mean estimated potency, expressed as a percentage of the mean estimated potency, should be within the required range given in the individual monographs.

Calculation of results

The following publications contain suitable methods that may be used to carry out the statistical evaluation of the microbiological assay of antibiotics:

1. Bliss CI.: *Statistics of bioassay.* New York, Academic Press, 1952.

2. Bliss CI.: *Statistics in biology.* vol. I, New York, McGraw Hill, 1967.

3. Bliss CI.: *Statistics in biology.* vol. II, New York, McGraw Hill, 1970.

4. Finney DJ.: *Statistical methods in biological assays.* London, Griffin, 1964.

5. Hewitt W.: *Microbiological assay.* New York, Academic Press, 1977.

6. Philippe J.: *Les methodes statistiques en pharmacie et en chimie.* Paris, Masson, 1967.

The methods of carrying out the statistical evaluation of the microbiological assay of antibiotics are also described in many national and regional pharmacopoeias.

TABLE 4. TEST ORGANISMS AND CONDITIONS OF ASSAY OF INDIVIDUAL ANTIBIOTICS

Antibiotic	Test organism	Culture medium; final pH	Phosphate buffer, sterile, pH [a], TS	Concentration (weight or International Units per mL) [b]	Incubation temperature (ir
Bacitracin	*Micrococcus luteus* NCTC 7743; ATCC 10240	Cm1; 7.0-7.1	7.0	1-4 IU	35-37
	Micrococcus luteus NCTC 7743; ATCC 10240	Cm1; 6.5-6.6	6.0	1-4 IU	30-31
Cefalexin	*Staphylococcus aureus* NCTC 6571; ATCC 9144	Cm1; 6.5-6.6	6.0	10-40 µg	32-35
	Staphylococcus aureus	Cm1;	6.0	10-40 µg	32-35

	ATCC 6538-P	6.5-6.6			
Cefalotin	*Staphylococcus aureus*	Cm1;	6.0	0.5-2 IU	32-35
	NCTC 6571;	6.5-6.6			
	ATCC 9144				
	Staphylococcus aureus	Cm1;	6.0	0.5-2 IU	32-35
	ATCC 6538-P	6.5-6.6			
Chlortetracycline	*Bacillus pumilus*	Cm1;	4.5	2-20 IU	37-39
	NCTC 8241;	6.5-6.6			
	ATCC 14884				
	Bacillus cereus	Cm1;	4.5	0.05-0.2 IU	30-33
	ATCC 11778	5.9-6.0			
Cloxacillin	*Bacillus subtilis*	Cm1;	7.0	5-20 µg	37-39
	NCTC 8236;	6.5-6.6			
	ATCC 11774				
	Staphylococcus aureus	Cm1;	6.0	2-8 µg	32-35
	ATCC 6538-P	6.5-6.6			
Dicloxacillin	*Staphylococcus aureus*				
	NCTC 6571;	Cm1;	6.0	2.5-10 µg	37-39
	ATCC 9144	6.6			
	Staphylococcus aureus	Cm1;	6.0	2-8 µg	32-35
	ATCC 6538-P	6.5-6.6			
Erythromycin	*Bacillus pumilus*				
	NCTC 8241;	Cm1;	8.0	5-25 IU	37-39
	ATCC 14884	8.0-8.1			
	Micrococcus luteus	Cm1;	8.0	0.5-1.5 IU	35-37
	ATCC 9341	8.0-8.1			
Kanamycin	*Bacillus subtilis*	Cm1;	8.0	5-20 IU	30-37
	ATCC 6633	7.9			
	Staphylococcus	Cm1;	8.0	10 IU	35-39

	aureus				
	ATCC 6538P	7.9			
Neomycin	*Bacillus pumilus*	Cm1;	8.0	2-14 IU	37-39
	NCTC 8241;	8.0-8.1			
	ATCC 14884				
	Staphylococcus aureus	Cm1;	8.0	2-20 IU	35-37
	ATCC 29737	7.8-8.0			
	Staphylococcus epidermidis	Cm1;	8.0	0.5-2 IU	35-37
	ATCC 12228	8.0-8.1			
Novobiocin	*Bacillus subtilis*	Cm1;	6.0	1-5 IU	30-33
	NCTC 10315;	6.5-6.6			
	Micrococcus luteus	Cm1;	6.0	10-50 IU	30-35
	ATCC 9341	6.5-6.6			
Nystatin	*Saccharomyces cerevisiae*	Cm3;	-[c]	25-300 IU	35-37
	NCYC 87;	6.0-6.2			
	ATCC 9763				
Oxacillin	*Bacillus subtilis*	Cm1;	7.0	2.5-10 µg	37-39
	NCTC 8236;	6.5-6.6			
	ATCC 11774				
	Staphylococcus aureus	Cm1;	6.0	2-8 µg	32-35
	ATCC 6538-P	6.5-6.6			
Oxytetracycline	*Bacillus pumilus*	Cm1;	4.5	2-20 IU	37-39
	NCTC 8241;	6.5-6.6			
	ATCC 14884				
	Bacillus cereus	Cm1;	4.5	0.5-2 IU	30-33
	ATCC 11778	5.9-6.0			
Polymyxin B	*Bordetella bronchiseptica*	Cm2;	6.0, TS3	20-100 IU	35-37
	NCTC 8344;	7.2-7.3			
	ATCC 4617				

	Bordetella bronchiseptica	Cm2;	7.2	50-200 IU	35-37
	NCTC 8344;	7.2-7.3			
	ATCC 4617				
	Escherichia coli	Cm1;	7.2	5-100 IU	35-37
	ATCC 10536	6.5-6.6			
Streptomycin	*Bacillus subtilis*	Cm1;	8.0	5-20 IU	37-39
	NCTC 8236;	7.9-8.0			
	ATCC 11774				
	Bacillus subtilis	Cm1;	8.0	3-15 IU	35-37
	ATCC 6633	8.0-8.1			
Tetracycline	*Bacillus pumilus*	Cm1;	4.5	2-20 IU	37-39
	NCTC 8241;	6.5-6.6			
	ATCC 14884				
	Bacillus cereus	Cm1;	4.5	0.5-2 IU	30-33
	ATCC 11778	5.9-6.0			

[a] Phosphate buffers, sterile, of suitable pH. Buffers designated as TS, TS1, or TS2 may be used.

[b] Range within which suitable concentrations may be found.

[c] The preparation of the solution of the reference material and of the corresponding dilution of the test substance is done as described in the monograph with the aid of dimethylformamide R and phosphate buffer, sterile, pH 6.0 TS3.

Culture media

The formulae for the culture media (Cm) referred to in Table 4 are described in Reagents, test solutions and volumetric solutions. In each instance the final pH is adjusted to that stated in the table.

Preparation of inoculum

Bacillus cereus; Bacillus pumilus; Bacillus subtilis. The test organism is grown for 7 days at a temperature of 37-39°C on the surface of culture medium Cm1 (pH 6.5-6.6 after sterilization) to which has been added 1 µg of manganese sulfate R per mL. Using sterile water, the growth, which consists mainly of spores, is washed off, heated for 30 minutes at 70 °C, and suitably diluted - for example, to give between 10^7 and 10^8 spores per mL. The spore suspension may be stored for long periods at a temperature not exceeding 4 °C.

Bordetella bronchiseptica. The test organism is grown overnight on culture medium Cm2 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth and diluting with sterile water or saline TS to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (1 g/l) TS2 that has been stored frozen at -70 °C and subsequently thawed.

Micrococcus luteus. The test organism is grown overnight on culture medium Cm1 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth and diluting with saline TS to a suitable opacity, for example, such that a 1-cm layer of a 1 in 50 dilution transmits 80% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile

peptone (1 g/l) TS2 that has been stored frozen at -70 °C and subsequently thawed.

Saccharomyces cerevisiae. The test organism is grown overnight on culture medium Cm3 (pH 6.0-6.2 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth with saline TS and diluting to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (1 g/l) TS2 that has been stored frozen at -70 °C and subsequently thawed.

Staphylococcus aureus. The test organism is grown overnight on culture medium Cm1 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth with saline TS and diluting to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (5 g/l) TS that has been stored frozen at -70 °C and subsequently thawed.