standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualisation and characterisation of immunoprecipitation lines

These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterisation of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

VALIDATION OF THE METHOD

Validation criteria

A quantitative immunochemical method is not valid unless:

1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound,

2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity,

3) The limit of quantitation is below the acceptance criteria stated in the individual monograph,

4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs,

5) The order in which the assay is performed does not give rise to systematic errors.

Validation methods

In order to verify these criteria, the validation design includes the following elements:

1) The assay is performed at least in triplicate,

2) The assay includes at least 3 different dilutions of the standard preparation and 3 dilutions of sample preparations of presumed activity similar to the standard preparation,

3) The assay layout is randomised,

4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared,

5) The test includes the measurement of non-specific binding of the labelled reactant,

6) For displacement immunoassay:

(a) maximum binding (zero displacement) is determined,

(b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

STATISTICAL CALCULATION

To analyse the results, response curves for test and standard may be analysed by the methods described in *5.3. Statistical Analysis of Results of Biological Assays and Tests*.

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

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2.7.2. MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the 2 log dose-response (or transformed response) lines of the preparation to be examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated.

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by method A or method B.

A. DIFFUSION METHOD

Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example 48 °C to 50 °C for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic to be examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2 mm to 5 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 2.7.2.-1, prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of

suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Table 2.7.2.-1. — *Diffusion assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Amphotericin B	*Amphotericin B CRS*	*Dimethyl sulphoxide R*	pH 10.5 (0.2 M)	*Saccharomyces cerevisiae* ATCC 9763 IP 1432-83	F - pH 6.1	35-37 °C
Bacitracin zinc	*Bacitracin zinc CRS*	*0.01 M hydrochloric acid*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 7743 CIP 53.160 ATCC 10240	A - pH 7.0	35-39 °C
Bleomycin sulphate	*Bleomycin sulphate CRS*	*Water R*	pH 6.8 (0.1 M)	*Mycobacterium smegmatis* ATCC 607	G - pH 7.0	35-37 °C
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617 *Escherichia coli* NCIB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Dihydrostreptomycin sulphate	*Dihydrostreptomycin sulphate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 8236 CIP 1.83 *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9 A - pH 7.9	30-37 °C 30-37 °C
Erythromycin estolate	*Erythromycin A CRS*	*Methanol R* (see the monographs)	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18 *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
Framycetin sulphate	*Framycetin sulphate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 *Bacillus pumilus* NCTC 8241 CIP 76.18	E - pH 7.9 E - pH 7.9	30-37 °C 30-37 °C
Gentamicin sulphate	*Gentamicin sulphate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18 *Staphylococcus epidermidis* NCIB 8853 CIP 68.21 ATCC 12228	A - pH 7.9 A - pH 7.9	35-39 °C 35-39 °C
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Kanamycin monosulphate Kanamycin acid sulphate	*Kanamycin monosulphate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 *Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	A - pH 7.9 A - pH 7.9	30-37 °C 35-39 °C
Neomycin sulphate	*Neomycin sulphate for microbiological assay CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18 *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9 E - pH 7.9	30-37 °C 30-37 °C
Netilmicin sulphate	*Netilmicin sulphate CRS*	*Water R*	pH 8.0 ± 0.1	*Staphylococcus aureus* ATCC 6538P CIP 53.156	A - pH 7.9	32-35 °C
Nystatin	*Nystatin CRS*	*Dimethylformamide R*	pH 6.0 (0.05 M) containing 5 per cent *V/V* of *dimethylformamide R*	*Candida tropicalis* CIP 1433-83 NCYC 1393 *Saccharomyces cerevisiae* NCYC 87 CIP 1432-83 ATCC 9763	F - pH 6.0 F - pH 6.0	30-37 °C 30-32 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 6.6	35-39 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-32 °C
Streptomycin sulphate	*Streptomycin sulphate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 8236 CIP 1.83 *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9 A - pH 7.9	30-37 °C 30-37 °C
Tylosin for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	A mixture of 40 volumes of *methanol R* and 60 volumes of *0.1 M phosphate buffer solution pH 8.0 R*	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 8.0	32-35 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Bacillus subtilis* NCTC 8236 CIP 52.62 ATCC 6633	A - pH 8.0	37-39 °C

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than 6 solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 h. A period of diffusion prior to incubation, usually 1 h to 4 h, at room temperature or at about 4 °C, as appropriate, may be used to minimise the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters with a precision of at least 0.1 mm or the areas of the circular inhibition zones with a corresponding precision and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

B. TURBIDIMETRIC METHOD

Inoculate a suitable medium with a suspension of the chosen micro-organism having a sensitivity to the antibiotic to be examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 h.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 2.7.2.-2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number 3 consecutive doses, using corresponding doses for the reference substance and the antibiotic to be examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 ml of the solution and 9 ml of the medium). For the assay of tyrothricin add 0.1 ml of the solution to 9.9 ml of inoculated medium.

Prepare at the same time 2 control tubes without antibiotic, both containing the inoculated medium and to one of which is added immediately 0.5 ml of *formaldehyde R*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomised block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3 h to 4 h, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the micro-organisms by adding 0.5 ml of *formaldehyde R* to each tube or by heat treatment and measure the opacity to 3 significant figures using suitable optical apparatus. Alternatively use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Calculate the potency using appropriate statistical methods.

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

Table 2.7.2.-2. – *Turbidimetric assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 7.0	*Escherichia coli* NCIB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Dihydrostreptomycin sulphate	*Dihydrostreptomycin sulphate CRS*	*Water R*	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C
Erythromycin estolate Erythromycin ethylsuccinate	*Erythromycin A CRS*	*Methanol R* (see the monographs)	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031	D - pH 7.0	35-37 °C
				Staphylococcus aureus NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Framycetin sulphate	*Framycetin sulphate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gentamicin sulphate	*Gentamicin sulphate CRS*	*Water R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Gramicidin	*Gramicidin CRS*	*Methanol R*	pH 7.0*	*Enterococcus hirae* CIP 58.55 ATCC 10541 *Staphylococcus aureus* ATCC 6538 P	C - pH 7.0	35-37 °C
	*Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions, for example 0.1 mg/ml of *polysorbate 80 R*					
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Kanamycin monosulphate Kanamycin acid sulphate	*Kanamycin monosulphate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Neomycin sulphate	*Neomycin sulphate for microbiological assay CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0	*Escherichia coli* NCIB 8879 CIP 54.127 ATCC 10536	C - pH 7.0	35-37 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Streptomycin sulphate	*Streptomycin sulphate CRS*	*Water R*	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C
Tylosin for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	pH 7.0	*Staphylococcus aureus* NCTC 6571 ATCC 9144 CIP 53.154	C - pH 7.0	37 °C
Tyrothricin	*Gramicidin CRS*	*Alcohol R*	*Alcohol R*	*Enterococcus hirae* ATCC 10541	C - pH 7.0	37 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P	C - pH 7.0	37-39 °C

The following section is published for information.

Recommended micro-organisms

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH. The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

Preparation of inocula. *Bacillus cereus* var. *mycoides*; *Bacillus subtilis*; *Bacillus pumilus*. Spore suspensions of the organisms to be used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/l of *manganese sulphate R*. Using sterile *water R*, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually 10×10^6 to 100×10^6 per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 26 °C for 4-6 days, then adding, aseptically, sufficient *manganese sulphate R* to give a concentration of 0.001 g/l and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile *water R* to give a concentration of 10×10^6 to 100×10^6 spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

Bordetella bronchiseptica. Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile *water R* and dilute to a suitable opacity.

Staphylococcus aureus; *Klebsiella pneumoniae*; *Escherichia coli*; *Micrococcus luteus*; *Staphylococcus epidermidis.* Prepare as described above for *B. bronchiseptica* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

Saccharomyces cerevisiae; *Candida tropicalis.* Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/l solution of *sodium chloride R*. Dilute to a suitable opacity with the same solution.

Buffer solutions. Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 ml of *0.2 M potassium dihydrogen phosphate R* with the quantity of *0.2 M sodium hydroxide* indicated in Table 2.7.2.-3. Dilute with freshly prepared *distilled water R* to produce 200.0 ml.

Table 2.7.2.-3.

pH	*0.2 M Sodium hydroxide* (ml)
5.8	3.72
6.0	5.70
6.2	8.60
6.4	12.60
6.6	17.80
6.8	23.65
7.0	29.63
7.2	35.00
7.4	39.50
7.6	42.80
7.8	45.20
8.0	46.80

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.-1 with the exception of bleomycin sulphate and amphotericin B.

For bleomycin sulphate, prepare the buffer solution pH 6.8 as follows: dissolve 6.4 g of *potassium dihydrogen phosphate R* and 18.9 g of *disodium hydrogen phosphate R* in *water R* and dilute to 1000 ml with *water R*.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 ml of *water R*, add 20 ml of *1 M sodium hydroxide* and dilute to 1000.0 ml with *water R*.

Culture media. The following media or equivalent media may be used.

Medium A

Peptone	6 g
Pancreatic digest of casein	4 g
Beef extract	1.5 g
Yeast extract	3 g
Glucose monohydrate	1 g
Agar	15 g
Water to produce	1000 ml

Medium B

Pancreatic digest of casein	17 g
Papaic digest of soya bean	3 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Agar	15 g
Polysorbate 80	10 g
Water to produce	1000 ml

The polysorbate 80 is added to the hot solution of the other ingredients after boiling, and immediately before adjusting to volume.

Medium C

Peptone	6 g
Beef extract	1.5 g
Yeast extract	3 g
Sodium chloride	3.5 g
Glucose monohydrate	1 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Water to produce	1000 ml

Medium D

Heart extract	1.5 g
Yeast extract	1.5 g
Peptone-casein	5 g
Glucose monohydrate	1 g
Sodium chloride	3.5 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Potassium nitrate	2 g
Water to produce	1000 ml

Medium E

Peptone	5 g
Meat extract	3 g
Disodium hydrogen phosphate,12H_2O	26.9 g
Agar	10 g
Water to produce	1000 ml

The disodium hydrogen phosphate is added as a sterile solution after sterilisation of the medium.

Medium F

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Glucose monohydrate	10.0 g
Agar	23.5 g
Water to produce	1000 ml

Medium G

Glycerol	10 g
Peptone	10 g
Meat extract	10 g
Sodium chloride	3 g
Agar	15 g
Water to produce	1000 ml

pH 7.0 ± 0.1 after sterilisation.

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2.7.4. ASSAY OF HUMAN COAGULATION FACTOR VIII

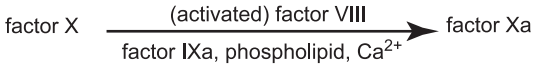
Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipid. The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VIII activity of a stated amount of the International Standard, which consists of a freeze-dried human coagulation factor VIII concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

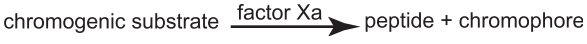
Human coagulation factor VIII BRP is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of 2 consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme.

Step 1



Step 2



Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the International Standard for human blood coagulation factor VIII concentrate as the standard, that the results obtained do not differ significantly.

It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa generation in order to determine the time taken to reach 50 per cent of the maximal factor Xa generation.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay. Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least 2 separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The 2nd step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between 3 and 5 amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

ASSAY PROCEDURE

Reconstitute the entire contents of 1 ampoule of the reference preparation and of the preparation to be examined; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5-2.0 IU/ml.

The prediluent consists of haemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma. The prediluted materials must be stable beyond the time required for the assay.

Prepare further dilutions of the reference and test preparations using a non-chelating, appropriately buffered solution, for example, tris(hydroxymethyl)aminomethane or imidazole, containing 1 per cent of human or bovine albumin. Prepare at least 2 dilution series of at least 3 further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU/ml, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.