10. READING AND INTERPRETATION OF THE RESULTS

Minute amounts of endotoxin in the water for BET, or in any other reagent or material to which the lyase is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lyase. However, they may raise the amount of endotoxin in the solution containing the product under examination to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lyase available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a ‘false positive result’ cannot be ruled out completely. It should be realised, however, that in this respect the test design is ‘fail-safe’ in contrast to a test design permitting a false negative result, which could lead to the release of an unsatisfactory product, thus endangering the patient’s health.

11. REPLACEMENT OF THE RABBIT PYROGEN TEST BY A TEST FOR BACTERIAL ENDOTOXINS

Monographs on pharmaceutical products intended for parenteral use that may contain toxic amounts of bacterial endotoxins require either a test for bacterial endotoxins or a rabbit pyrogen test. As a general policy:

11.1. In any individual monograph, when a test is required, only one test is included, either that for pyrogens or that for bacterial endotoxins.

11.2. In the absence of evidence to the contrary, the test for bacterial endotoxins is preferred over the test for pyrogens, since it is usually considered to provide equal or better protection to the patient.

11.3. Before including a test for bacterial endotoxins in a monograph, evidence is required that one of the tests described in chapter 2.6.14 can be applied satisfactorily to the product in question.

11.4. The necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the test for bacterial endotoxins to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the test for bacterial endotoxin is appropriate, must be provided.

Additional requirements are defined in the following sections.

12. USE OF A DIFFERENT BACTERIAL ENDOTOXIN TEST FROM THAT PRESCRIBED IN THE MONOGRAPH

When a test for bacterial endotoxins is prescribed in a monograph and none of the six methods (A to F) described in chapter 2.6.14 is specified, then method A, the gel-clot method limit test, has been validated for this product. If one of the other methods (B to F) is specified, this is the one which has been validated for this product.

13. VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test by a bacterial endotoxin test, or replacement of a stated or implied method for bacterial endotoxins by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopeial test, as described in the General Notices:

“The test and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative.”

The following procedures are suggested for validating a method for bacterial endotoxins other than the one implied or indicated in the monograph.

13.1. The procedure and the materials and reagents used in the method should be validated as described for the test concerned.

13.2. The presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least three production batches. It should be borne in mind that methods D and E, using a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

14. VALIDATION OF THE TEST FOR NEW PRODUCTS

The procedures described under 13.1 and 13.2 should be applied to all new products intended for parenteral use that have to be tested for the presence of bacterial endotoxins according to the requirements of the Pharmacopoeia.

01/2005:20615 corrected

2.6.15. PREKALLIKREIN ACTIVATOR

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard which consists of freeze-dried prekallikrein activator. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Prekallikrein activator in albumin BRP is calibrated in International Units by comparison with the International Standard.

PREPARATION OF PREKALLIKREIN SUBSTRATE

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or 38 g/l sodium citrate R) to which 1 mg/ml of hexadimethrine bromide R has been added. Centrifuge the mixture at 3600 g for 5 min. Separate the plasma and centrifuge again at 6000 g for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing agarose-DEAE for ion exchange chromatography R which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 ml/cm²/h. Collect the eluate in fractions and record the absorbance at 280 nm (2.2.25). Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.
Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 1 g/1 of sodium chloride R and filter using a membrane filter (porosity 0.45 μm). Freeze the filtrate in portions and store at −25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

ASSAY
The assay is preferably carried out using an automated enzyme analyser at 37 °C, with volumes, concentration of substrates and incubation times adjusted so that the reaction rate is linear at least up to 35 IU/ml. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, N-benzoyl-D-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide or D-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate R or D-prolyl-D-phenylalanyl-L-arginine-4-nitroanilide dihydrochloride R), dissolved in buffer B. Record the rate of change in absorbance per minute for 2 min to 10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Correct ΔA/min by subtracting the value obtained for the corresponding blank. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined.

Buffer A
Tris/hydroxymethylaminomethane R 6.055 g
Sodium chloride R 1.17 g
Hexadimethrine bromide R 50 mg
Sodium azide R 0.100 g
Dissolve the ingredients in water R, adjust to pH 8.0 with 2 M hydrochloric acid and dilute to 1000 ml with water R.

Buffer B
Tris/hydroxymethylaminomethane R 6.055 g
Sodium chloride R 8.77 g
Dissolve the ingredients in water R, adjust to pH 8.0 with 2 M hydrochloric acid and dilute to 1000 ml with water R.

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2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

In those tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (5.2.2).

VIRUS SEED LOT
Take samples of the virus seed lot at the time of harvesting and, if they are not tested immediately, keep them at a temperature below −40 °C.

Adult mice. Inoculate each of at least ten adult mice, each weighing 15 g to 20 g, intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the virus seed lot. Observe the mice for at least 21 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice which are observed for 21 days. The virus seed lot complies with the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Suckling mice. Inoculate each of at least twenty mice, less than 24 h old, intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the virus seed lot. Observe the mice daily for at least 14 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice which are observed daily for 14 days. The virus seed lot passes the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Guinea-pigs. Inoculate intraperitoneally into each of at least five guinea pigs, each weighing 350 g to 450 g, 5.0 ml of the virus seed lot. Observe the animals for at least 42 days for signs of disease. Carry out an autopsy of all guinea-pigs that die after the first 24 h of the test or that show signs of illness and examine macroscopically; examine the tissues both macroscopically and culturally for evidence of infection. Kill animals that survive the observation period and examine in a similar manner. The virus seed lot passes the test if no guinea-pig shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the guinea-pigs survive the observation period.

VIRUS SEED LOT AND VIRUS HARVESTS
Take samples at the time of harvesting and, if not tested immediately, keep them at a temperature below −40 °C.

Bacterial and fungal sterility. A 10 ml sample complies with the test for sterility (2.6.1).

Mycoplasmas. A 10 ml sample complies with the test for mycoplasmas (2.6.7).

Mycobacteria (2.6.2). A 5 ml sample is tested for the presence of Mycobacterium spp. by culture methods known to be sensitive for the detection of these organisms.

Test in cell culture for other extraneous agents. Neutralised samples equivalent, unless otherwise prescribed, to 500 human doses of vaccine or 50 ml, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures. If the virus is grown in human diploid cells, the neutralised virus harvest is also tested on a separate species.