

XV. Botulinum Antitoxin Immunoserum botulinicum

The current monograph stipulates one animal test for potency testing which is combined with the identification test.

XV.1 POTENCY TEST

XV.1.1 Requirements/practical application

The potency test prescribed for botulinum antitoxin is based on a classical toxin neutralisation test (TNT) in mice, i.e. the antibodies against toxin type A, type B and type E are determined by lethal *in vivo* titration in comparison to a reference preparation calibrated in International Units (IU). The test requirements are, in principle, similar to those of the TNTs stipulated by other monographs, although there are some differences between this test and the methods stipulated for diphtheria, tetanus, and gas-gangrene antitoxins (see Table XII-2 in chapter XII): four mice per dilution are inoculated intraperitoneally with the relatively high volume of 1 ml.

The sole manufacturer producing botulinum antitoxin in Germany tests three dilutions of the reference antitoxin and two dilutions of the test antitoxin, using five animals per dilution. The test is conducted on each of the three antitoxins or toxin types (A, B, E), thus giving a total of 75 (3 x 25) mice per test. Potency testing is carried out at least twice on each batch, therefore, at least 150 mice are used per batch.

The test toxin has been shown to be stable and therefore the dose of the test toxin is not determined prior to each test. Any change in the test toxin would be reflected in the results obtained with the reference antitoxin. The reference antitoxin is calibrated against the WHO reference antitoxin. This same procedure is also used in the testing of human tetanus immunoglobulins and tetanus antitoxin (chapters XII and XIII).

In 1991-1993, six batches of botulinum antitoxin were released in Germany. Since 150 animals are required on average for the testing of each batch, c. 900 mice were used in total in this period.

XV.1.2 Evaluation of the method

See chapter XII.1.2 (*Human Tetanus Immunoglobulin*).

In addition, the studies of Takahashi *et al.* [1] should be noted. Instead of using the very imprecise "all or none" titration, these authors tried to evaluate more-differentiated parameters (severity of symptoms, interval between challenge and death) and express them as "scores". This permitted the intermediate effects of the antitoxins to be determined as well as enabling the test results to be evaluated through a comparison of ED₅₀ values. In contrast to the current method, it was possible to evaluate the results statistically (with a 95% confidence interval) without using more animals [1].

XV.1.3 Reduction and refinement

Test size and precision:

See chapter XII.1.3 (*Human Tetanus Immunoglobulin*).

Number of animals used per dilution:

In chapter XII.1.2 it was shown that, theoretically, it would suffice to use one animal per dilution. There is no mathematical basis for requiring the use of four mice per dilution to test this particular antitoxin. This figure of "four" probably derives from the experience that the TNT is less reproducible in botulinum testing (personal communication). This is reflected in the number of animals (5) used by the manufacturer participating in our survey.

As already recommended for *Human Tetanus Immunoglobulin* (chapter XII.) and *Tetanus Antitoxin* (chapter XIII.), the requirement for animals in this monograph should also be modified to "1-3 animals per dilution".

Frequency of determination of the toxin:

As practice shows, it is possible to use stable toxins for testing (see XV.1.1), because any change would be reflected noticeably in the results obtained with the reference serum. Therefore, it should suffice to determine the test toxin dose on one occasion only. A note to this effect should be incorporated into the monograph. Furthermore, the monograph should also incorporate the requirement that the in-house reference preparation be regularly calibrated against the WHO reference preparation.

Distress:

The possibility of reducing the volume injected (1 ml intraperitoneally) should be investigated, because the use of this volume causes unnecessary suffering to the mice.

XV.1.4 Alternatives

By analogy with tetanus antitoxin, it should also be possible to use **immunochemical *in vitro* methods** to quantify neutralising antibodies in botulinum antitoxin. In principle, the same types of method as used for tetanus immunoglobulins (see chapter XII) should be suitable, namely **immunoelectrophoresis** (RIE, PICI), **nephelometry**, or **ELISA**.

ELISA procedures have already been developed for antibodies against toxins type A and type B, but, in comparison with the TNT, they proved unsatisfactory for determination of serum antibody titres in immunised humans [2]. However, it should be noted that the antitoxin used as the reference is not totally appropriate for the evaluation of human sera.

There are no reports of corresponding studies on antitoxin type E nor of further *in vitro* studies on botulinum antitoxin in comparison with the TNT. At present, there are no approaches to the development of functional *in vitro* tests (e.g. titration in cell cultures).

Recent studies on RIE (A. Zott, PEI, personal communication) have given promising results which indicate that quantification of all three antitoxin types may be possible.

As already recommended for tetanus immunoglobulin and antitoxin (chapter XII. 1.4), less sensitive methods such as RIE should be preferred to ELISA, because the test samples contain high levels of antibodies.

Provided that further comparative studies confirm a correlation between TNT results and antibody titres in botulinum antitoxins determined using *in vitro* methods (RIE, ELISA, nephelometry, etc.), revision of the monograph could be achieved in the medium term, in order to incorporate the possibility, as in the monograph for tetanus immunoglobulins, of using *in vitro* methods for routine quality control.

Once the suitability of an *in vitro* method has been confirmed, it is recommended, as a medium-term aim, to revise this monograph in the same way as the monograph *Human Tetanus Immunoglobulin* in order to permit the use of *in vitro* methods for routine potency testing once batch consistency has been demonstrated.

LITERATURE:

1. Takahshi M, Komiya T, Kameyama S, and Sakaguchi G, Titration of botulinum antitoxin of low levels by the score method. *Jpn J Med Sci Biol* 43:163-170 (1990).
2. Shone C, Appleton N, Wilton-Smith P, Hambleton P, Modi N, Gatlev S, and Melling J, *In vitro* assays for botulinum toxin and antitoxins. *Dev Biol Stand* 64: 141-145 (1986).

SUMMARY OF RECOMMENDATIONS

The ***in vivo* toxin neutralisation test** used for the potency testing of botulinum antitoxin should be modified as follows:

- A maximum size for the dilution interval (e.g. 20%) or a maximum error limit should be stated in order to enable titres to be determined with a comparable minimum precision.
- The use of only 1-3 instead of 4 animals should be stipulated per dilution.
- The error limits (calculated from the size of the dilution intervals) should be stated together with the results.
- It should be noted that the dose of test toxin should only be re-determined if a different toxin or animal strain is used or if the toxin is suspected of being unstable.
- The reference preparation (in-house reference) should be regularly calibrated against the WHO reference.
- For the purposes of standardisation and of increasing reproducibility, the initial weight of the mice should be defined (e.g. 18 g), although the maximum permitted difference of 5 g can be retained.

Furthermore, the possibility of reducing the injection volume (1 ml intraperitoneally) should be investigated, because the use of this volume causes unnecessary suffering to the mice.

Since the currently used test is performed at different stages of production, consideration should be given to whether it would suffice to use only one test dilution, at least for testing the finished product. In this way, it would be possible to confirm with little effort that the minimum required antibody content is present (“the antibody content is greater than...”).

A number of ***in vitro* methods** which are suitable for the potency testing of highly concentrated tetanus immunoglobulins or antitoxins, e.g. methods such as immunoelectrophoresis (e.g. RIE), nephelometry or ELISA could also serve for the potency testing of botulinum antitoxins and therefore should be validated for this purpose. However, the results of studies on the use of ELISA have not been promising. Less sensitive methods, such as immunoelectrophoresis or nephelometry, should be preferred over highly sensitive methods, such as ELISA.

