PHARMEUROPA BIO 2001-1

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Collaborative Study for the Establishment of a European Pharmacopoeia Biological Reference Preparation for Serological Potency Testing of Tetanus Vaccines for Human Use:

Clostridium Tetani Guinea Pig Antiserum (Human)

Collaborative Study for the Establishment of a European Pharmacopoeia Biological Reference Preparation for Serological Potency Testing of Tetanus Vaccines for Human Use: *Clostridium Tetani* Guinea Pig Antiserum (Human)

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1. INTRODUCTION

A test on potency is required for the batch consistency quality control of the finished product of tetanus vaccines for human use.

According to the specifications of the monograph *tetanus vaccine (adsorbed*) (*0452*) and to the Section *2.7.8. Assay of tetanus vaccine (adsorbed)* of the European Pharmacopoeia (Ph. Eur.)[4] 3rd Edition, the potency of each batch of vaccine is determined by a direct challenge test in mice or guinea pigs. In this test, groups of mice or guinea pigs are immunised with serial dilutions of the tetanus vaccine under study and a reference vaccine, and after an immunisation period of 4 weeks challenged with *Clostridium (Cl.) tetani* neurotoxin. The end-point used is paralysis or death due to incomplete neutralisation of tetanus toxin.

In vitro serological assays have been described as potential alternatives to the challenge procedure with the tetanus toxin. These tests include Enzyme Linked Immunosorbent Assay (ELISA) and Toxin Binding Inhibition test (ToBI).

As part of the Biological Standardisation Programme (BSP) of the Ph. Eur. Commission, a collaborative study has been performed to evaluate *in vitro* serological methods (ELISA and ToBI) for potency testing of tetanus vaccines for human use. The ultimate goal of the study is to replace the multi-dilution challenge test on mice and guinea pigs with a single dilution test in guinea-pigs, based on serology and using a 6 week immunisation period. Interim and final reports of the collaborative study have been published [1, 2, 3].

In view of the outcome of the study it has been concluded that a prerequisite for the introduction of a serological potency test is the in-house validation of either ELISA or ToBI. Regardless of the method (the choice of either ELISA or ToBI resting with the manufacturer) the monitoring of consistency in animals' response to vaccination and in performance of the *in vitro* assays requires the following, respectively:

- an in-house reference vaccine having the same formulation as the test vaccine and calibrated against the current World Health Organisation (WHO) International Standard (IS)/Ph. Eur. Biological Reference Preparation (BRP), and
- positive and negative run controls (serum samples).

For establishment and calibration of run controls by ELISA and ToBI, the current IS for tetanus antitoxin - the equine hyperimmune *Cl. tetani* antitoxin serum (2nd WHO IS 1964, 1400 IU/ampoule, code TE) is not appropriate. Therefore, it was decided to develop a homologous guinea pig reference preparation, to be used as a working standard in the *in vitro* part of serological assays for tetanus vaccines batch potency testing.

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[4] Abbreviations: **AU**: Antibody unit; **BRP**: European Pharmacopoeia Biological Reference Preparation; **BSP**: Biological Standardisation Programme; **c**: Candidate; ***Cl. Clostridium***; **EDQM**: European Directorate for the Quality of Medicines; **ELISA**: Enzyme Linked Immunosorbent Assay; **GMP**: Good Manufacturing Practice; **IS**: International Standard; **IU**: International Unit; **Ph. Eur.**: European Pharmacopoeia; **RIVM**: Rijksinstituut voor Volksgezondheid en Milieu; **SLV**: Statens Legemiddelverket; **TN**: Toxin neutralisation; **ToBI**: Toxin binding Inhibition test; **SPF**: Specific pathogen free; **WHO**: World Health Organization.

2. SUMMARY OF STUDY

The European Directorate for the Quality of Medicines (EDQM) organised a study aimed at establishing the *Cl. tetani* guinea pig antiserum (human) BRP batch 1.

The development of the candidate (c) BRP consisted of four phases:

- the production of the bulk reference material (Phase I);
- the characterisation of the bulk material (Phase II);
- the processing and characterisation of the finished product (Phase III)
- and the assignment of potency of the finished product (Phase IV).

3. PARTICIPANTS

Three public sector laboratories participated in this collaborative study. The study was performed at the RIVM laboratories, at the EDQM laboratory, and at the SLV laboratories under the responsibility of Dr. C. Hendriksen, Dr. G. Rautmann, Dr. J. Miller and Dr. R. Winsnes.

4. SUMMARY OF THE PRODUCTION AND CHARACTERISATION OF cBRP

The bulk material processing, the filling of sterile vials and the bio- and physical-chemical characterisation of the finished product were performed at the EDQM.

The bulk material production, the immuno-chemical characterisation and microbiological screening of the cBRP were performed at the RIVM. The antitoxin titrations using Toxin neutralisation (TN) test, ToBI and ELISA were performed both at the RIVM (bulk and finished product) and SLV (finished product).

4.1. Preparation of bulk material

For the purpose of the study 400 Specific Pathogen Free (SPF) guinea-pigs (50% of both sexes, HsdPoc:DH, Harlan UK Ltd), within a weight range of 250-350g, were immunised with 0.5 ml of tetanus vaccine (adsorbed) Ph. Eur. BRP batch 1 (ref. T0400000), diluted to a concentration of 14.71 International Units (IU)/ml.

Animals were bled by cardiac puncture at day 42 and blood was collected in 250 ml bottles. The day after blood collection, serum was separated by centrifugation and the bulk volume of 4 l was pooled and divided over 8 bottles of 0.5 l each and stored at – 20 °C.

4.2. Antitoxin titration of bulk material

The antitoxin concentration of the bulk material was determined in duplicate by TN test, ToBI and ELISA. The mean antitoxin concentration obtained in TN test against the WHO IS, was 1.0 IU/ml. This titre was confirmed in ToBI and ELISA. Sterility testing of the bulk did not reveal any evidence of contamination.

4.3. Pilot filling and antitoxin titration

The 8 bottles of the bulk material were sent to the EDQM. A volume of 116 ml was filled in ampoules of 0.4 ml and 0.5 ml and freeze-dried. The samples were tested at the RIVM using ToBI and ELISA. After reconstitution of the contents of each vial in 1 ml distilled water, the antitoxin concentration assigned to these trial fills, was 0.42 Antibody Units (AU)/ml (ToBI) and 0.41 AU/ml (ELISA) for the 0.4 ml ampoules; and 0.49 AU/ml (ToBI) and 0.51 AU/ml (ELISA) for the 0.5 ml ampoules.

4.4. Processing in final containers

In order to obtain a sufficient number of vials of the cBRP, the bulk material was diluted with an equal volume of guinea-pig serum (Charles River, F) that was shown to be negative for tetanus antitoxin. Finally, the resulting bulk was aseptically poured into aliquots with 0.50 ml of serum per vial and subsequently the product was freeze-dried in two sessions.

Using this approach, 2 sublots, referred to as sublots 1 and 2, consisting respectively of 8256 and 5377 vials were produced under GMP conditions by a sub-contractor commissioned by the EDQM.

Sampling of these ampoules for the purpose of quality control has been performed by the EDQM and 2 sets were sent to RIVM and SLV, for further characterisation and antitoxin determination. The sublots are stored at a temperature of – 20 °C at the EDQM.

4.5. Characterisation of the finished product

The characterisation of the finished product of the cBRP has been carried out in accordance with Good Quality Control Laboratory Practice [4] at the EDQM, RIVM and SLV laboratories. The EDQM was in charge of the physical and physical-chemical tests (visual appearance, dissolution time, vacuum, pH of the reconstituted solution and moisture content) and the RIVM was responsible for the immuno-chemical tests, bioassays and sterility testing (identity, potency, stability and sterility of the reference preparation). In addition, the SLV was requested to confirm potency results of the finished product by TN test.

For the purpose of performing controls, the final lyophilised product was reconstituted by addition of 500 µl of *water for injections* per vial.

4.6. Physical-chemical tests

As regards visual inspection, dissolution time (sublot 1: mean time 38 s, sublot 2: mean time 35 s), vacuum testing, pH determination (indicator strips: 9.5; pH meter: 9.2) and moisture content (2 %), the cBRP sublots 1 and 2 were considered to be of satisfactory quality.

4.7. Identification

The identity of the guinea-pig origin of cBRP sublots 1 and 2 was confirmed by using ELISA.

4.8. Sterility testing

As regards bacterial and fungal sterility testing, it was found that about half of the vials of sublots 1 and 2 examined were contaminated. The contaminants were identified as *Staphylococcus epidermis* (sublot 1) and an unidentified *Staphylococcus* species (sublot 2). The contamination was confirmed by additional testing. However, contamination level appeared to be low. Nevertheless, a storage at – 20 °C of the un-reconstituted freeze-dried BRP was recommended and storage of the reconstituted cBRP should be avoided.

4.9. Potency testing by immuno-chemical methods and TN testing

Detailed protocols for TN test, ToBI or ELISA are available from the EDQM upon request.

ToBI and ELISA have been performed on 25 vials of each sublot on two separate occasions. For both tests the same vials have been used; after reconstitution the samples were titrated in ToBI, stored at + 4 °C and the next day titrated in ELISA. In ToBI, the 2nd WHO IS, diluted to 1 IU/ml was used, while in the ELISA, the liquid form of the starting guinea pig immune serum at 1.0 IU/ml was used as a reference.

The mean antitoxin content obtained by ToBI (RIVM and SLV) and ELISA (RIVM only) was 0.22 AU per vial. The results of the ELISA and ToBI testing are given below.

	Potency (AU/vial)			
Method	**ToBI***		**ELISA***	
Laboratory	**RIVM**	**SLV**	**RIVM**	**SLV**
Sublot 1	0.19	0.27	0.21	**
Sublot 2	0.185	0.24	0.20	**

* mean of two assays
** not determined

The TN was performed on sublots 1 and 2 in triplicate. First, the rough antitoxin titre was obtained in a pilot study, using 100 % dilution steps. Based on these results, the exact titre was determined in a TN, using 10 % dilution steps and including duplicate testing.

The results of the TN titrations at RIVM and SLV are given below.

		Potency (IU/vial)	
Laboratory		**RIVM**	**SLV**
Sublot 1	Assay 1	0.25	0.18
	Assay 2	0.16	0.18
Sublot 2	Assay 1	0.25	0.18
	Assay 2	0.25	0.18

Based on the results of the TN tests, the antitoxin content assigned to both sublots 1 and 2 was 0.20 IU per vial.

4.10. Stability testing

The stability of the *Cl. tetani* antitoxin content of sublots 1 and 2 cBRP was investigated by an accelerated degradation test using storage temperatures at – 20 °C, + 4 °C, + 37 °C and + 50 °C, for 30, 60 and 90 days of storage. Antitoxin concentrations were determined by ELISA and by ToBI and TN test (TN for 90 days samples only). All samples were monitored against the – 20 °C stored samples. At 60 and 90 days, + 50 °C stored samples appeared to be gelatinous and no antitoxin concentrations could be estimated.

The antitoxin concentration of the – 20 °C stored sublots 1 and 2 at 90 days, determined by TN, was 0.21 IU/vial. As can be seen, there appears to be a tendency to a slight decrease in antibody titre at + 37 °Cstorage after a period of 90 days, for both sublots. However, TN test was only performed once. Furthermore, different vials were used for ELISA/ToBI and for TN test. No deviation of antibody titre was seen in the + 4 °C storage samples and at days 30 and 60 at + 37 °C storage.

	Potency of Sublot 1								
Storage temperature	**ELISA* Days of storage**			**ToBI* Days of storage**			**TN# Days of storage**		
	30	**60**	**90**	**30**	**60**	**90**	**30**	**60**	**90**
+ 4^{0}C	0.27	0.24	0.24	0.20	0.21	0.25	n.d.	n.d.	0.21
+ 37^{0}C	0.21	0.20	0.19	0.20	0.21	0.18	n.d.	n.d.	0.15
+ 50^{0}C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* mean of two values, expressed in AU/vial
# single value expressed in IU/vial
n.d. = not determined

Storage temperature	Potency of Sublot 2								
	ELISA* Days of storage			ToBI* Days of storage			TN# Days of storage		
	30	60	90	30	60	90	30	60	90
+ 4^0C	0.24	0.24	0.27	0.21	0.20	0.23	n.d.	n.d.	0.21
+ 37^0C	0.21	0.21	0.20	0.19	0.21	0.21	n.d.	n.d.	0.12
+ 50^0C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* mean of two values, expressed in AU/vial
# single value expressed in IU/vial
n.d. = not determined

5. CONCLUSION

Based on TN titration, confirmed by ELISA and ToBI testing, the tetanus antitoxin content of the proposed reference antiserum is of 0.20 IU per vial. It has been concluded that the proposed reference antiserum is suitable for its intended purpose, i.e. serving as a positive run control for the validation of the *in vitro* part of serological potency assays of tetanus vaccines for demonstration of batch consistency. As no significant differences between the two sublots could be demonstrated, it was decided to merge them in a single batch. This candidate batch has been adopted as the "*Clostridium tetani* guinea pig antiserum, (human) Ph. Eur. BRP Batch 1" [5] at the 108th session of the Ph. Eur. Commission in November 2000.

6. ACKNOWLEDGMENTS

The organisers express their sincere thanks to the project leader, Dr. C. Hendriksen, RIVM (NL) and to all participants for their contributions to this study. The study was run by the EDQM with the support of the Council of Europe and of the European Community in the framework of the Biological Standardisation Programme (internal project number BSP045) with Dr. Marie-Emmanuelle Esposito-Farese acting as project monitor.

7. REFERENCES

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