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ECVAM European Centre for the Validation of Alternative Methods

STATEMENT ON THE APPLICATION OF THE ELISA PROCEDURE FOR BATCH POTENCY TESTING OF TETANUS VACCINES FOR HUMAN USE

At its 15th meeting, held on 5-6 December 2000 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)¹ unanimously endorsed the following statement:

The results obtained with the ELISA procedure in the international validation study on alternative methods for batch potency testing of tetanus toxoid vaccines for human use were reproducible, both within and among the participating laboratories that performed the tests. The ELISA procedure proved applicable to testing a diverse group of tetanus toxoid vaccines for human use, of different potencies, composition and combinations. The concordances between the potencies derived from the *in vitro* serological (ELISA) data and from the *in vivo* data were very good. The ELISA was able to distinguish between highly potent and less potent vaccines. The Committee therefore agrees with the conclusion from this formal validation study that the ELISA procedure is scientifically validated for use as a replacement for the challenge procedure in the batch potency testing of tetanus toxoid vaccines for human use, and that it is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team.²

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT³/ERGATT³ workshop held in 1990,⁴ guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,⁵ criteria laid down by ECVAM and the ECB,^{3,6} criteria recommended at an OECD³ workshop held in 1996,⁷ the US ICCVAM³ report on validation and regulatory acceptance⁸, the ICH^{3,9,10} guidelines and WHO^{3,11} guidance on the validation of analytical test methods, and the recommendations of ECVAM/AGAATI³ workshop held in 1997.¹²

The outcome of a collaborative study on the use of the ELISA procedure for the batch potency testing of tetanus vaccines for veterinary use was published in 1995,¹³ and its regulatory acceptance is *in progress*.¹⁴

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1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 5-6 December 2000:

Dr B Blaauboer (ERGATT)	Mr M Balls (ECVAM – Chairman)
Professor J Castell (Spain)	Ms B Lucaroni (DG RTD)
Dr D Clark (UK)	Mr L Nørgaard (DG ENTR)
Dr B Garthoff (EFPIA)	Mr J Riego Sintes (ECB)
Professor A Guillouzo (France)	Mr E Sabbioni (ECVAM)
Professor C Hendriksen (The Netherlands)	Mr F Mc Sweeney (IHCP)
Professor G Koptopoulos (Greece)	Mr G Willmott (DG ENV)
Professor V Rogiers (Belgium)	Mr A Worth (ECVAM)
Dr B Rusche (EUROGROUP for Animal Welfare)	
Dr O de Silva (COLIPA)	
Professor H Spielmann (Germany)	
Professor O Svendsen (Denmark)	
Professor H Tritthart (Austria)	
Dr M Viluksela (Finland)	
Professor E Walum (Sweden)	
Dr F Zucco (EUROGROUP for Animal Welfare)	

2. Final report (contract No 11274-95-10F1ED ISP NL): *Alternative methods to replace, reduce and/or refine the use of laboratory animals in vaccine production, quality control and assessment. Subproject I: The validation of a serological approach as an alternative to the lethal or paralytic challenge procedure in the potency testing of tetanus toxoid vaccines for human application.*
3. AGAATI: Advisory Group on Alternatives to Animal Testing in Immunobiologicals; CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; CPMP: ECB: European Chemicals Bureau, Ispra, Italy; ICH: International Committee of Harmonisation; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; ICH: International Committee of Harmonisation; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations, New York, USA; WHO: World Health Organization, Geneva, Switzerland.
4. Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Roberfroid M, Rosenkranz H, Schmid B, Spielmann H, Stamatii AL & Walum E (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* **18**: 303-337.
5. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* **23**: 129-147.
6. Balls M & Karcher W (1995) The validation of alternative test methods. *ATLA* **23**: 884-886.
7. OECD (1996) Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. 60pp. Paris: OECD.

8. NIEHS (1997) Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. 105pp. Research Triangle Park, NC: NIEHS.
9. EMEA (1995) CPMP/ICH/281/95: Note for Guidance on Validation of Analytical Procedures: Methodology (CPMP adopted December 96).
10. EMEA (1995) CPMP/ICH/381/95: Note for Guidance on Validation of Analytical Methods: Definitions and Terminology (CPMP adopted Nov. 94)
11. WHO (1997) WHO/VSQ/97.02 A WHO guide to good manufacturing practice requirements. Chapter 15. Validation of analytical assays. pp. 65-69.
12. Hendriksen CFM, Spieser J-M, Akkermans A, Balls M, Bruckner L, Cussler K, Daas A, Descamps J, Dobbelaer R, Fentem JH, Halder, M, van der Kamp M, Lucken R, Milstien J, Sesardic D, Straughan D, Valadares A (1998) Validation of alternative methods for the potency testing of vaccines. The report and recommendations of ECVAM Workshop 31. *ATLA* **26**, 747-761.
13. Hendriksen CFM, Woltjes J, Akkermans AM, van der Gun JW, Marsman FR, Verschure MH & Veldman K (1994) Interlaboratory validation of *in vitro* serological assay systems to assess the potency of tetanus toxoid in vaccines for veterinary use. *Biologicals* **22**, 257-268.
14. Council of Europe (1996) Tetanus vaccine for veterinary use. *Pharmeuropa* **8**, 238-240.

General information about the validation of serological methods for the potency testing of tetanus toxoid vaccines for human use:

- A. The study was a joint activity of ECVAM and the European Directorate for the Quality of Medicines (EDQM; European Pharmacopoeia, Council of Europe, Strasbourg, France). Members of the Management Team (MT) were: Dr Coenraad Hendriksen (coordinator; RIVM, Bilthoven, The Netherlands), Dr Guy Rautman (EDQM), Dr Thea Sesardic (National Institute for Biological Standards and Control [NIBSC], London, UK), Dr Jean-Marc Spieser (EDQM), and Dr Randi Winsness (co-coordinator; Statens Legmiddelkontroll [SL], Oslo, Norway). Two biostatisticians, Dr Arnold Daas (EDQM) and Arnoud Akkermans (RIVM), attended the MT meetings. The study was jointly funded by ECVAM, under the terms of a contract with RIVM, and by EDQM within the framework of the second Biological Standardisation Programme of the European Pharmacopoeia and the EU. In addition to RIVM, EDQM, NIBSC and SL, three vaccine manufacturers participated in the study: Chiron-Behring AG (Marburg, Germany), Pasteur-Merieux Connaught (Val de Reuil, France) and Pasteur-Merieux Connaught (Marcy l'Etoile, France).
- B. This study began in 1996. The main objectives were to replace: a) the toxin challenge of guinea pigs with *in vitro* estimation of serum antibodies to tetanus toxin; b) the multi-dilution (quantitative) test with a single-dilution (qualitative) test; and c) to use guinea pigs instead of mice for the immunisation with tetanus toxoid vaccine. The serological tests selected for inclusion in the validation study were the ToBI test and an ELISA procedure.

The study was divided into three phases. Phases 1 and 2 focused on the relevance of the ELISA and ToBI test for tetanus vaccine potency testing and on intra-laboratory variation. In Phase 3, inter-laboratory variation of the ELISA and ToBI test were studied.

The parameters that were evaluated in Phase 1 and 2 included: a) estimates of vaccine potency obtained with the challenge test and the serological tests; b) the correlation between antibody concentration of individual animals and protection after challenge; c) the correlation between antibody concentrations of pooled and individual serum samples obtained with the ELISA and the ToBI test and antitoxin concentration obtained with *in vivo* toxin neutralisation test (TNT; three laboratories), and d) the intra- and (e) the inter-laboratory variation in the ELISA procedure and ToBI test.

Study design: a) immunisation and challenge of the guinea pigs were carried out according to the protocol agreed prior to the study: animals were immunised on day 0 and challenged with tetanus toxin on day 44; b) blood samples of each individual guinea pig were taken 42 days after immunisation; their tetanus antibody levels were estimated with the ToBI test and the ELISA (for both, in three independent test runs); c) in three laboratories, the tetanus antibody levels of pooled serum samples were estimated with the TNT in mice.

- C. The vaccine samples were coded and distributed by EDQM. At the beginning of the study a set of five combined tetanus vaccines was available which originated from different manufacturers and had been calibrated against the *Ph. Eur.* BRP for Tetanus Vaccine (adsorbed). The potency of these five vaccines was estimated in six laboratories with the guinea pig challenge test, the ELISA procedure and the ToBI test. At a later stage of the study, a tetanus vaccine of borderline quality and a new combined tetanus vaccine became available; they were tested in two laboratories with the guinea pig challenge test, the ELISA procedure and the ToBI test.

One laboratory produced non-valid results (both in the challenge test and in the serological tests), and one laboratory provided only valid data for the serological tests. The data were analysed at the RIVM and at EDQM.

- D. The ELISA procedure estimates *in vitro* the quantity of tetanus antibodies in the serum of immunised guinea pigs. Serial dilutions of the test serum and a standard serum (produced by RIVM prior to this study) are incubated on tetanus toxoid-coated microtiter plates. After addition of a peroxidase conjugated goat-guinea pig IgG, the amount of tetanus antibody bound to the tetanus toxoid is visualised by adding a substrate. The antibody titre is estimated by comparing the dose-response curves of the test and standard serum. The results of a validation study carried out in 1992-1993 showed that the ELISA and the ToBI test are suitable methods for replacement of the *in vivo* TNT test in mice, which is performed for the potency testing of tetanus vaccine for veterinary use (1, 2).
- E. In Phase 3, inter-laboratory variation was assessed for the ELISA and the ToBI test. Twenty-eight serum samples, obtained after immunisation with various dilutions of several types of tetanus toxoid vaccines, were titrated in duplicate in 23 laboratories worldwide.

Results:

- a) Within each laboratory, vaccine potencies obtained with the challenge test were in agreement with potencies obtained with the ELISA procedure, also for the borderline product. The confidence intervals (95%) of potencies estimated with the ELISA procedure were generally smaller than those estimated with challenge test.
- b) The results indicated a very good correlation between the antibody concentration assessed by ELISA procedure and death/survival after the challenge (predictive range: 91 - 95%, for six laboratories).
- c) An overall good correlation between the TNT in mice and the ToBI test ($r = 0.96$, range 0.925 - 0.986, for three laboratories) was also seen for serum pools of the guinea pigs injected with equal vaccine doses.
- d) The correlation between ELISA and ToBI test was $r = 0.92$ (range 0.88 - 0.97), for seven laboratories). The slope of line of agreement between ELISA and ToBI test was below 1 for all laboratories, indicating that there is no 1 to 1 relationship over the whole range of titres measured. This was particularly noticeable for titres below 0.0025 IU/ml. Antibody concentrations determined by ELISA and ToBI were generally in the same range.
- e) Information on intra-laboratory variation was based on assessment of the test repeatability (relative standard deviations [RSD] of antibody concentrations) and on assessment of the distribution of intra-laboratory precision (relative width of confidence interval from individual triplicate assays). In general, RSD and precision were within 20%-50% and are considered to be acceptable.
- f) Potencies obtained between the laboratories sometimes differed substantially, both in the challenge test and in the serological tests. This might be related to the guinea pig strains used.
- g) Information on inter-laboratory variation was based on assessment of relative standard deviations. Inter-laboratory standard deviations were considered to be acceptable for ELISA (on average 0.14), and generally did not exceed 0.50.

- h) The correlation between the ELISA procedure and the ToBI test in the Phase 3 study was 0.9. The ELISA/ToBI test ratio deviated from 1, indicating that there is no one-to-one relationship over the whole range, a finding similar to the Phase 1 and 2 studies. However, this ratio divergence is not believed to be relevant, as it did not interfere with correlations as presented in a), b) and c).
- F. In order for the ELISA procedure to be considered for use for legislative and other purposes, measures will be taken to press for the updating of *Directive 89/342/EEC*, *Directive 90/677/EEC*, and the European Pharmacopoeia monograph on Tetanus Vaccine (absorbed) (1997: 0452).
- G. A statement on the scientific validity of the ToBI test for batch potency testing of tetanus vaccines for human use was also endorsed by the ESAC on 5-6 December 2000.

References:

1. Hendriksen CFM, Woltjes J, Akkermans AM, van der Gun JW, Marsman FR, Verschure MH & Veldman K (1994) Interlaboratory validation of *in vitro* serological assay systems to assess the potency of tetanus toxoid in vaccines for veterinary use. *Biologicals* **22**, 257-268.
2. Council of Europe (1996) Tetanus vaccine for veterinary use. *Pharmeuropa* **8**, 238-240.