

The Potential of Physicochemical and Immunochemical Assays to Replace Animal Tests in the Quality Control of Toxoid Vaccines

The Report and Recommendations of ECVAM Workshop 61^a

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Preface

This is the 61st report of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). The main goal of ECVAM, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which have scientific relevance and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which groups of invited experts would review the current status of non animal-based tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on the potential of physicochemical and immunochemical assays to replace animal

tests in the quality control of toxoid vaccines was held at ECVAM on 14–15 April 2005, under the co-chairmanship of Coenraad Hendriksen and Gideon Kersten. The objectives of the workshop were: a) to review the state-of-the-art of physicochemical, biochemical and immunochemical tests for the quality control of diphtheria and tetanus toxoid vaccines; and b) to identify possibilities for and limitations to their use in the regulatory framework, i.e. for replacing the use of *in vivo* tests. The outcome of the discussions and the recommendations of the workshop participants are summarised in this report.

Introduction

Reasonable progress has already been made in the past, toward reducing and refining several animal tests for quality control of human toxoid vaccines (2). A great deal of effort has been exerted by the European Directorate for the Quality of Medicines (EDQM, Council of Europe, Strasbourg, France) and ECVAM, to validate serological assays incorporating the principles of reduction and refinement,

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for the potency testing of diphtheria and tetanus vaccines. Nevertheless, the quality control of toxoid vaccines still requires the use of large numbers of animals (Figure 1).

It is the primary concern of the vaccine industry and the regulatory authorities to produce and release vaccines of a consistently high quality. For the release of a vaccine lot, control tests have to be performed during production and on the final product. Nevertheless, manufacturers, regulatory authorities and ethical committees wish to minimise animal use, because of a number of serious drawbacks, including cost, imprecision and ethical concerns (3). The numerous disadvantages of the *in vivo* tests justify the quest for alternative methods. During the last decade, the concept of quality control testing of biologicals has been changing from final lot testing toward monitoring consistency in production. Various authorities, including the EDQM, the US Food and Drug Administration (FDA) and the World Health Organisation (WHO), recommend the use of analytical chemical tests for in-process controls to assess the consistency of production (4, 5). The WHO recommendations, for example, state that “there is a need to support the data generated by a simple potency assay with physical/chemical methods, in order to ensure overall consistency in production” (5). Monitoring consistency in production is a prerequisite for guaranteeing a high quality product.

Physicochemical and immunochemical tests are the ultimate tools for demonstrating consistency of production: these test methods can be used to study many properties of the antigen, including identity, size, structure, purity, amino acid modifications, and antigenicity (2, 6). A combination of several non-related techniques can provide a fingerprint of the antigen, which can be compared with a reference preparation of proven potency or with previous batches. The resulting fingerprint can demonstrate that a vaccine lot is essentially the same as previous production lots, indicating that the production process is robust and consistent.

Currently, physicochemical and immunochemical tests are not used for quality control before the release of toxoid vaccines. This is due to the fact that toxoid vaccines are among the least well-defined of the available biologicals. Formaldehyde inactivation results in a heterogeneous product. In contrast to the classical toxoid vaccines for some new-developed vaccines, such as Haemophilus type b vaccines, release is based on the results of physicochemical and immunochemical tests. Control tests for the Haemophilus type b vaccine have been performed on intermediate products at all stages of manufacture.

As a result of new developments, a large panel of analytical techniques which are possibly suitable for the quality control of toxoid vaccines, have become available.

Toxoid Vaccines: Structure and Composition

Toxoid is a collective name for chemically inactivated toxin antigens. Several toxoid vaccines have been developed, such as diphtheria, tetanus, botulinum and other clostridial vaccines. The first toxoid vaccine to be developed was diphtheria toxoid. In the 1920s, Glenny and Hopkins in England, and Ramon in France, successfully developed a method to prepare a vaccine from diphtheria toxin (7, 8). Although the production process differs from manufacturer to manufacturer, current diphtheria vaccine production is essentially based on their method. Important production steps are the inactivation of the toxin by formaldehyde and the adsorption of the toxoid to a colloidal aluminium salt. Differences in production involve growth media, purification before and/or after inactivation, preservatives, adjuvants, and combination with other vaccines in the final formulation. The production processes of many other toxoid vaccines are basically similar to that of diphtheria vaccine.

The treatment with formaldehyde has a large effect on the structure of the antigen, and affects the toxicity, antigenicity, immunogenicity and stability of the protein. The formaldehyde reaction yields intramolecular cross-links, which stabilise the protein structure, but also causes the loss of some epitopes. In general, the toxoids remain very immunogenic, and induce a protective immune response. Six different types of amino acid residues and the N-terminal amino acid of a protein are reactive to formaldehyde (9). The result of the inactivation is a very heterogeneous product with many modified amino acid residues. Although the chemistry is rather well known, it remains very difficult and laborious to identify all the modified residues in toxoid molecules.

In the final vaccine formulation, toxoid vaccines for human use are predominantly adsorbed with aluminium hydroxide or aluminium phosphate. The adsorption has a strong adjuvant effect on the immune reaction (10–12), but makes the physicochemical and immunochemical characterisation of the adsorbed toxoids difficult, although not impossible (13, 14).

The identification of critical factors for potency of toxoid vaccines

The type of potency test used for diphtheria and tetanus toxoid vaccines varies between countries. Since 2005 and 2007, respectively, the European Pharmacopoeia (Ph. Eur.) has recommended the use of serological methods for the potency testing of tetanus and diphtheria vaccines, although the immunisation challenge assays are still included in the general texts (15, 16). Many countries outside

Europe probably use the immunisation challenge assays described in the WHO report series (5). They are assumed to be the 'gold standards', ensuring the clinical performance of the toxoid vaccines. For diphtheria and tetanus vaccines, the potency estimates are substantially above the minimum protective levels, and only a few problems are encountered with respect to a lower potency of diphtheria toxoid as a result of the interference of multiple antigens (17). Interestingly, clinical trials and post-marketing surveillance of these products show that the vaccines are fully protective in humans.

There are large differences in the quality control testing of well-characterised new vaccines and of the older toxoid vaccines. These differences can be explained on a historical basis: supportive data obtained by immunochemical and physicochemical tests were generated during the development of new vaccines. No animal tests are needed for batch release of well-characterised products, e.g. polysaccharide vaccines. Immunochemical and physicochemical tests have proved to be valid for the demonstration of lot-to-lot consistency. Animal potency assays have been shown to be unnecessary, if comparability is shown for a new vaccine lot and a 'gold standard' with a proven efficacy and safety in (pre)clinical trials. On the other hand, toxoid vaccines require a relatively high number of animals for the quality control of final lots. These vaccines are poorly characterised, immunochemically and physicochemically, whereas a lot of data have been collected over the years and much experience has been acquired via the traditional potency assays.

Several factors might influence the potency of a toxoid vaccine, including the detoxification process, the presence of impurities, the adsorption with aluminium salts, and the addition of other vaccine components. Firstly, the concentration of formaldehyde used for detoxification may have a substantial effect on the immunogenicity of toxoids, as has been shown for diphtheria and pertussis toxoids (18, 19). Secondly, the presence of impurities, e.g. medium components and cell debris, in the toxoid vaccine can also change the potency of the vaccine (Albrecht Zott, personal communication). However, the role of such impurities has never been fully investigated. Thirdly, adjuvants improve the potency of toxoid vaccines, but which characteristics of the adjuvant, e.g. degree of adsorption, immunogenic properties of the adjuvant, etc, exert the greatest influence, is still not known. Finally, the presence of other antigens can change the potency of the toxoid component. They might interfere with the adsorption or affect the immune response. For instance, the presence of whole-cell pertussis vaccine increases the immunogenicity of diphtheria and tetanus toxoids, whereas, the addition of Hib conjugate decreases their immunogenicity in humans.

Animal-based potency assays are recommended for toxoid vaccines in pharmacopoeial monographs, as well as in WHO guidelines. A panel of physicochemical and immunochemical tests could act as an alternative for the potency assay, if bridging studies demonstrated that the tests could discriminate between potent and inferior products. The minimum requirement is that the physicochemical and immunochemical tests ensure that the potency is above the acceptable lower limit. The same holds true for the current, one-dose potency assays. The ability of the test panel to show relevant potency differences has to be established in a series of artificially changed bulk vaccines. This can only be achieved, if the gaps in our knowledge are filled (see *Development and validation of physicochemical and immunochemical methods*, below).

The Safety of Toxoid Vaccines

Safety is one of the most important requirements for all vaccines. In the case of toxoid vaccines, ensuring the absence of active toxin is therefore paramount, and as a result, safety testing is generally considered to be more important than potency testing. Vaccines are usually given to healthy children and older people. The recipient will not necessarily discern the beneficial effects of the vaccines, but any side-effects, e.g. fever or pain, will be noticed. The Ph. Eur. recommends three different tests for determining the safety of toxoid vaccines: specific toxicity, absence of toxicity, and irreversibility of toxoid. Since 2001, the tests for absence of toxicity and irreversibility of toxoid have been combined in a single assay, whereas the test for specific toxicity is no longer required for lot release, but only during production (20, 21). These changes have resulted in a reduction of animal use for the safety testing of toxoid vaccines.

A very sensitive *in vitro* Vero cell assay for determining the presence of diphtheria toxin has now been developed and validated, and is described in the Ph. Eur. Monograph under *Absence of toxin and irreversibility of toxoid* (20). This *in vitro* assay is also more sensitive than the previous animal model, but it cannot be used for testing final vaccine lots with adsorbed diphtheria toxoids. No similar test is available for tetanus toxin. However, an ELISA to detect tetanus toxin-specific induced cleavage of synaptobrevin has recently been described (22). The detection limit of this assay and its precision is better than those of the animal model. This assay system appears to be very promising, but will require further validation and collaborative studies, in order for its applicability to be established for a wide range of tetanus toxoid-containing vaccines. Enzymatic assays have also been described for the detection of specific toxicities associated with pertussis and botulinum vaccines (23, 24).

Physicochemical and Immunochemical Techniques for the Characterisation of Toxoid Vaccines

A large variety of analytical techniques are available for the characterisation of toxoid vaccines (13, 14, 19, 25–29). Commonly-used techniques for biologicals are listed in Table 1. The table indicates the capacity of the individual techniques to assess the identity, purity, stability and structure of the non-adsorbed toxoid. The possibility of analysing adsorbed toxoids is also indicated. Important considerations in the selection of a set of analytical techniques are that they should be complementary, robust, have high precision, and be inexpensive and, preferably, easy to perform. Suitability for the assessment of particular structural aspects of the toxoid has not yet been established for several of the techniques listed in Table 1 (indicated as 0, “possibly useful”). This is particularly true for adsorbed toxoids. Furthermore, there is a need to collect more data on *in vitro* functional tests, such as cytokine induction assays, and to establish their role in the characterisation of toxoid vaccines.

Several physicochemical and immunochemical techniques can be used for the quality control of toxoid vaccines (Table 1), but the choice of the technique depends mainly on the objectives: for in-process controls (demonstrating consistency of production) or for quality control before lot release. The next sections focus on the use of the techniques for these two purposes.

Techniques for in-process control

In-process controls of a vaccine are intended to monitor the intermediate production steps. Not only the active component, but also any impurities,

may be subject to analysis. Several suitable physicochemical and immunochemical tests are used today for in-process control of toxoid vaccines, including SDS-PAGE, size exclusion chromatography, colorimetric assays, flocculation test and ELISA (Table 1). With a suitable combination of tests, a new toxoid batch can be compared with a ‘gold standard’ (e.g. a reference toxoid) to demonstrate equivalence. Preferably, this ‘gold standard’ should have been tested, and shown to be acceptable, in a clinical study. The toxoid should meet the specified criteria to demonstrate consistency of production. The test results can be presented in a radar plot, with one axis for each assay (Figure 2). These types of plots have already been used to demonstrate proof of principle for non-adsorbed toxoid bulks (10, and Satish Ravetkar personal communication). If the results of one test are outside the specification, a deviation in the process is indicated, in which case additional (non-routine) assays can be performed, in order to permit an informed decision on whether the batch should be discarded. For each test, the criteria that the products have to meet must be determined, but these can be both product-specific and manufacturer-specific. However, this does not imply that there should be no intention to harmonise the test battery.

Techniques for quality control before lot release

The testing of final vaccine lots for release requires the use of large numbers of animals, compared with the testing of toxoids in bulk. If the overall number of animals used in toxoid vaccine control is to be reduced, reduction of animal use for testing the final lot is essential. However, in contrast to toxoid bulk concentrates, the application of physicochemical and immunochemical tests for the quality con-

Figure 1: Animal consumption for quality control of vaccines



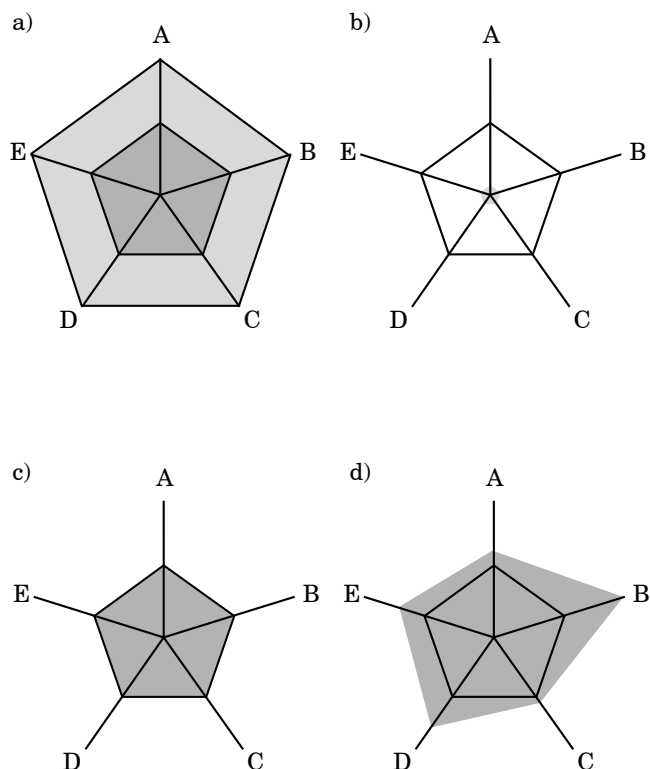
Currently, the highest proportion of animals are used for quality control of commercial lots. In the future, this will probably change to the highest proportion of animals being used for development lots (Omer Van Opstal, personal communication).

Table 1: Potential physicochemical and immunochemical techniques for quality control of toxoid vaccines

Technique	Information provided	Identity	Purity	Stability	Structure	Adsorbed	Easy to perform	Costs	Currently used
Biosensor	Antigen concentration; epitope integrity	+	0	+	(3°) ^b	0	—	—	+
Circular dichroism (CD)	Secondary and tertiary structure; extent of formaldehyde treatment; cross links; thermodynamics of toxoid unfolding	—	—	0	2°, 3°	0	+	—	0
Colorimetric assays (primary amino groups)	Extent of formaldehyde treatment	—	—	0	1°	0	+	+	+
2D-electrophoresis	Size; pI; protein modifications; degradation; aggregation; profile of protein impurities	(+) ^a	+	+	1°	0	0	+	+
Differential scanning calorimetry	Extent of formaldehyde treatment; cross links; thermodynamics of toxoid unfolding	—	—	+	3°	0	0	0	+
ELISA	Antigen concentration	+	0	+	(3°) ^b	+	0	+	+
Fluorescence	Tertiary structure; extent of formaldehyde treatment; cross links; thermodynamics of toxoid unfolding	—	—	0	3°	+	+	0	0
Flocculation (Kf and Lf)	Antigen concentration; extent of formaldehyde treatment	+	—	+	—	0	+	+	+
Immunoblotting	Size; protein modifications; degradation; aggregation	+	0	+	1°, 4°	0	+	+	+
Infrared spectroscopy	Excipients	—	0	—	—	0	—	—	—
Ion exchange chromatography	Protein modifications; degradation; aggregation; purity	0	+	0	1°	—	+	0	0
Isoelectric focussing	pI; protein modifications; degradation; aggregation; purity	0	+	+	1°	0	+	+	+
Light scattering (DLS/SLS)	Aggregation	—	—	+	4°	—	0	—	0
NMR	Excipients	—	0	—	—	0	—	—	—
Peptide mapping (CE)	Primary structure; protein degradation	0	0	+	1°	—	—	0	+
Peptide mapping (LC-MS)	Primary structure; protein modifications; degradation	+	0	+	1°	—	—	—	+
SDS-PAGE	Size; protein modifications; degradation; aggregation; purity	0	+	+	1°, 4°	0	+	+	+
Size exclusion chromatography	Hydrodynamic size; aggregation; purity; oligomeric repartition	0	+	+	1°, 4°	—	+	0	0
Synaptobrevin test	Specific toxicity of tetanus toxin	—	—	0	0	0	0	0	0

+ = yes; 0 = neutral/possibly; — = no/unfavourable. Abbreviations: CE = capillary electrophoresis; LC-MS = liquid chromatography; MS = mass spectrometry; CD = circular dichroism; NMR = nuclear magnetic resonance; DLS = dynamic light scattering; SLS = static light scattering; Kf = flocculation time; Lf = flocculation titre. ^aIn combination with mass spectrometry or N-terminal sequencing by Edman degradation. ^bIf monoclonal antibodies are used that recognise conformational epitopes.

Figure 2: Radar plot representation of the quality of a toxoid vaccine



Five physicochemical and/or immunochemical tests are indicated by letters A–E. Toxoids have a satisfactory quality when the grey area completely crosses the black borderline (adapted from 19).

a) = control; b) = toxin; c) = borderline toxin; d) = toxoid.

□ = satisfactory quality; ■ = insufficient quality.

trol of the final lot is more complicated, because of several factors. For example, the antigen concentrations in the final vaccine lot are normally low. Furthermore, the antigens are adsorbed onto aluminium adjuvants, often in the presence of several other antigens. Consequently, these issues may make some physicochemical and immunochemical testing impractical.

The adsorption onto aluminium salts can affect the immunogenicity and stability of the antigen. It is not clear from present knowledge, how adsorption affects the structure of the toxoids in the final lot. Adsorption has been shown to affect the pyrogenicity of vaccines, and may affect the reactogenicity to endotoxin in certain combination vaccines, either positively or negatively. An additional problem is variability in the degree of adsorption, which is affected by the composition of the product. Nevertheless, the importance of the degree of adsorption for the potency is still under discussion, as adsorbed antigen may de-adsorb

quickly *in vivo* (30). Thus, the final lot vaccine is quite different from the bulk toxoid vaccine, so the results of potency and safety tests with the bulk toxoid may not apply to the final lot.

The tests employed should provide information about the stability and the structure of the adsorbed toxoid and the quality of the adsorbent. The reproducible preparation of the colloidal aluminium salt and the adsorption process, while important, may not be sufficient for guaranteeing quality. For instance, changes in adsorption may occur over time in complex mixtures. There is a need to assess the effects of these changes on the immunogenicity of the toxoids. It would be useful to know what range of variability in the degree of adsorption can occur without affecting potency.

At present, no physicochemical and immunochemical tests can be used directly as surrogates for potency assays of toxoid vaccines. Nevertheless, recent publications have shown that these techniques can be used to characterise adsorbed antigens (13, 14). In the near future, physicochemical and immunochemical tests should be developed for demonstrating the structural integrity, stability and composition of adsorbed toxoids.

The Development and Validation of Physicochemical and Immunochemical Methods

Several physicochemical and immunochemical tests are currently available and could be used for quality control of toxoid vaccines (Table 1). Nevertheless, toxoid vaccines have not been fully characterised by applying these techniques. The following information gaps were identified:

- *Adsorption*: More information is needed on the structure and quality of the antigen after adsorption, and about the level of adsorption in the context of the potency (12). In combination vaccines, the effects of additional antigens on the toxoid–adjuvant complex should be studied.
- *Dilution*: In the final lot, the antigen concentration might be below the minimum level required for some of the physicochemical and immunochemical tests. Attempting to solve this problem by concentrating the product again could introduce anomalies in the results obtained.
- *Protective epitopes*: More knowledge is needed about the protective epitopes, and about how they may be modified during toxoid inactivation and formulation of the final vaccine.
- *Purity*: Since product impurities may affect the potency of the vaccine (Albrecht Zott, personal communication), more information about the

nature and immunomodulating effects of the impurities is needed.

- *Preservatives*: Preservatives are not used for the storage of toxoids. However, residual traces of the formaldehyde used to inactivate toxoids may act as a preservative. Preservatives are mainly used as bacteriostatic/bactericidal agents in multidosed formulations of the final vaccine (thiomersal, 2-phenoxyethanol). It should be noted that some of these compounds, such as thiomersal and formaldehyde, are not chemically inert and will react with protein (toxoid) side-chains.
- *Stability*: As a result of adsorption, the formation of antigen–adjuvant complexes might affect the stability of the vaccine (31). The stability of adsorbed toxoids has not been studied by using physicochemical and immunochemical tests.
- *Interaction with other vaccine antigens*: It is known that some vaccine antigens have immunomodulating effects on other antigens. It is likely that the existing physicochemical and immunochemical tests cannot determine this modulating effect.
- *Functionality*: The potential of a vaccine to induce an immune response will require the use of an *in vitro* functional test.

Studies aimed at filling these gaps should be initiated, preferably involving collaboration between the industry and the regulatory authorities. The complete characterisation of the toxoid vaccines will require funding from external organisations. The quantification and validation of several physicochemical and immunochemical tests should also be performed. This quantification and validation will require the production of artificial toxoids of inferior or borderline quality, and the analysis of these products with the new tests and the current standard methods. Such borderline products could be obtained by: a) producing toxoids with unusually large amounts of impurities; b) preparing toxoids under alternative detoxification conditions; or c) maltreating toxoids by using heat or chemicals. Furthermore, the quantification and validation of tests will require parallel analyses of routinely produced vaccines, which should be performed with both the new and the standard methods.

Conclusions and Recommendations

General aspects

1. Monitoring consistency of production is an accepted approach for the release of recently-

developed, well-defined vaccines. The consistent production of successive batches is monitored by the use of physicochemical and immunochemical tests. There is now sufficient reason to believe that this strategy is also feasible for the traditional toxoid vaccines.

2. The introduction of this approach for toxoid vaccines should be discussed with manufacturers and with the regulatory authorities. Agreed guidelines might be useful in promoting the application of this approach.
3. The currently-available physicochemical and immunochemical techniques have great potential for in-process testing and for consistency monitoring. However, further information and the development of additional methods will be needed for batch release testing.

Specific aspects

4. Toxoid vaccines are not very well characterised.
5. A panel of complementary tests which are easy to perform, should be selected. These tests should include physicochemical, immunochemical and functional assays, which together should provide information on different structural levels (primary, secondary, tertiary and quaternary), antigenicity, and functionality.
6. More data need to be collected on several analytical techniques, in order to fully establish their suitability.
7. The quantification and validation of the suitable physicochemical and immunochemical tests must be performed.
8. The introduction of physicochemical and immunochemical tests as alternatives to *in vivo* assays should be phased, as follows: phase 1 — validation of currently-available physicochemical and immunochemical tests for toxoids; phase 2 — parallel testing in which all vaccine batches are tested *in vitro* and *in vivo*; phase 3 — regulatory acceptance; and phase 4 — *in vitro* testing only.

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