Chapter 6
Analytical Control Strategy of Bacterial Subunit Vaccines

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6.1 Introduction

The success of vaccines and their positive impact on public health cannot be overstated. Vaccines have evolved through history and can be classified into two broad groups by composition, which generally relates to their chronology of discovery. Traditional vaccines are based on the administration of either live, attenuated organisms, or inactivated/killed organisms. Vaccines in this group include well-recognized products such as cowpox (for preventing smallpox), varicella (chickenpox), MMR (measles, mumps, rubella), IPV (polio), hepatitis A, and the seasonal flu (influenza) vaccines. These attenuated or killed whole microbe type of preparations helped to establish the early notion of immune prophylaxis, which gave rise to modern vaccinology beginning in the nineteenth century with the famous early studies of microbial pathogens, infectious diseases, and new medical treatments. New treatments appearing in this period included a second type of

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vaccine developed against epidemic bacterial microbes including cholera (caused by *Vibrio cholerae*), chicken cholera (*Pasteurella munticoda*), and anthrax (*Bacillus anthracis*). This second group of vaccines is based on the administration of inactive microbial components, which were originally classified as toxoid-type vaccines as they principally contained inactivated bacterial toxins. Their development coincides with the discoveries of serum-derived treatments (antitoxin; i.e., immune therapy) against the devastating childhood diseases of diphtheria and bacterial influenza. Today, the general heading of subunit vaccines includes products comprising purified protein antigen, sometimes covalently bound to microbe-specific carbohydrate, and nearly always including an adjuvant of some type to enhance immune activity. Products for immune therapy such as monoclonal antibodies have grown into the largest class of biological products, and are not generally used as prophylactic vaccines (however, see Chap. 17 for one exception which targets immune regulators called dendritic cells). This chapter concerns a few of the most prominent subunit and toxoid type vaccine products.

There are several types of subunit bacterial vaccines, and all employ extracted, purified, or engineered components from one or more pathogens. These are purified substances that range from secreted proteins, membrane proteins, outer vesicles membranes, capsular polysaccharides, or inactive toxin or toxin fragments (Levine 2004; Plotkin et al. 2008; Hilleman 2000). For toxoid-type vaccines, the immunogenic components are chemically or genetically modified so as to remove toxic activity (Galazka 1993). The recombinant subunit approach has become popular for toxoid and nontoxin antigens, wherein a gene of the target vaccine is transferred to a non-pathogenic expression organism, expressed, purified, and tested (Liljeqvist and Ståhl 1999a, b; Gentschev et al. 2001). Pertussis toxoid, produced by *Bordetella pertussis* with specific mutations in its toxin gene, is included as an active component of an acellular Pertussis (aP) vaccine (Del Giudice et al. 1998; Del Giudice and Rappuoli 1999). A recent and effective type of bacterial derived subunit vaccine is the surface protein from the outer membrane vesicles (OMV) of Gram-negative bacteria (Rinaudo et al. 2009; Rappuoli 2007). Production occurs via fermentation, either a recombinant expressed or purified subunit protein vaccine in a heterologous expression system (e.g., bacteria or yeast) or purified from large amounts of the pathogenic organism (Plotkin et al. 2008). In general, with bacterial derived protein vaccines both universal and strain specific, such as in Meningitides serogroup B vaccine design have been pursued (Rinaudo et al. 2009). Compared to traditional whole-cell preparations, subunit vaccines contain fewer antigens and simpler total composition. This well-defined and pure composition is intended to improve the safety profile, analytical testing, and regulatory review of a subunit vaccine as a new drug entity (Levine 2004). However, the loss of multiple antigens often compromises immunogenicity in scope and longevity. Consequently, subunit products require additional immune stimulation in the form of an adjuvant, such as aluminum salts (Newman and Powell 1995).

In this chapter, the analytical control strategy supporting bacterial subunit derived proteins is described using a few licensed and new products as examples. General descriptions and details of the critical assays are presented for the well-established licensed vaccines of Diphtheria, Tetanus, acellular Pertussis toxoid

vaccines (DTaP), and DTaP combination vaccine, plus more recent Cholera and Meningococcal B OMV products, as well as the existing and next generation subunit Anthrax vaccines.

6.2 Diphtheria, Tetanus, Acellular Pertussis Toxoid Vaccines, and DTaP Combination Vaccine

This section summarizes testing principles and strategy for diphtheria, tetanus, aP toxoid vaccines, and DTaP combination vaccine. DTP is the first combination vaccine used to immunize infants and children in 1948. Over the years, other vaccines were added to the combination and whole-cell pertussis antigen was replaced with less reactogenic aP antigens (Skibinski et al. 2011).

The testing of toxoid vaccines presents a challenge; chemical inactivation creates heterogeneity in the product and it is difficult to characterize biochemically, even using state-of-the-art techniques. Thus, the analytical strategy supporting the qualification of toxoid vaccines is generally composed of classical methods given their reliability of performance.

Analysis of diphtheria and tetanus vaccines includes testing of toxin and toxoid. Toxin tests include measurement of antigen content by limit of flocculation (Lf), toxicity of the purified toxin before inactivation (integrity), total protein by protein nitrogen, and purity based on antigen content and protein nitrogen. Toxoid tests include residual formaldehyde inactivation agent, residual process impurities (ammonium and sulfate), potency, specific toxicity to verify the completeness of inactivation and irreversibility to assure toxoid does not revert back to be toxic upon storage. Most of the methods are available in the European Pharmacopeia (EP), WHO technical reports and NIH guidance documents. In some instances, a comparison of these three references is provided. The aP vaccine described here is a single-component pertussis toxoid. Testing of pertussis toxin (PT) with respect to purity and biological activity includes SDS-PAGE, HPLC, Western blot, process-related impurities (blue dye and fetuin), hemagglutination, ADP-ribosylation, and Chinese Hamster Ovary (CHO) toxicity. Testing of unadsorbed pertussis toxoid includes measurement of residual hemagglutination, inactivation kinetics, CHO toxicity, and residual inactivation agent, peroxide. Testing of adsorbed toxoid includes histamine sensitization assay (HIST), reversion to toxicity, potency, and percent adsorption. Testing of DTaP combination includes percent adsorption, identity, specific toxicity, general safety, formaldehyde, aluminum, endotoxin, and thimerosal. Thimerosal as a preservative for human formulations was removed or reduced to trace amounts in all vaccines for children 6 years of age and younger (CDC 2012). However, the preponderance of available evidence has failed to demonstrate serious harm associated with thimerosal in vaccines (Orenstein et al. 2013).

The ongoing effort to establish international reference standards and to standardize assays for vaccine evaluation is also summarized.

6.2.1 Tetanus Toxoid Vaccine

Tetanus vaccine is used to prevent a deadly tetanus disease characterized by convulsive tonic contractions of voluntary muscles. The causative agent is a Gram-positive, spore-forming, strict anaerobic microorganism, *Clostridium tetani*. Tetanus toxin is an exotoxin with a heavy chain of about 100 kDa and a light chain of about 50 kDa linked by disulfide bonds. The most widely used chemical detoxification reagent is formaldehyde; the treatment results in intramolecular and intermolecular cross-linking (Wirz et al. 1990).

The production of tetanus toxoid vaccine involves the following steps:

1. Production of tetanus toxin by fermentation of *C. tetani.*
2. Purification of tetanus toxin; ammonium sulfate fractionation is the most common method.
3. Inactivation of toxin by formaldehyde treatment.

A list of testing for tetanus vaccine is outlined in Table 6.1.

Table 6.1 Testing for tetanus vaccine intermediate (toxin), bulk, final bulk, and final containers

Material	General tests	Product specific tests
Toxin	Bioburden	Antigen concentration by limit of flocculation (Lf/mL)[a]
		Toxicity of purified toxin (L+/10 or L+)
		Minimum lethal dose (MLD)
		Antigen purity (Lf/mg protein nitrogen)
Bulk purified toxoid	pH	Antigen concentration by Lf/mL[a]
	Endotoxin	Characterization (SDS-PAGE)
	Sterility	Antigen purity (Lf/mg protein nitrogen)
	Appearance	Process-related impurities (e.g., ammonium and sulfate)
		Residual inactivation agent (formaldehyde)
		Absence of toxin and irreversibility of toxoid
		Potency (tested as adsorbed toxoid)[a]
Adsorbed toxoid (final bulk and containers)	Aluminum	Potency[a]
	pH	Identity[a]
	Sterility	% Adsorption[a]
	Thimerosal (if applicable)	Specific toxicity
	General safety	

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b)

6.2.1.1 Antigen Content by Limit of Flocculation (Lf), Ramon Assay (Ph. Eur. 2.7.27)

The content of toxin or toxoid in a sample can be expressed as a flocculation value using the Ramon assay. In this assay, antitoxin is added in increasing concentrations to series of tubes containing a constant amount of toxin or toxoid. At the equivalence point of toxin/toxoid and antitoxin, flocculation occurs in one or more tubes. The first tube in which flocculation occurs is used to determine the Lf value of the sample.

Lf refers to the amount of toxin or toxoid that when reacted with 1 international unit of antitoxin gives a flocculation in the shortest period of time. The Lf value shall be determined by comparison with a reference material calibrated against the International Reference Reagent of Tetanus Toxoid for flocculation test or approved equivalent (WHO 1990).

6.2.1.2 Toxicity of Purified Tetanus Toxin (L+/10, L+ and MLD)

L+/10 is defined as the minimum amount of toxin which, when mixed with 0.1 IU of antitoxin kills an animal of a defined weight in 4 days. L+ is defined as the minimum amount of toxin which when combined with 1 IU of antitoxin kills an animal of a defined weight in 4 days (WHO 1965). MLD is the smallest amount of toxin that, when injected by a given route, causes death of the treated animals within a stated period of time (Wirz et al. 1990).

6.2.1.3 Antigen Purity (Lf/Mg Protein Nitrogen)

Purity (or specific activity) is expressed as Lf/mg protein nitrogen and WHO requirement is a minimum value of 1,000 (WHO 2014a).

Protein nitrogen is determined by the micro-Kjeldahl method. The proteins are first precipitated with 5 % trichloroacetic acid (WHO 1965) and subsequently, the precipitate is collected and washed. The Kjeldahl method starts with a sulfuric acid digestion to convert nitrogen in the sample to ammonium hydrogen sulfate. Upon alkaline addition, the liberating ammonium is distilled into HCl and titrated with NaOH (Ph. Eur. 2.5.9). Without precipitation, the micro-Kjeldahl determines the total nitrogen.

6.2.1.4 Characterization by SDS-PAGE

SDS-PAGE for tetanus toxoid can only be used qualitatively for purity assessment due to cross-linking to itself and to the impurities during formaldehyde inactivation. Figure 6.1 shows tetanus toxoid under reducing conditions. The dominant band on the gel is a tetanus toxoid monomer which migrated between 200 and 116 kDa

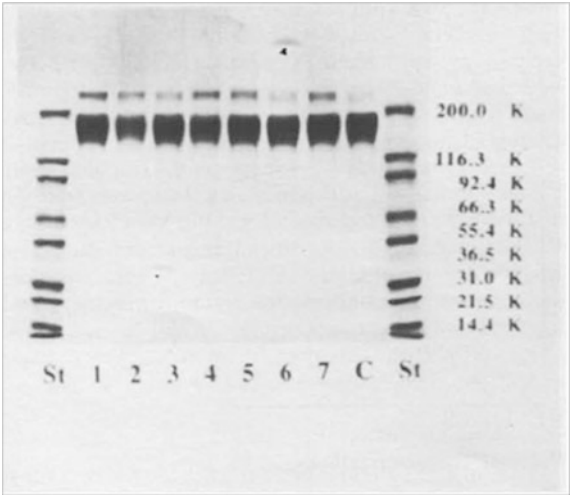


Fig. 6.1 SDS-PAGE analysis of tetanus toxoid samples under reducing conditions. Tetanus toxoid before (*lane 1*) and after treatment (*lanes 2–8*) under various experimental conditions for microencapsulation. Reproduced with permission from Elsevier (Alonso et al. 1994)

molecular weight standards. The reducing conditions did not separate the heavy and light chains because they have been cross-linked during formaldehyde treatment. The high molecular weight cross-linked molecules are also visible in most of the preparations.

6.2.1.5 Process-Related Impurity: Ammonium and Sulfate

Ammonium forms colored complex with basic tetraiodine mercurate. A limit assay is established by comparing color intensity of test sample with 1 ppm ammonium standard after complex formation (Ph. Eur. 2.4.1 Method A).

Sulfate is precipitated with barium chloride in acid. After precipitation, the opalescence of test sample and 10 ppm of sulfate standard (potassium sulfate) is compared (Ph. Eur. 2.4.13).

6.2.1.6 Absence of Tetanus Toxin and Irreversibility of Toxoid

The bulk-purified TT should be tested to ensure that reversion to toxicity cannot take place on storage. The purified bulk toxoid should be diluted in order to obtain the same concentration as that present in the final bulk except the presence of adjuvant. Divide the dilution in two equal parts. Keep one part at 2–8 °C and the other at 37 °C for 6 weeks. Both dilutions are tested in the same way. A total of 15 guinea pigs, each weighing 250–350 gm and that have not previously been treated with any material that will interfere with the test, is used in the test. Five milli Litre of each dilution is injected subcutaneously into two groups of five guinea pigs. The additional five guinea pigs should receive subcutaneously at least 500 Lf of the

Table 6.2 Comparison of three guidelines for the absence of tetanus toxin and irreversibility of toxoid

	Ph. Eur. 0452	WHO (2014a)	US (NIH 1952)
Irreversibility of toxoid	Toxoid tested at the same concentration as final bulk (e.g., 12 Lf/mL) and kept at 37 and 5 °C for 6 weeks. Each of 5 guinea pigs receives 5 mL of toxoid. Observe for 3 weeks	Section A 3.4.5: For bulk purified toxoid, similar to EP	Not described
Absence of toxin	Guinea pigs are injected with 1 mL of 500 Lf of toxoid and observed for 21 days for sign of tetanus intoxication	Section A 3.4.4: Bulk purified toxoid follows the same procedure as Ph. Eur.	Section 2.2: Inject subcutaneously into at least 4 guinea pigs weighing 300–400 gram ≥5 single human doses (≥2.0 mL) produced no symptoms of tetanus toxin poisoning for 21 days

nonincubated bulk purified toxoid in a volume of 1 mL (to test for the absence of toxin). The bulk-purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than one animal dies from nonspecific causes, the test may be repeated; if more than one animal dies in the second test, the toxoid does not comply with the test (Ph. Eur. 0452). The procedure varies slightly in different guidelines; however, they follow the same general principles (WHO 2014a; NIH 1952). A comparison is provided in Table 6.2.

6.2.1.7 Specific Toxicity

This method is included in Ph. Eur. 0452 under production, general provisions, specific toxicity for adsorbed tetanus toxoid vaccine. It is also cited in Section A 3.5.2.5 of WHO document (2014a) for final bulk. Inject subcutaneously at least five single human doses (SHDs) to each of the five guinea pigs and observe 21 days for paralysis. The specific toxicity test for final bulk may be omitted once consistency of manufacturing is established (WHO 2014a).

6.2.1.8 Potency for Adsorbed Tetanus Toxoid

Potency is determined by administration of the vaccine to animals (guinea pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of antitoxin in the serum of the guinea pigs (method C). Methods

A or B are used during development and whenever revalidation is needed following significant manufacturing process changes. Method C is used in the interest of animal welfare. The potency of the vaccine should be determined by comparison with an appropriate reference material calibrated against the International Standard for tetanus toxoid adsorbed (Ph. Eur. 2.7.8).

The WHO International Standard preparation of tetanus toxoid adsorbed has been defined in IU based on the results obtained in guinea pig challenge assays. The 4th International Standard for Tetanus Toxoid adsorbed was established in October 2010 to have a unitage of 490 IU/ampoule (Tierney et al. 2011).

Method A: Challenge test in guinea pigs

For each assay, three groups of animals (sufficient number of animals to obtain a valid assay) are immunized subcutaneously with three twofold to 2.5-fold dilutions of the vaccine under test and three groups are given three analogous dilutions of the standard. The dilutions were made in saline. After 28 days, animals are given a paralytic or lethal challenge with 1 mL of toxin containing 50 LD_{50} per animal. Dilutions of both preparations must be such that the intermediate dose protects about 50 % of the animals. The number of guinea pigs without paralysis should be counted 5 days after injection of the challenge toxin. Results of the assay are then statistically analyzed for determination of vaccine potency.

Method B: Challenge test in mice.

Method B is very similar to method A, except 5-week-old mice are used, the immunization dose is 0.5 mL and the challenge dose is 50 LD_{50} in 0.5 mL, given after 28 days. The number of mice without paralysis is counted 4 days after challenge.

Method C: Determination of antibodies in guinea pigs

One mL of the vaccine or reference standard dilutions are injected subcutaneously to each guinea pig, a blood sample is obtained 35–42 days later, and the relative antibody titer is determined by (1) Enzyme-linked immunosorbent assay (ELISA) or (2) toxin-binding inhibition assay.

Method C (1) ELISA plates are coated with tetanus toxoid and peroxidase–conjugated rabbit or goat antibody against guinea pig IgG is used in detection.

Method C (2) For toxin–binding inhibition assay, tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. The unbound toxin or toxoid is determined by ELISA with plates coated with antitoxin and peroxidase-conjugated equine anti-tetanus IgG is used for detection.

All the three above methods are multi-dilution assays, which should be used to establish production consistency, product shelf life, and calibrate reference preparation. Potency for lot release following licensing can be simplified as a one-dilution assay. Multiple-dilution assay is carried out only periodically, or when manufacturing changes occur with the need to justify to the regulatory authorities.

The single dilution assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (SHD), that elicits a minimal protective effect and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine, the potency of the test vaccine is satisfactory (WHO 2014a).

The approach taken by the USA is described in the "Minimum Requirements," often referred to as the NIH method (NIH 1952). Minimal acceptable potency in these documents is defined as the capacity of a test dose of vaccine to induce an antibody response that reaches or surpasses the threshold of 2 units/mL. A suitable reference antitoxin, to which "units/mL" have been assigned, is used to control the activity of the test toxin, which is used to measure the neutralizing activity of the sera induced in guinea pigs, in an in vivo assay. The US potency assay development history was reviewed, together with an overview of the possibility of using alternative, nonanimal methods to measure neutralization in the assay. Toxin neutralization in vivo is correlated with biological protection, but antigen antibody binding by ELISA in vitro could not distinguish between neutralizing antibody and nonneutralizing antibody (Keller 2011). The EP, WHO, and US methods are briefly summarized in Table 6.3. For routine lot release, validated serological assays are being used that offer significant advantages in terms of reduction in animal numbers (Stickings et al. 2011). The test methods are generally proposed by the manufacturer and evaluated by the regulatory authorities; there are no universally accepted methods.

Table 6.3 Summary of potency assay for adsorbed tetanus vaccine from different guidelines

EP (Ph. Eur. 2.7.8)	WHO (2013b, 2014a)	USA (NIH 1952)
Immunize groups of guinea pigs or mice with test and reference vaccines. After 28 days, challenge animals with tetanus toxin and observe tetanus paralysis for 5 days (guinea pigs) or 4 days (mice). Alternatively, at 35–42 days, test sera by ELISA or toxin binding inhibition assay. Compare results with reference standard calibrated in international units	Same as Ph. Eur.	Immunize four or more guinea pigs with 1 SHD of vaccine and collect the immune serum 6 weeks later. A defined dilution of immune serum pool is combined with a standardized quantity of tetanus toxin and then injected into test animals. Protection of test animals, which equates to toxoid potency, is determined using two guinea pigs or three mice. The two control guinea pigs injected with toxin must die in 4 days, whereas the test animals should survive for 96 h or more
	The potency of tetanus vaccine used for the immunization of children should be ≥40 IU/single human dose	Minimum requirement is ≥2 antitoxin units/mL

6.2.2 Diphtheria Toxoid (DT)

Diphtheria is an acute, often fatal bacterial disease caused by toxigenic strains of *Corynebacterium diphtheriae*. Diphtheria toxin is the major virulence factor of these microorganisms and the clinical manifestations of the disease are due mainly to the presence of circulating toxin in the bloodstream of the infected individuals. Active immunization against diphtheria is based on the use of formaldehyde-detoxified preparation of diphtheria toxoid (DT). The production of Diphtheria vaccine utilizes similar methods as those described in Sect. 6.2.1.

Diphtheria toxin is secreted by *C. diphtheriae* as a single polypeptide proenzyme of 58,342 Da, but purified preparations of toxin frequently contain a heterogeneous array of molecular species. In most preparations, a fraction of the toxin has been cleaved by contaminating trypsin-like proteases, yielding a nicked form containing two fragments: A (21.2 kDa) and B (37.2 kDa), held together by a disulfide bridge. It is the nicked form that is believed to be responsible for the biological effects of diphtheria toxin. Fragment A is a potent ADP-ribosylating enzyme and fragment B recognizes mammalian cell surface receptors. Formaldehyde detoxification cross-links the two fragments and stabilizes the molecule (Carroll et al. 1988; Pappenheimer 1984). Diphtheria toxin has the capacity to block protein synthesis in cultured mammalian cells, thus causing cell death. This capacity was utilized for an in vitro neutralization assay to determine antibody titer using Vero cells. Table 6.4 outlines the testing strategy for diphtheria vaccine.

6.2.2.1 SDS-PAGE of Diphtheria Toxin and Toxoid

Diphtheria toxin and 10 experimental toxoids were analyzed on a 10 % reducing SDS-PAGE (Fig. 6.2). The diphtheria toxin used in this study was almost completely nicked. The bands of the A and B fragments appeared at higher apparent masses (27 and 43 kDa, respectively). Higher formaldehyde concentrations increased the intensity of the 58-kDa toxoid band by cross-linking of the A and B fragments and broadened the band. In the meantime, formaldehyde shifted the A and B fragments to lower molecular weight species (Metz et al. 2003)

6.2.2.2 Antigen Content by Limit of Flocculation (Lf)

The quantitation is based on flocculation of toxoid and antitoxin at the equivalence point. The test is carried out with antiserum calibrated against the WHO reference reagent of DT for flocculation test. The Lf dose of toxin is equivalent to about 2.5 μg protein (Pappenheimer 1984).

Table 6.4 Summary of testing for diphtheria vaccine including toxin intermediate and final product

	General test	Product specific test
Toxin	Bioburden	Antigen concentration by Lf (Lf/mL)[a]
		Toxicity (L+/10 or L+)
		Minimal lethal dose (MLD)
		Antigen purity (Lf/mg protein nitrogen)
		SDS-PAGE
Bulk purified toxoid	pH	SDS-PAGE
	Endotoxin	Antigen concentration by Lf (Lf/mL)[a]
	Sterility	Antigen purity (Lf/mg protein nitrogen)
	Appearance	Process-related impurities (ammonium and sulfate)
		Inactivation agent (formaldehyde)
		Specific toxicity
		Absence of toxin and irreversibility of toxoid[a]
		Potency[a] (tested adsorbed)
Adsorbed toxoid (final bulk and containers)	Aluminum	Potency[a] (if not already performed)
	Thimerosal (if applicable)	Identity[a]
	pH	% Adsorption[a]
	Sterility	Specific toxicity (if not performed earlier)

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b)

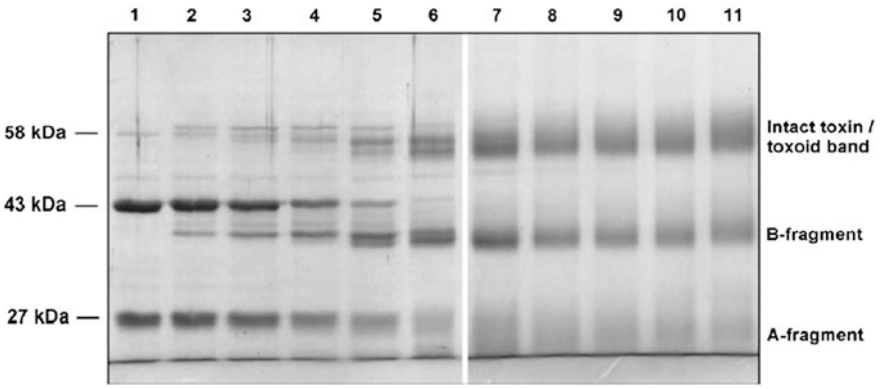


Fig. 6.2 SDS-PAGE of diphtheria toxin and toxoid. *Lane 1* Dipththeria toxin, *Lanes 2–11* Experimental toxoids prepared with increasing formaldehyde concentrations. Reproduced with permission from Elsevier (Metz et al. 2003)

6.2.2.3 Toxicity of Diphtheria Toxin

L+ is the minimum amount of diphtheria toxin that, when mixed and injected with 1 IU of specific antitoxin, kills an animal with a defined weight in 4 days (WHO 1965).

Minimum lethal dose (MLD) refers to the minimal amount of toxin that, when inject into a 250-g guinea pig, will cause death on the 4th or 5th day. The best purified preparation of toxin contains about 25 MLD/μg protein (Pappenheimer 1984).

The cell toxicity effect of diphtheria toxin can also be measured in Vero cell culture by titration of toxin with a fixed amount of antitoxin.

In the Vero cell culture, Phenol red is added to the medium as pH indicator. When pH drops from alkaline to acidic, this indicator changes color from red to orange to yellow. In the presence of excess of toxin cells are rapidly killed and the medium remains red. In the wells with excess antitoxin, the Vero cells survive and secrete acid metabolites, which induce a visible change of color from red to yellow. The point of equivalence is determined by twofold titration of the crude toxin. Using a spectrophotometer, the equivalence point can be determined more accurately (Aggerback and Heron 1991).

6.2.2.4 Antigen Purity

Purity (or specific activity) is expressed as Lf/mg protein nitrogen and a minimum value of 1,500 Lf/mg protein nitrogen is the WHO requirement (WHO 2014b).

6.2.2.5 Specific Toxicity (Ph. Eur. 0443)

Five SHDs of vaccine are injected subcutaneously into each of five healthy guinea pigs, each weighing 250–350 gm, that has not previously been treated with any material that will interfere with the test. If within 42 days of the injection, any of the animals show signs or dies from diphtheria toxemia, the vaccine does not comply with the test. If more than one animal dies from nonspecific causes, the test can be repeated once; if more than one animal dies in the second test, the vaccine does not comply with the test.

If this in vivo method is used for validation during the production stage of the vaccine, it may not be necessary to test the product at the final stage.

6.2.2.6 Absence of Toxin and Irreversibility of Toxoid (Ph. Eur. 0443)

A solution of bulk purified toxoid containing 100 Lf/mL is prepared using the same buffer solution as for the final vaccine without adsorbent. The solution is divided into two equal parts; one is maintained at 2–8 °C, while the other is kept at 37 °C

Table 6.5 Comparison of three guidelines for diphtheria toxoid specific toxicity and irreversibility

	Ph. Eur. 0443	WHO (2014b)	US (NIH 1947)
Specific toxicity	Inject five single human doses into at least five guinea pigs and observe 42 days	A 3.4.4: For bulk purified toxoid use 1 mL containing at least 500 Lf for each of the five guinea pigs, observe 42 days	Inject subcutaneously ≥5 SHDs (≥2.0 mL) into guinea pigs and observe 30 days without evidence of diphtheria toxin poisoning
		Cell culture test system may be used	
		A.3.5.2.5: For final bulk, use the same procedure as Ph. Eur.	
Absence of toxin and irreversibility of toxoid	Prepare diphtheria toxoid at 100 Lf/mL and incubate for 6 weeks at 2–8 °C or at 37 °C. Study toxicity in Vero cell, with and without antitoxin	A3.4.5: Dilute diphtheria toxoid to the same concentration as final bulk; incubate for 6 weeks at 2–8 °C or at 37 °C. Inject 10 SHDs (5 mL) to each of the five guinea pigs and observe 42 days. Only required for purified toxoid bulk but not for final bulk	Not described
		Intradermal test in guinea pigs and cell culture toxicity test are also considered suitable	

for 6 weeks. A Vero cell cytotoxicity assay is used to detect active diphtheria toxin in the presence and absence of diphtheria antitoxin. The toxoid sample fails the irreversibility test if toxicity neutralizable by antitoxin is present in either sample.

The guidelines vary slightly as outlined in Table 6.5.

6.2.2.7 Potency for Adsorbed Diphtheria Toxoid (Ph. Eur. 2.7.6)

This section describes potency method for DT per Ph. Eur. 2.7.6.

The potency is determined by administration of the vaccine to guinea pigs, followed either by challenge with diphtheria toxin (method A or B) or by determination of the titer of antibodies against diphtheria toxin or toxoid in the sera of guinea pigs (method C). The potency of the vaccine is calculated by comparison with a reference preparation, calculated in International Units.

Method A or B is used during development of a vaccines or whenever revalidation is needed following a significant change in the manufacturing process. In the interest of animal welfare, method C is used whenever possible.

Method A: Intradermal challenge test in guinea pigs
At least six groups of guinea pigs are injected subcutaneously with 1.0 mL of graded doses of test vaccine and reference preparation. The dilution should not be more than 2.5-fold from each other and the intermediate dilution should result in an intradermal score of approximately 3 (3 of the 6 challenge sites free from reactions) when 1.0 mL dose is used. After 28 days, shave both flanks of each guinea pig and inject 0.2 mL of each of the six toxin dilutions (containing 0.0512, 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf) intradermally into six separate sites on each of the vaccinated animals in such a way as to minimize interference between adjacent sites. Examine all injection sites 48 h after and record incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intradermal challenge score. Obtain relative potency by parallel-line quantitative analysis using skin test scores to calculate dose-response curve.

Method B: Lethal challenge test in guinea pigs
Groups of guinea pigs are immunized with test samples and reference vaccine dilutions for 28 days and challenged subcutaneously with 1.0 mL of 100 LD_{50} of toxin. Count the number of survived animal after 4 days. Calculate relative potency using statistical methods based on animal survival.

Method C: Inject subcutaneously to each guinea pig 1 ml of the diluted vaccine. Take a blood sample from each vaccinated and control animals 35–42 days later. Determine antibody titer by a sandwich ELISA or Vero cell assay.

The Vero cell assay relied on metabolic inhibition (method 1) or on cytotoxicity (method 2) as the endpoint and the cells are inspected microscopically or visually.

Method 1: The diphtheria toxin causes a cytopathogenic effect on Vero cells leading to cellular lysis. Antibody directed against diphtheria toxin may inhibit this effect. Consequently, the potency of a diphtheria vaccine may be indirectly determined with the help of this cell culture system if different serum dilution from immunized animals is cultured with a constant toxin concentration.

Method 2: Thiazolyl blue MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is reduced to a blue/black formazan product by the mitochondrial dehydrogenase of viable cells, and thus serves as a quantitative measure of living cells present, indicating when toxin has been neutralized by the antitoxin.

The Ph. Eur. challenge model in guinea pigs was used in establishing the 4th International Reference Standard for DT Adsorbed with a unitage of 213 IU/ampoule by the WHO Expert Committee on Biological Standardization in October 2009 (WHO 2014b). Additional collaboration study was performed using guinea pigs and mouse serological assays. Results suggest that Vero cell assay may be suitable for future replacement standards (Stickings et al. 2010) (Table 6.6).

Table 6.6 Summary of diphtheria potency assays from different guidelines

EP (Ph. Eur. 2.7.6)	WHO (2014b and 2013b)	USA (NIH 1947)
Immunize groups of guinea pigs with test and reference vaccines. At 28 days, challenge the animals with diphtheria toxins lethally (4-day read) or by skin test (2-day read). Alternatively, at 35–42 days, test sera by ELISA or by its ability to neutralize diphtheria toxin inhibition of Vero cell culture. Compare results with reference standard calibrated in international units	A.3.5.2.6: Same principle as EP. In addition to guinea pig challenge assays, WHO also allows immunization in mice with antibody levels titrated by toxin neutralization tests such as Vero cell assay (mice are not sensitive to diphtheria toxin challenge).	Immunize at least four guinea pigs with no more than one-half of the total human immunizing dose of the test vaccine and collect the immune serum 4 weeks later. A defined dilution of the immune serum is combined with a standardized quantity of diphtheria toxin and then injected into two naïve guinea pigs. Protection of test animals equates to toxoid potency. The L+ dose of the toxin is confirmed in the death of at least two control guinea pigs
	Potency used in immunization of children should be ≥30 IU/single human dose	Minimum requirement is ≥ 2 antitoxin units/mL for the pediatric dose

6.2.3 Acellular Pertussis Vaccine

Pertussis vaccine is used to prevent a childhood disease, whooping cough, caused by infection with *B. pertussis*. The first generation of the vaccine is the whole-cell pertussis vaccine, which contains inactivated bacteria (see Chap. 5) and is still used in most parts of the world. In higher-income countries, the whole-cell pertussis vaccine has been replaced with the less reactogenic aP vaccine.

The licensed aP vaccine contains up to five purified pertussis antigens, namely, pertussis toxoid, filamentous hemagglutinin (FHA), pertactin (69 kDa protein), and fimbrial agglutinogens 2 and 3. Pertussis toxoid is the essential component of the vaccine and a single-component pertussis toxoid vaccine is used to demonstrate the testing principle and strategy in this chapter, even though there is still debate on what components are required for protection (Tondella et al. 2009; Poolman and O Hallander 2007). The multiple component pertussis vaccine is discussed further in Sect. 6.2.3.13.

The production of the single-component aP vaccine involves the following steps:

1. Fermentation of *B. pertussis*
2. Purification of PT by affinity columns (Sekura et al. 1983)
3. Detoxification of PT by hydrogen peroxide treatment in the presence of ferric sulfate and EDTA (Sekura 1988).

Testing of PT, hydrogen peroxide inactivated pertussis toxoid and adsorbed toxoid (final bulk) is summarized in Table 6.7.

Table 6.7 Testing of a single-component acellular pertussis vaccine

Stages	General test	Product specific test
Purified toxin	Protein	Purity and characterization (SDS-PAGE, Western blot, and reverse-phase HPLC)
		Process-related impurities (blue dye and fetuin)
		CHO toxicity[a]
		ADP-ribosylation
		Hemagglutination (HA)
Unadsorbed toxoid	Protein	Inactivation kinetics
	UV spectrum	Residual HA
	Endotoxin (LAL)	Residual CHO toxicity[a]
		SDS-PAGE
		ADP-ribosylation
		Residual inactivation agent (hydrogen peroxide)
Adsorbed toxoid	Sterility	Histamine sensitization[a]
	Endotoxin	Irreversibility of toxoid
	Aluminum	Potency
	Thimerosal (if applicable)	% Adsorption
	pH	

[a] The step-by-step procedure is included in a WHO publication (WHO 2013b) for whole-cell pertussis vaccine, which is also applicable to acellular vaccine

6.2.3.1 Purity of Pertussis Toxin and Toxoid by SDS-PAGE

Pertussis toxin is the major virulence factor of *B. pertussis*. After being purified by Affi-Gel blue and fetuin-Sepharose 4 B affinity columns, its purity was verified by SDS-PAGE shown in Fig. 6.3 (Sekura et al. 1983).

Once PT is chemically detoxified by hydrogen peroxide in the presence of Fe^{+3} and EDTA (Sekura 1988), the structure becomes heterogeneous and characterization became difficult. Corbel et al. (1999) showed that the bands on the SDS-PAGE became fussy and broadened for the detoxified protein (Fig. 6.4).

6.2.3.2 Product-Related Impurities

The contaminant level of pertussis antigens had to be established as part of the product characterization, because trace amount of some pertussis antigens may play a role in protection or toxicity. Using Western blot and monoclonal antibodies specific to FHA, adenylate cyclase, pertactin, and fimbriae, the assay sensitivities are defined and the purified toxin is tested. The other potential toxic contaminants include heat labile (dermonecrotic) toxin and tracheal cytotoxin. Heat labile toxin can be detected by observing dermonecrotic activity after subcutaneous injection of

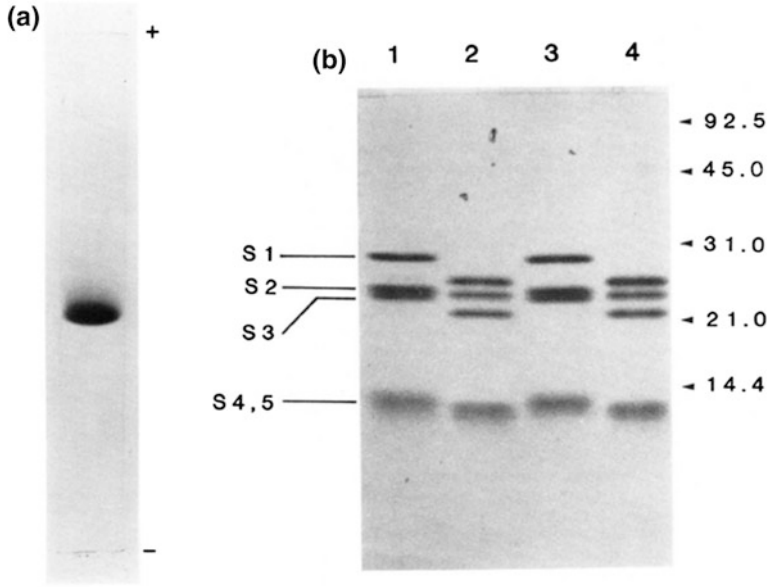


Fig. 6.3 Panel **a** Purified pertussis toxin on 5 % native gel. Panel **b** Pertussis toxin on 15 % SDS-PAGE. Reproduced with permission from The American Society for Biochemistry and molecular biology (Sekura et al. 1983). *Lanes 1* and *3* reducing samples, *Lanes 2* and *4* nonreducing samples

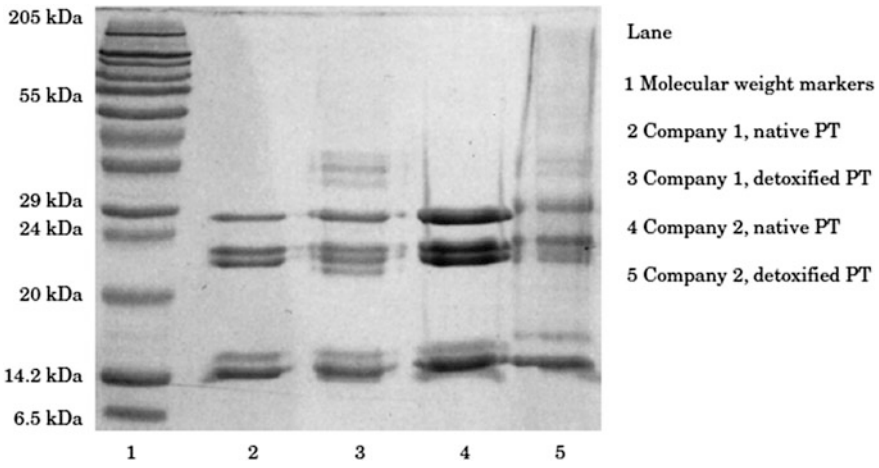


Fig. 6.4 Comparison of native and detoxified pertussis toxin on a 15 % SDS-PAGE with Coomassie blue stain. Reproduced with permission from Elsevier (Corbel et al. 1999)

the test material into the nuchal area of suckling mice (WHO 2013b). Tracheal cytotoxin, a disaccharide-tetrapeptide with molecular weight of 921 Da, can be tested by HPLC (Cookson et al. 1989).

The limits for pertussis impurities are defined in the EP (Ph. Eur. 1356, pertussis vaccine, acellular, component, adsorbed). The limit of adenylate cyclase is 500 ng per dose, for tracheal cytotoxin, no more than 2 pmol per dose and for dermonecrotic toxin, three unweaned mice each injected with 0.1 mL, containing the equivalent amount of antigenic fraction or component present in one dose, should show no dermonecrotic reaction within 48 h.

The active ingredient of one commercial aP bulk vaccine is a hydrogen peroxide inactivated pertussis toxoid at 40 μg per dose. The pertussis antigen contamination level verification was a critical part of the comparability study when postlicensure process changes were submitted for regulatory approval.

6.2.3.3 Process-Related Impurities

Assays for residual levels of ligands used in affinity purification (e.g., Blue dye and fetuin) are included in the impurity profile. Cibacron Blue F3G-A absorbs at 610 nm with an extinction coefficient of 13,600 M^{-1} cm^{-1} (Lowe and Pearson 1984) and a limit assay can be established based on UV/Visible absorbance scan. Residual fetuin can be tested by a suitable immunological method (ELISA or rocket electrophoresis) using purified rabbit anti-fetuin antibody.

6.2.3.4 HPLC Characterization of Pertussis Toxin

PT contains 6 subunits with an A-B structure and molecular weight of 117 kDa. The A component is composed of a single S1 subunit with ADP-ribosyl transferase activity. The B oligomer, consists of single S2, S4, and S5 subunits and two S3 subunits, mediates receptor binding and intracellular translocation (Tamura et al. 1982). Reverse-phase HPLC has been used to separate the subunits and LC-MS/MS used in the subunit sequence determination (Tummala et al. 2008). The subunits 1, 2, and 5 of PT were observed to undergo oxidation under normal storage conditions as ammonium sulfate suspension at 2–8 °C (Fig. 6.5).

After detoxification with hydrogen peroxide, there was no clear separation of toxoid subunits in the reverse-phase HPLC. Two-dimensional liquid chromatography-tandem mass spectrometry, ultra performance liquid chromatography/Mass spectrometry, and capillary liquid chromatography/MALDI tandem mass spectrometry were used to analyze the tryptic digest of pertussis toxoid. Hydrogen peroxide treatment in the presence of Fe^{+3} modified nearly all the methionine and tryptophan residues and a large percentage of cysteine, aspartic acid, and histidine. Some tyrosine, Phenylalanine, proline, and glutamine were also modified (Tummala et al. 2010, 2013). This type of product characterization is laborious and unpractical for routine analysis. Instead, loss of biological activities (histamine sensitization, CHO toxicity, and hemagglutination) is measured routinely following detoxification.

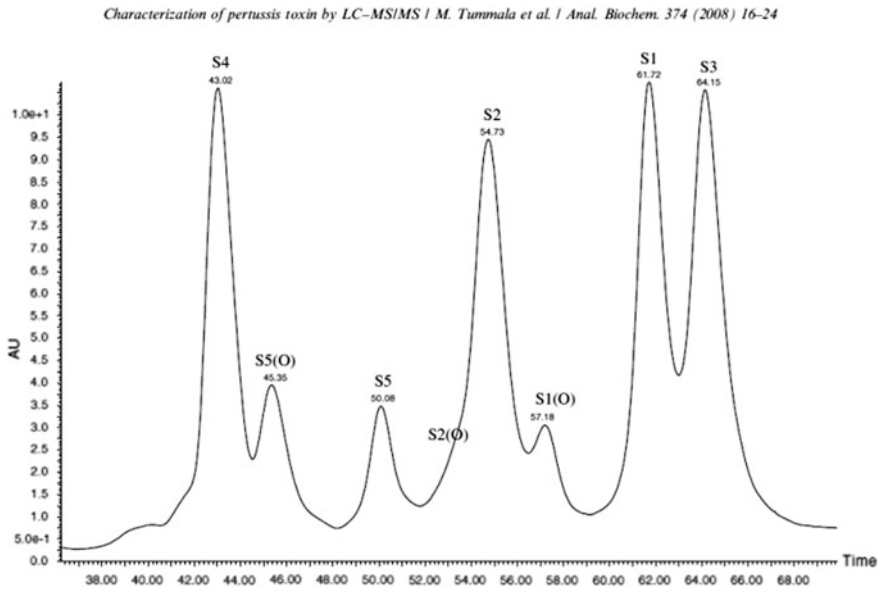


Fig. 6.5 Reverse-phase HPLC separation of pertussis toxin. Reproduced with permission from Elsevier (Tummala et al. 2008)

Of the components used in the formulation of multicomponent aP vaccines, only PT has significant intrinsic toxicity. The other components, FHA, pertactin, Filbriae 2 and 3 have not been shown to exert toxic effects. The safety testing is implemented to regulate the residual PT activity in the product. There is no general agreement on choice of assay or acceptable limits for residual toxicity.

6.2.3.5 Hemagglutination Assay for Toxin and Unadsorbed Toxoid

PT causes goose red blood cell to agglutinate; it is a rapid assay used for process monitoring during PT purification (Sekura et al. 1983). Hemagglutination activity requires only the B subunit of PT, which provides the binding function. Detoxification of PT with hydrogen peroxide in the presence of EDTA and Fe^{+3} brings hemagglutination activity down to <1 % (Sekura 1988).

6.2.3.6 CHO Cell Toxicity for Toxin and Unadsorbed Toxoid

Exposing CHO cells in culture to PT results in a clustered growth pattern (Hewlett et al. 1983). The clustering of CHO cells correlates with ADP-ribosylation of a 41-kDa protein in CHO cells and thus a true measure of PT activity for both A and B subunits (Burns et al. 1987). The CHO cell assay has been used as an in vitro test

to detect residual active PT in the vaccine. It has been suggested that toxoid aggregation could give false-negative results with formaldehyde-inactivated vaccines (Kataoka et al. 2002). Moreover, adjuvant can cause CHO cell death (Corbel and Xing 2004) and a porous cell culture insert is used to prevent vaccine adjuvant from directly contacting the CHO cells (Isbrucker et al. 2014).

6.2.3.7 ADP-Ribosylation

The S1 subunit of PT harbors ADP-ribosyl transferase activity with a GTP-binding protein (G protein) being the natural substrate; this enzymatic assay can be used to quantify PT and monitor the extent of detoxification. A fluorescent-tagged synthetic peptide homologous to the carboxy-terminal 20 amino acid sequence of the alpha subunit of G protein was used as the substrate for enzyme assay. The tagged peptide and the ADP-ribosylated product were separated by HPLC with fluorescent detection (Cyr et al. 2001). The assay could detect as little as 2 ng toxin and worked well with adsorbed product to detect the residual toxin activity (Yuen et al. 2002).

6.2.3.8 Kinetics of Inactivation

Kinetics of inactivation should be established as part of the manufacturing process validation (WHO 1998). Hemagglutination assay and ADP-ribosyl transferase assay have been used to establish the inactivation kinetics to demonstrate the consistency of inactivation (Sekura 1988).

6.2.3.9 Histamine Sensitization Test (HIST) and Irreversibility of Pertussis Toxoid (Ph. Eur. 2.6.33)

HIST is the official Pharmacopeia test used for detecting residual PT activity in the adsorbed vaccine. Groups of at least 10 mice, of suitable strain, age, and weight, are injected intraperitoneally with between 1 and 2 human doses of the adsorbed test vaccine. The first group receives vaccine stored at 2–8 °C (test for residual PT); the second group receives vaccine incubated at 37 °C for 4 weeks (test for irreversibility of toxoid), and the third group receives diluent (negative control).

In addition, a positive control group of mice is injected with an equivalent volume of reference PT preparation at a dose that has been defined in the validation stage as demonstrating the assay sensitivity.

If a reference group of mice is used, it may be injected with a reference PT preparation at a dose previously set as the allowable upper limit of PT in the product, according to historical safety data. Alternatively, a reference vaccine with established clinical safety may be used instead of the reference toxin preparation.

Five days later, animals are challenged intraperitoneally with histamine solution (2 mg histamine base in ≤0.5 mL) and the number of mice dying within 24 h is

recorded. If a reference group is included, the vaccine complies with the test for residual PT if the percentage of deaths in the first group is not greater than that in the reference group. The vaccine complies with the test for irreversibility of pertussis toxoid if the second group also complies with these criteria. If no reference group is included and no animal dies, the preparation complies with the test.

The mice strain is suitable if its LD_{50} is between 6 and 50 IU. The test is not valid if one or more mice in the negative control group die following histamine challenge and the histamine sensitivity does not meet the defined limit (e.g., at least 30 % of the mice die in the positive control group).

The aP vaccine licensed in the United States follows a slightly different protocol and acceptance criteria. The USA, WHO and EP procedures together with HIST theory and history were reviewed by Arciniega et al. (2011). The key differences between the three guidelines are summarized in Table 6.8.

The assay procedure varies from one laboratory to the other. In an international collaborative study involving six laboratories, HSD_{50} values obtained in this study differ significantly between laboratories (Xing et al. 2002).

Table 6.8 Comparison of HIST protocol between USA, WHO, and Ph. Eur.

	Ph. Eur. 2.6.33	WHO (2013a) Appendix 3	USA (Arciniega et al. 2011)
Mice	≥10 mice	Appropriate number	20 mice 15–20 gm 4–5 weeks old, female
Amount injected	1–2 human doses intraperitoneally	1–2 SHDs intraperitoneally	One human dose in 0.5 mL intraperitoneally
Histamine formula	2 mg histamine base/ ≤ 0.5 mL	1–2 mg histamine base	1 mg histamine base/ 0.5 mL
Challenge date after injection	5 days	4–5 days	5 days
HSD_{50}	6–50 IU	Not specified	10–100 ng
Allowed death in control	0 (No control mouse should die for the assay to be valid)	≤5 %	≤10 %
Allowed death in sample	0 (no test mouse should die for the assay to pass)	PT activity ≤ clinical lots	≤10 %
Retest criteria after initial failure	Could be repeated if one mouse dies	Two consecutive assays must pass	Retest two consecutive times and both must pass
	The assay passes if total death in all valid assays ≤ 5 %		

In the subsequent international collaboration study involving ten laboratories for the aP and the combination vaccines, use of a validated procedure and inclusion of a common reference standard were shown to greatly improve HIST between laboratory agreements and reduce repeat testing. A potency of 7,500 IU/vial was assigned to the Ph. Eur. Biological reference preparation for PT (Xing et al. 2010).

Concerns about the reliability of the lethal endpoint have been reported (Xing et al. 2010) and alternative endpoint measurement has been proposed. Jensen et al. (2012) developed a dermal temperature-based HIST assay and estimated a sensitivity of 5 ng PT per human dose of pertussis vaccine using this modified procedure. In this method, the change in body temperature after histamine challenge is measured and sensitized mice show a sharp decrease in body temperature following histamine challenge. The dermal temperature-based method, which has been incorporated to the HIST procedure used routinely in Japan, has been shown to be as sensitive as the method based on rectal temperature measurement (Ochiai et al. 2007). The use of a histamine-sensitization test based on body temperature measurements as endpoints in mice has been approved in Ph. Eur. 2.6.33.

HIST is difficult to standardize, its precise mechanism is unknown, the amount of PT that is unsafe for human is also unknown, and therefore it was regarded as a priority for replacement by in vitro methods. A new in vitro test system was shown to be a potential alternative to the current HIST. The in vitro test system is based on two assays, the HPLC assay measuring ADP-ribosyl transferase activity of the S1 subunit of PT described in Sect. 6.2.3.7 and a fetuin binding ELISA based on the activity of the B protomer. A mathematical formula showed a good agreement with HIST results, based on dermal temperature reduction after histamine challenge. A regression factor should be established for each type of vaccine (Yuen et al. 2010). Arciniega et al. (2011) have expressed concerns about how an acceptance limit could be set for residual PT activity using the dual in vitro assay. Subsequent international collaborative study did not establish a direct correlation between the in vitro assays and the in vivo HIST (Xing et al. 2012). Several workshops have been held to discuss alternatives to the histamine sensitization test and a working group was established for ongoing discussions (Bache et al. 2012; Isbrucker et al. 2014).

6.2.3.10 Potency

According to Ph. Eur. 2.7.16, two serological potency assays are available for aP vaccine.

Method A: Serology in mice

Six groups of 5-week-old healthy mice are injected intraperitoneally or subcutaneously with 0.5 mL of three dilutions of either the test vaccine or a reference vaccine (for example, a batch shown to be effective in clinical trials, or a batch representative thereof). The mice are bled 4–5 weeks later and antibody titer is determined by ELISA using plates coated with each pertussis antigen (if the test is

for multicomponent aP vaccine). Antibody titer is calculated in relative terms to a reference serum.

Method B: Serology in guinea pigs

Immunize at least six groups (three dilutions each for test and reference vaccines) of guinea pigs with 1.0 mL of vaccine subcutaneously. Bleed the animal 35–42 days later. Determine antibody titer by ELSA coated with purified antigen.

No completely satisfactory method is available for monitoring pertussis potency. Immunogenicity assays are useful for checking consistency in comparison to a clinical trial lot, but do not necessary correlate with protection.

A modified intracerebral challenge assay (MICA, modified Kendrick test) has been used in Japan, Korea, and China as the potency assay for release with a specification of ≥4 unit/dose; vaccines regulated using this approach have been shown to be effective in controlling pertussis (Tondella et al. 2009). MICA appears to be a suitable model to assess the potency of aP vaccines of different antigen compositions (Knezevic et al. 2008), but it is also a consistency test whose correlation to protection has not been demonstrated.

The modified Kendrick test is described here. Groups of mice are injected intraperitoneally with 0.5 mL serial dilutions of the reference and the test vaccines. At 3 weeks after immunization, mice are challenged intracerebrally with a *B. pertussis* suspension prepared from a 20–24 h culture grown on Bordet-Gengou agar or other suitable medium. The bacteria suspension is adjusted in such a way that each challenge dose of not more than 0.03 mL contains 100–1,000 times of LD_{50}. The mice are observed for lethal effects over the next 14 days. The potency is estimated in terms of international units by parallel-line assay. The modification from the original Kendrick assay includes a longer interval between immunization and challenge (3 weeks instead of 2 weeks) and using a special mouse strain (Corbel and Xing 2004).

In an international collaborative study initiated in September 2006, JNIH-3 aP vaccine reference was found to give similar dose-response lines to a variety of aP vaccines and DTaP formulations, irrespective of the differences in aP components. The WHO working group established it as the First International Standard for aP vaccine in MICA. (WHO 2013a) JNIH-3 contains lyophilized PT and FHA adsorbed onto aluminum phosphate with an assignment of 34 IU per ampoule (Gaines-Das et al. 2009).

6.2.3.11 Percentage Adsorption

For low or moderate level of adsorption, there is sufficient amount of antigen in the supernatant (unadsorbed material) to be determined by a protein assay. For high level of adsorption, a more sensitive immunological assay is used to determine the unadsorbed antigen in the supernatant.

6.2.3.12 Residual Inactivation Agent (Hydrogen Peroxide)

Residual hydrogen peroxide is tested with Quantofix Peroxide test sticks purchased from Sigma Aldrich. It is a limit assay with 10 ppm sensitivity (10 µg/mL solution).

6.2.3.13 Process and Testing of Multicomponent Acellular Pertussis Vaccine

Two approaches have been followed to produce multicomponent aP vaccines. In the first approach, vaccine components have been co-purified and the purified material is detoxified with formaldehyde. The second approach is to purify each component individually, detoxify when required and blend to produce the vaccine bulk (Corbel and Xing 2004). For example, DAPTACEL, a DTaP combination vaccine licensed in the USA, contains five pertussis antigens. PT, FHA, and pertactin are purified separately from *B. pertussis* culture supernatant and fimbrial agglutinogens 2 and 3 are co-purified from the bacteria cells. PT is detoxified with glutaraldehyde and FHA is treated with formaldehyde. The individual antigens are adsorbed separately onto aluminum phosphate and combined.

Due to the toxoiding process, these vaccines do not have a defined chemical composition. To monitor consistency, biological testing is required. Ph. Eur. 1595 describes testing for adsorbed, co-purified aP vaccine and Ph. Eur. 1356 describes testing for adsorbed, individually prepared and purified aP vaccine. Most of the methods have been discussed earlier in Sect. 6.2.3. The testing not mentioned in subsections of Sect. 6.2.3 is outlined below.

a. Specific properties of the additional components.

(1) FHA: Hemagglutination and inhibition by specific antibody. PT and FHA both have hemagglutinating activities and they are discerned with FHA specific antibody.

(2) Pertactin and fimbrial agglutinogens 2 and 3: reactivity with specific antibody.

(3) Pertussis toxoid: The toxoid induces in animals the production of antibodies capable of inhibiting all PT activities. For example, CHO neutralization assay demonstrates CHO activity of PT neutralized by the antibody produced from pertussis toxoid.

(4) For individually purified antigen, the purified components must be characterized separately by physicochemical, immunological, or biological assays before detoxification.

b. Antigen content: Immunochemical methods are used to determine quantitative antigen composition. The ratio of each antigen to total protein is within the limits established for the product.

c. Potency: Antibodies to each individual pertussis antigen are measured and compared to the reference vaccine (Ph. Eur. 2.7.16). For example, potency of DAPTACEL is determined by the antibody response of immunized mice to detoxified PT, FHA, pertactin, and fimbrial agglutinogens as measured by ELISA.
d. Final container identity testing: Specific antisera to the components are used.

6.2.4 Testing of DTaP Combination

6.2.4.1 Percent Adsorption

The supernatant of the adsorbed vaccine is tested for D, T, or aP component using suitable immunological methods. In the DTaP vaccine reported by Bergfors et al. (2003), the manufacturer used Rocket electrophoresis to determine % adsorption for D and T toxoids and used ELISA for aP adsorption. Coombes et al. (2009) developed a capture ELISA and a direct ELISA to measure percent adsorption for Diphtheria component in combination vaccines. Another capture ELISA for percent adsorption of tetanus toxoid in combination vaccines was reported by Coombes et al. (2012). In addition, the step-by-step procedure for D and T capture ELISA was provided by WHO (2013b).

6.2.4.2 Identity

Each component is identified by a suitable immunological method. Although Ph. Eur. 1931 and WHO guidelines (1990, 2013b) describe dissolving adjuvant or eluting toxoid, the ELISA identity test can work without dissolving adjuvant or eluting toxoid.

6.2.4.3 General Safety (Innocuity)

Half a human dose (<1 mL) is intraperitoneally injected into each of 5 mice (17–22 gm) and at least one human dose (<1 mL) is injected into each of 2 guinea pigs (250–350 gm); all animals should survive at least 7 days without showing significant signs of toxicity (WHO 1990).

The USA procedure uses 0.5 mL vaccine for each of the 2 mice and 5.0 mL for each of the 2 guinea pigs (21CFR610.11).

6.2.4.4 Specific Toxicity (Ph. Eur. 1931)

For Tetanus, see Sect. 6.2.1.7. Inject subcutaneously at least 5 SHDs to each of the 5 guinea pigs and observe 21 days for paralysis.

For Diphtheria, see Sect. 6.2.2.5. Inject subcutaneously at least 5 SHDs to each of the 5 guinea pigs and observe 42 days for diphtheria toxemia.

The same animals are used for both DT and TT tests and are observed for 6 weeks to cover the observation period specified for DT.

For pertussis component, see Sect. 6.2.3.9 for histamine sensitization.

6.2.4.5 Aluminum Adjuvant

The aluminum adjuvant in the vaccine is first digested with a mixture of nitric and sulfuric acid at high temperature and then measured by atomic absorption (May et al. 1984) or by titration (Ph. Eur. 2.5.13).

6.2.4.6 Thimerosal

Thimerosal is determined by a spectrophotometric assay (Shrivastaw and Singh 1995b).

6.2.4.7 Formaldehyde

A colorimetric reaction was described by Nash (1953), which depends on the synthesis of diacetyldihydrolutidine from acetylacetone and formaldehyde in the presence of excess ammonium salt (Hantzsch reagent). The diacetyldihydrolutidine formed is yellowish-green and highly absorptive at 412 nm. A standard curve with formaldehyde can be established as a quantitative assay. Ph. Eur. 2.4.18 (method A) described it as a limit assay by visually comparing vaccine dilution with 20 μg/mL formaldehyde solution after the reaction. The limit established by WHO (1990) was 0.2 gm/L.

Another formaldehyde colorimetric assay was described by Shrivastaw and Singh (1995a). In this system, formaldehyde reacts with phenyl hydrazine/potassium ferricyanide and chloroform in the presence of methanol to form a colored complex. The adsorbed product is tested without having to remove the adjuvant.

6.2.4.8 Endotoxin (Ph. Eur. 2.6.14)

To quantify endotoxins in the vaccine samples, amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) is used. There are three techniques for this test: the gel-clot technique, which is based on gel

formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex.

6.2.4.9 PH, Sterility, Appearance

These compendial methods are not described here.

6.2.4.10 Potency

The animal potency tests currently required by WHO, the EP, and FDA differ. Despite these differences, the potency tests have been adequate to ensure sufficient immunogenic activity of the vaccines to induce protective immunity in target populations.

It is recommended to perform potency testing after combination of the individual diphtheria and tetanus toxoid components in Diphtheria and Tetanus Toxoid vaccines for pediatric use (Federal register 2005)

For TT potency, see Sect. 6.2.1.8 and for DT potency, Sect. 6.2.2.7

The US method allows D and T potency to be tested in the same group of guinea pigs (NIH 1953; Keller 2011).

For aP potency see Sect. 6.2.3.10.

6.3 MenB OMV: Outer Membrane Vesicles

The epidemiology of endemic meningococcal disease in many developed countries is currently caused by serogroup B meningococcus, responsible for septicemia, meningitis, and killing children and young adults in few hours (Feavers et al. 2012). In the past, the vaccine approach to group B meningococci has largely focused on OMPs (Outer Membrane Proteins) as part of OMVs (Outer Membrane Vesicles) that were prepared by detergent extraction from the bacterium. Antigen presentation in the vesicle structure has the main advantage to preserve the physicochemical organization of the OMPs and consequently their immunogenicity (Holst et al. 2009).

Until recently, OMV vaccines were the only formulations licensed against serogroup B meningococcal disease. Efficacy has been demonstrated by their ability to control clonal epidemics, and strain-specific vaccines, such as MenBvac (NIPH) and MeNZB (Chiron, later Novartis Vaccines) have been successfully used to combat outbreaks in Norway and New Zealand, in the 1990s and early 2000s, respectively (Fredriksen et al. 1991; Holst et al. 2005).

Unfortunately, this approach is very successful in providing protection against homologous strains but not against heterologous strains due to sequence and antigenic variability of the main antigenic components. During the last decade, a

new generation of vaccines has been developed with the aim of providing broader coverage and the first multivalent serogroup B (4CMenB) vaccine named Bexsero® has been recently licensed for human use in Europe starting from 2 months of age. The vaccine includes the OMV MeNZB vaccine as one of the components (Serruto et al. 2012).

The concentrated OMV antigen is a stable colloidal suspension that consists of small membranous spherical vesicles in which the native complex antigen composition of the subcapsular cell surface of *Neisseria meningitidis* serogroup B is highly conserved. As a result, the OMV particles contain the most abundant surface-exposed proteins, (PorA and PorB are the main components), several less abundant proteins, and low amounts of LPS, embedded in the vesicles as presented in Fig. 6.6. Additional components such as phospholipids and CPS (Capsular Poly Saccharides) are also part of the vesicle structure.

The OMV particles are produced by fermentation of the appropriate *N. meningitides* strain, followed by concentration and bacterial inactivation with DOC (deoxycholate), also mediating OMV formation. The OMVs are then separated, sized, and purified by centrifugation and sonication steps, followed by a final filtration step using a 0.22-μm membrane (Frasch et al. 2001).

Quality and consistency of OMV concentrated bulk production is monitored by adherence to release the specifications as listed in Table 6.9.

Due to the complex structure and presence of several antigenic components, all with different highly informational structures, the OMV particles present many analytical challenges. Dedicated analytical methods are required to characterize the OMV particles on a molecular level and to monitor key components of these complex structures.

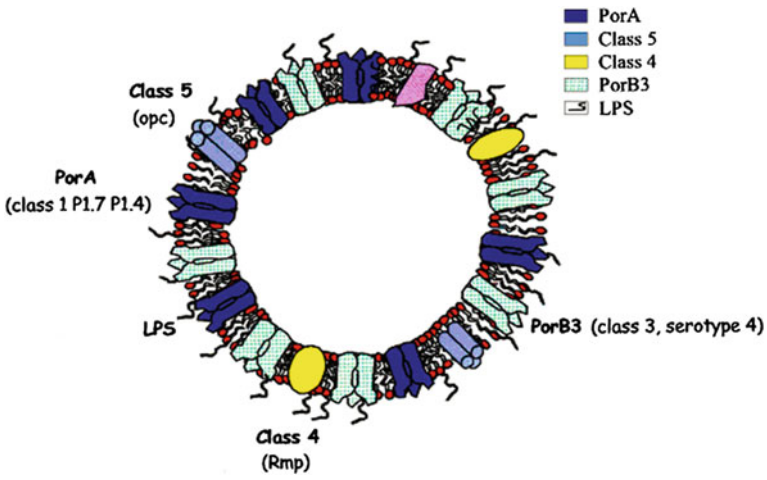


Fig. 6.6 Schematic model of OMV (strain NZ 98/254 -MeNZB): OMVs are portions of the outer membrane of the MenB, extracted with detergent in the form of vesicles [Figure adapted from Holst et al. (2009) with permission]

Table 6.9 Example of OMV (as component of MeNZB) bulk release specifications

Critical quality attribute	Analytical method	Release specification	Stability indicating	Rationale
Identity	Western blot	Presence of class 1 (P1.4), class 3 (ST 4), class 5 (OPC), LPS 3, 7, 9	Yes	The test indicates positive reaction to antibodies directed against LPS and the most crucial protein antigens eliciting responses in humans (the most important is PorA or Class 1)
Protein content	Lowry	450–1,240 μg/ml	Yes	The test measures the total amount of proteins present in OMV, including the major immunogenic antigens PorA and PorB
Protein pattern	Coomassie SDS-PAGE	Purity: conform 70 kDa: 1–12 %; 80 kDa: 1–4 %; Class 1: 13–25 %; Class 3: 20–55 %; Class 4: 7–15 %; Class 5: 1–5 %; NspA: 1–7 %	Yes	The test measures by electrophoresis the presence and relative percentage of the main protein antigens. Moreover, it evaluates (as purity) their content respect total lane proteins
DOC/protein	Enzymatic reaction followed by colorimetric test	0.1–0.4 μg/μg	No	The test measures the presence of residual DOC impurity in the OMV bulk
LPS/protein	RP-HPLC	0.04–0.12 μg/μg	No	The test evaluates the LPS total amount in the OMV bulk by chromatographic determination of 3OH 12:0 fatty acid component of Lipid A
Endotoxin/protein	Kinetic chromogenic LAL	<20,000 IU/μg	Yes	Due to the presence of LPS in the vesicles extracted from the bacteria, the OMV purified

(continued)

Table 6.9 (continued)

Critical quality attribute	Analytical method	Release specification	Stability indicating	Rationale
				bulk presents some endotoxicity. The test evaluates the LPS bioavailability, influenced by Lipid A insertion in the vesicle bilayer
DNA/ protein	Fluorimetry	≤0,035 μg/μg	No	The test measures the presence of residual DNA impurity in the OMV bulk
pH	Potentiometry (Ph. Eur.)	7.0–8.3	Yes	The test measures the pH of the OMV bulk suspension
Sucrose %	Spectrophotometry	2.7–4.1 %	No	The test measures the quantity of sucrose, added to the OMV suspension as preservative
Sterility	Membrane filtration (Ph. Eur.)	Sterile	No	The test measures sterility of the OMV drug substance, required before vaccine formulation

Four quality attributes of the OMVs requiring specific analytical focus were explored by extended physicochemical characterization and are described in the subsequent sections. These analytics are aimed to antigen structural understanding and can be used as additional tools in case of comparability studies.

6.3.1 OMV (MenB NZ) Dimensions and Morphology

Chemical composition and quantity of the single constituents are not the only parameters sufficient for characterizing the OMVs as vaccine component. Vesicle dimensions, morphology, and homogeneity are important structural features that may influence immunogenicity and thus need to be controlled. One example of the importance of vesicle integrity is related to the LPS component of the OMV. LPS

endotoxicity is reduced when the Lipid A moiety is inserted into the vesicle lipid bilayer (Tsai et al. 1989). Consequently, evaluation of the OMV bulk vesicle integrity is important as it relates to pyrogenicity.

Here, we show three different and complementary analytical methods to study OMV size, morphology, and dimensional distribution: DLS (Dynamic Light Scattering), TEM (Transmission Electron Microscopy), and SE-HPLC (Size Exclusion Chromatography).

OMV dimensions are in the subvisible range (0.001–100 μm) and can be measured by in-batch DLS able to determine the vesicles average diameter and the population polydispersity in a fast and simple manner (Dahneke 1983).

DLS was applied to OMV bulk characterization and comparability studies between different production campaigns and during manufacturing development to evaluate in-process parameters of the sonication step necessary to improve material filterability and homogeneity.

The average vesicle diameter of OMV bulk production (determined from data on more than 40 lots) resulted between 97 and 154 nm. PdI (polydispersity Index, distribution confidence ratio related to Z-average measure), estimating the width of the distribution was typically determined to be in the range of 0.1 and 0.3 and indicated, as expected, presence of a multimodal distribution.

Figure 6.7 shows a representative DLS output dataset for OMV bulk. The upper panel reports the input parameters for the analysis. In the lower panel, a Gaussian

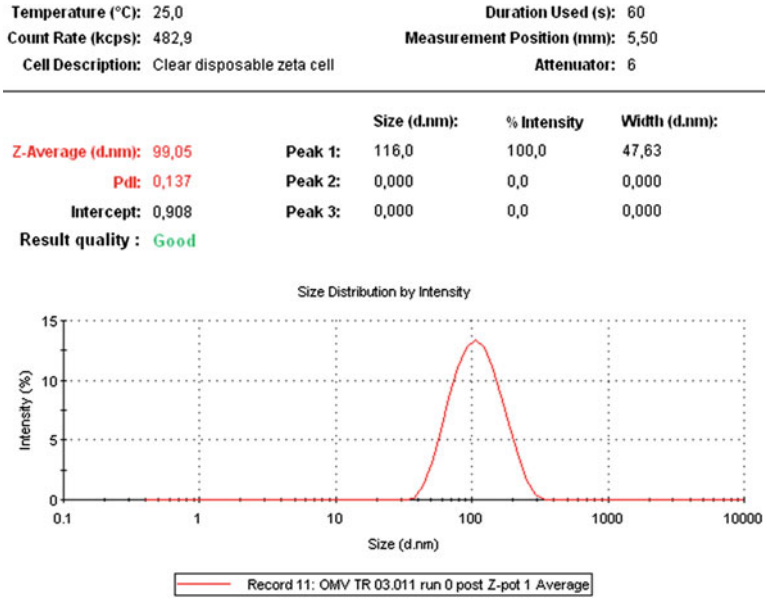


Fig. 6.7 Representative DLS output data for OMV bulk production. Highlighted in *red* are Z-average diameter (d.nm, average diameter in nanometers of the populations) and PdI (distribution confidence ratio related to Z-average measurement)

distribution is visible for the vesicles population with an average diameter (Z-average diameter (d.nm)) of 99 nm and a polydispersity index (PdI) of 0.14.

Dimensional data obtained by DLS were integrated and confirmed by use of complementary TEM analysis. TEM provides information on vesicle morphology and integrity. In addition, TEM can be used to localize antigens on the vesicle surface.

OMVs were analyzed by TEM in negative staining mode by using uracil acetate and 4 % glutaraldehyde. The negative staining TEM allowed examination of the vesicles' external surface by visualizing shape and integrity.

A set of 14 OMV concentrated bulk lots were analyzed. The vesicles consisted mainly of rounded shaped vesicles, with dimensions in the range of 50–200 nm (in agreement with DLS data), presenting light or dark tonalities, possibly connected to concavity/convexity. A representative OMV TEM picture is shown in Fig. 6.8.

The TEM images can be used to evaluate vesicle integrity. TEM images can also be applied to gain information on antigens localization on the OMV surface. By utilizing anti-LPS (3, 7, 9) antibodies and immunogold staining, LPS can be localized on the vesicle surface as small dark dots (Fig. 6.9).

SE-HPLC was applied to separate chromatographically the dimensional components of the OMV bulk solution to complement the DLS in-batch analysis. The results showed that SE-HPLC is able to discriminate up to 4–5-dimensional populations differing in size when applied to the OMV batches.

The chromatographic separation resulted not completely quantitative due to retention of liposomal vesicles by the column matrices tested. However, the

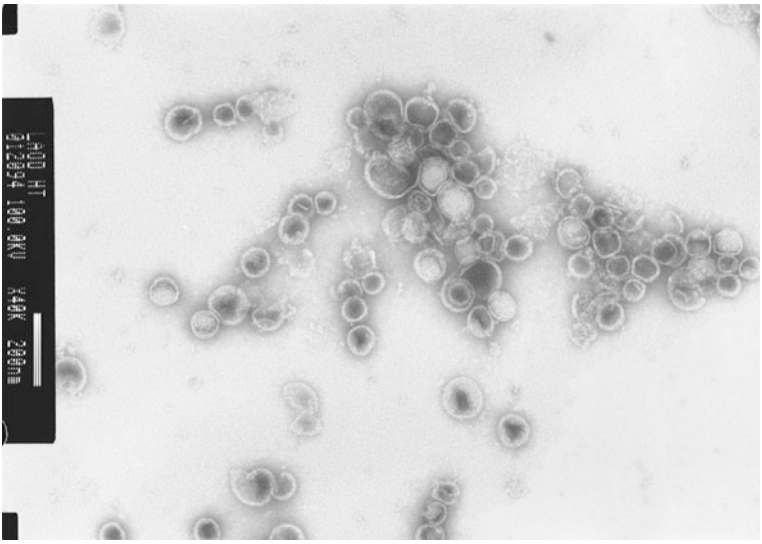


Fig. 6.8 Representative TEM image of OMV concentrated bulk. The *bar* on the *left* represents 200 nm

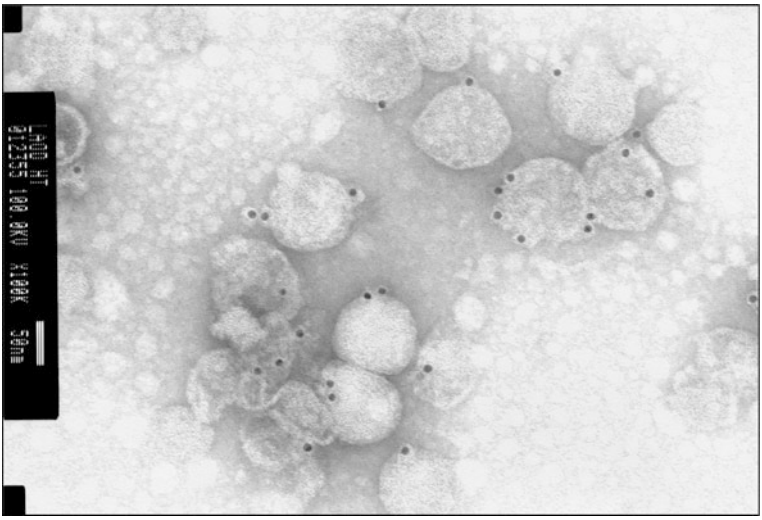


Fig. 6.9 Immunogold staining of LPS (3, 7, 9) on the OMV surface. The *bar* on the *left* represents 50 nm

SE-HPLC analysis remains a relevant complementary method to DLS for the investigation of vesicle degradation.

Figure 6.10 shows the chromatographic profiles of two OMV bulk lots produced at different times and analyzed by SE-HPLC. The individual peaks were fractionated and analyzed by DLS and TEM for characterization. Compared to the unfractioned sample (about 100 nm size average and 86 % integrity by visual TEM evaluation), the main peak in lot 1 (Fig. 6.10 upper panel, red frame) was shown to contain intact OMVs of about 100 nm size average and 97 % integrity and the second fraction (Fig. 6.10 upper panel, blue frame) smaller vesicles with 82 % integrity. In the third fraction (Fig. 6.10 upper panel, green frame), no intact vesicles were detected by TEM, suggesting presence of membrane fragments and cellular debris. Lot 2 (Fig. 6.10 lower panel) was found to be similar to the lot 1 but with minor differences in the vesicle populations of smaller size as well as in the membrane fragments/cellular debris content. In this case, the main peak (Fig. 6.10 lower panel, gray frame), was found to consist of a homodisperse population of vesicles of about 100 nm average diameter when analyzed by DLS.

The effect of physical and chemical stress on vesicle integrity, dimensions, morphology, and homogeneity was investigated to identify conditions that may induce the release of free LPS from the membrane. LPS forms stable spherical membrane structures with proteins and phospholipids where the toxic lipid part of LPS (Lipid A) is buried into the interior of the membrane. Approximately 4–8 % of LPS (calculated as LPS/protein ratio) remains as an integrated part of the vesicle (Lyngby et al. 2002). This structural organization is extremely important because the toxicity of LPS is substantially reduced when integrated in the OMV compared to free LPS in solution (Lyngby et al. 2002; Tsai et al. 1989).

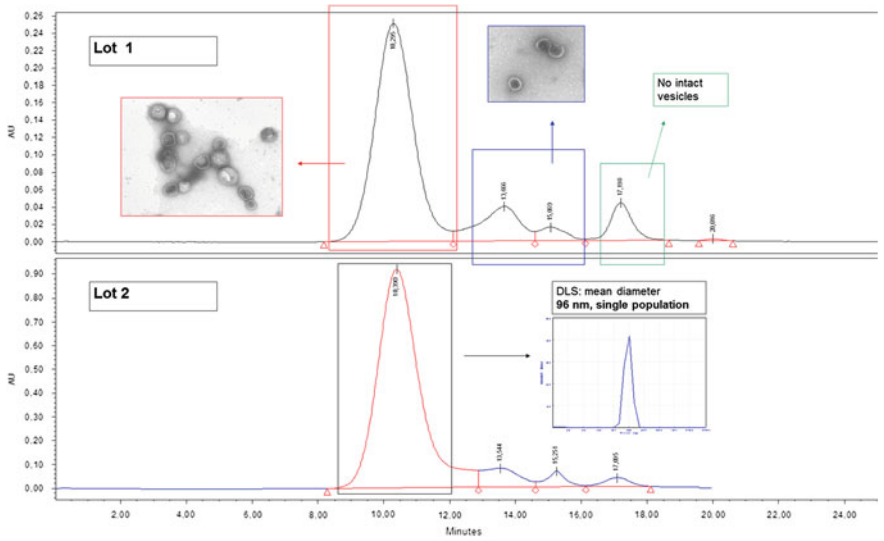


Fig. 6.10 Separation of OMVs on the basis of dimensions by SE-HPLC

Studies of OMVs subjected to different types of stress were used to evaluate whether the described analytical methods (DLS, TEM, SE-HPLC) were also able to monitor vesicles degradation.

Most of the stress conditions applied, i.e., thermal, pH, or physical stress, did not cause any degradation of the OMV particles demonstrating indeed the high degree of stability of this type of particles. Degradation of the vesicular structure was observed by the use of detergents. Zwittergents (3-(N,N-Dimethylpalmithylammonium propansulfonate) were found to be particularly effective in degrading the OMV membrane. The detergent was used as a 2 % solution in H_2O mixed with the OMV sample in a 1:1 ratio. The resulting solution was incubated at room temperature for at least 2 h before being analyzed by TEM and SE-HPLC analysis.

The analytical data on the OMV bulk material before and after the stress induced by Zwittergent are shown in the Figs. 6.11 and 6.12 and listed in Table 6.10. The Zwittergent caused a change in vesicle morphology as observed by TEM (Fig. 6.11). In Fig. 6.12, the main SE-HPLC peak (yellow frame) represents the integral OMV MenB NZ vesicles, while the additional peaks represent vesicle fragments. Addition of Zwittergent caused a major change in the chromatographic profile with complete absence of the peak representing the intact OMV particles. Batch DLS data presented in Table 6.10 show differences in the average OMV diameter before and after stress, but more significantly, a large difference in the polydispersity index caused by vesicle disruption.

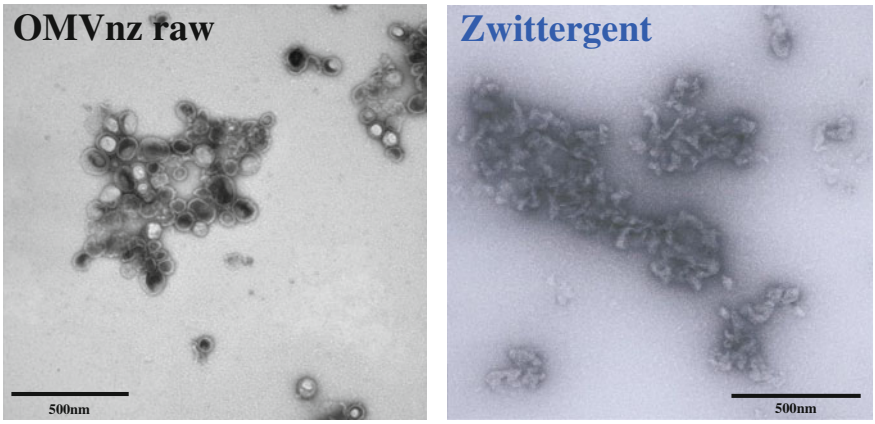


Fig. 6.11 TEM analysis of OMV before (*left*) and after (*right*) Zwittergent treatment. The bar at the *lower* part of the images represents 500 nm

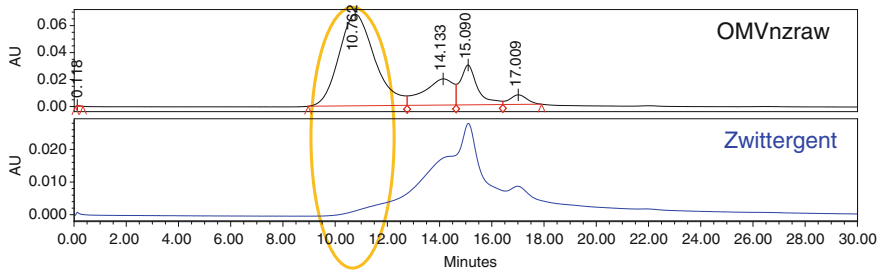


Fig. 6.12 SE-HPLC analysis of OMV before (*top*) and after (*bottom*) Zwittergent treatment. The *highlighted area* indicates the main vesicle peak, disappearing upon stress treatment

Table 6.10 DLS dimensional data on OMV before (top) and after (bottom) Zwittergent treatment

	Average diameter (nm)	PdI[a]
OMVnz raw	134	0.2
Zwittergent	114	1.1

The results represent the average of three repetitions
[a] Polydispersity index

6.3.2 OMV (MenB NZ) Protein Pattern and Content

Characterization of the OMV proteome has been accelerated by the availability of the *neisseria* genome sequence and of improved proteomic technologies. In addition to the most abundant outer membrane proteins, a high number of periplasmic and cytoplasmic proteins have been identified by 2D-PAGE and mass spectrometric

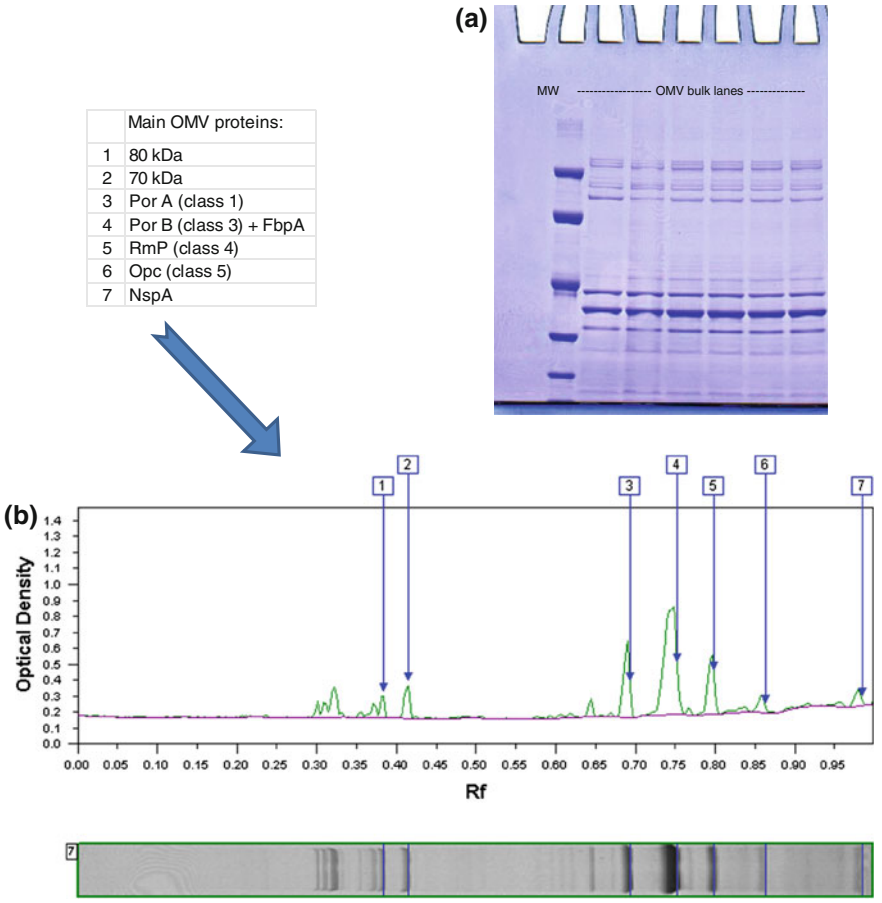


Fig. 6.13 Panel **a** SDS-PAGE separation of OMV bulk: *lane 1*, MW standard, other lanes, OMV bulk material; panel **b** single OMV lane electropherogram; other than relative ratio between target proteins (listed 1–7), their total percentage with respect to total proteins in the lane is evaluated [Figure adapted from Wassil et al. (2012) with permission]

analyses. In total, more than 100 proteins have been identified in DOC produced OMVs applying these technologies (Vipond et al. 2006; Ferrari et al. 2006).

The OMV protein pattern is quantitatively dominated by OMPs like PorA and PorB porins, RmpM (reduction modifiable protein), the OpcA invasin and iron-regulated proteins like FetA/FrpB, depending on iron-depleted growth conditions. Each of these proteins was suggested in the past to be possible vaccine candidates. Based on clinical study results, PorA is considered to be the immunodominant component of OMV following infant vaccination, although additional proteins may contribute to the immunogenic effect (Wassil et al. 2012).

SDS-PAGE with Coomassie Brilliant Blue staining and WB against specific OMPs have been the standard methods used to identify and determine the relative amount of the main protein antigens in the OMV bulk.

The SDS-PAGE release method monitors relative abundance of eight selected OMPs considered relevant both quantitatively and immunogenically in the presence of minimal quantities of several other less abundant proteins. Figure 6.13 shows a typical SDS-PAGE separation of the OMV proteins. Evaluation of the presence and relative quantities of the eight proteins identified as important for OMV immunogenicity with respect to the total OMV protein pattern is performed by densitometric evaluation of the gel lane electropherograms.

One of the specification criteria is based on the relative percentage of the single target proteins with respect to the total proteins based on band intensity in SDS-PAGE. A second specification criterion on total purity guarantees that the sum of the target proteins remains consistent with respect to nonspecified OMV proteins between different lots as ulterior assurance for standardized immunogenic response. The contribution of the nonspecified OMV proteins is distributed over a multitude of proteins that singularly are present in very small amounts and render accurate quantification challenging. These proteins are OMV derived and as such not impurities. Potentially they may be involved in the pathogenesis and thus providing immunity to meningococcal disease (Vipond et al. 2006).

RP-HPLC was applied on OMV bulk to develop a chromatographic method able to complement/confirm the SDS-PAGE separation method of the main OMV proteins. The chromatographic profile by UV detection showed the presence of several protein peaks (Fig. 6.14). To perform a preliminary identification of the observed peaks, total eluted volume plus three fractions of the RP-HPLC profile

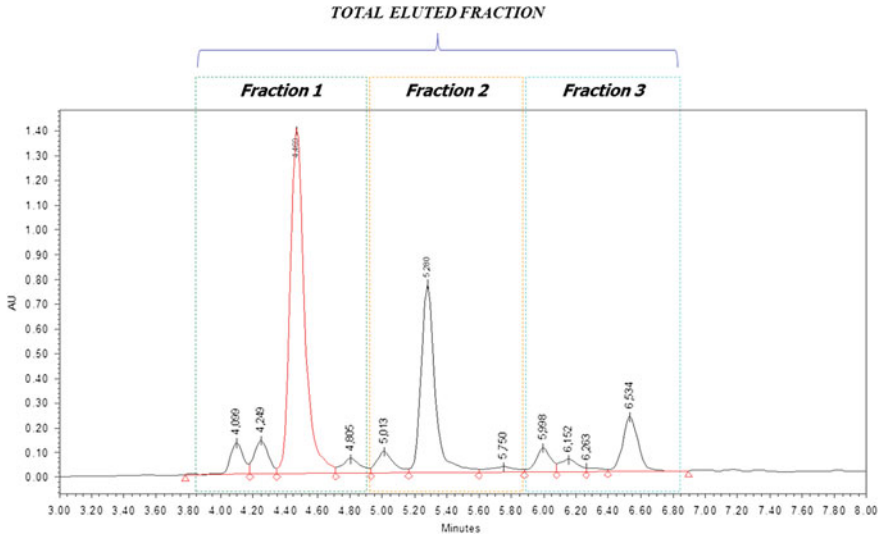


Fig. 6.14 RP-HPLC chromatographic profile of OMV and fractioning for peak identification

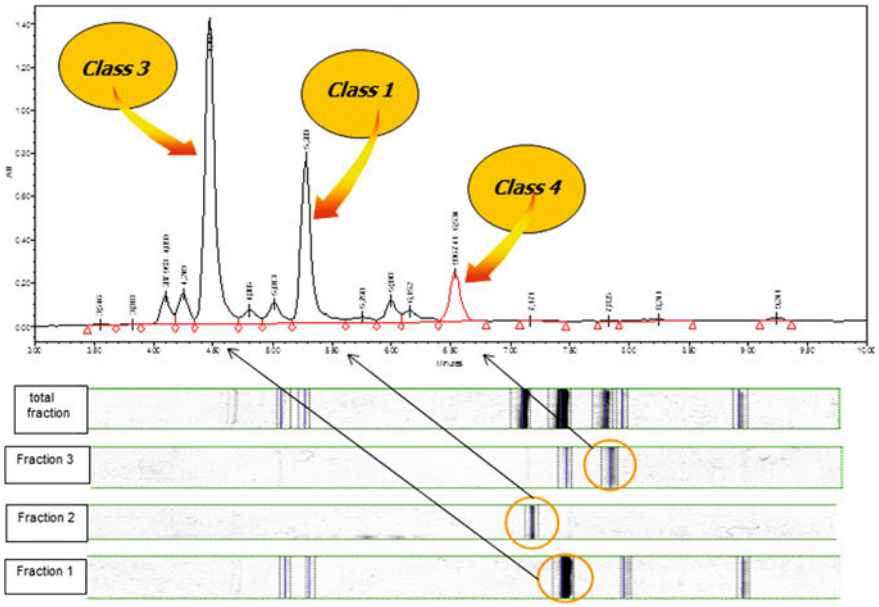


Fig. 6.15 The main protein peaks identified on the chromatogram (*top panel*) by comparison with SDS-PAGE of the corresponding fractions (*bottom panel*) are reported. The RP-HPLC method could provide advantages as potential release method. Identification of main OMPs in RP-HPLC separation (*top panel*) peak at 4.5 min assigned as *PorB class 3*; peak at 5.26 assigned as *PorA class 1*; and peak at 6.5 min *Rmp class 4*

were collected and analyzed by SDS-PAGE, reference SDS-PAGE characterization results were used for comparison (Fig. 6.15). Comparable profiles were obtained for OMV bulk directly loaded on SDS-PAGE and OMV total fraction, although differences in the relative protein concentration were observed, probably due to sample dilution with mobile phase during RP-HPLC elution.

In order to achieve a more comprehensive characterization of the OMV protein composition, mass spectrometric technologies are currently being developed. A large number of less abundant protein accounts for approximately 33 % of non-specified OMV proteins. These proteins are either difficult to detect or not detectable at all using SDS-PAGE or RP-HPLC. Consequently, more sensitive methods are required for their identification and quantification. Mass spectrometry is a powerful technology able to identify all proteins in very complex mixtures in a quantitative manner and represent an ideal tool for the analysis of the OMV proteome (Tani et al. 2014). The about 100 proteins of the OMV proteome and the limited dynamic range required to quantify most of these proteins allow for good coverage in a single LC-MS/MS run. Such a method will enable the assessment of lot-to-lot consistency on a proteome-wide level.

6.3.3 OMV (MenB NZ) LPS Quantification

LPS are structural constituents of OMV particles and cannot be avoided in detergent extracted material as mentioned earlier. However, endotoxicity of LPS in the final vaccine is reduced by two factors: first, the insertion of Lipid A into the vesicle membrane bilayer, and second, by adsorption of the OMV particles to Alum adjuvant in the final formulation.

Potential endotoxicity needs to be strictly monitored and therefore OMV bulk material is analyzed for LPS content in terms of total content via chromatographic determination and bioavailability via a chromogenic LAL test. In both cases, the values are expressed in relation to the total protein content.

The release method used for quantification of total LPS is based on the one developed by Lyngby et al. (2002). This analytical method determines the total quantity of LPS present in OMV independently of its bioavailability.

Quantification of LPS in the OMV is performed via determination of a fatty acid, 3-hydroxy-lauric acid (3-hydroxy-lauric, 3-OH-12:0), considered a marker molecule for the analysis because it is a specific part of the Lipid A component of *N. meningitidis* (Fig. 6.16). The method is based on precipitation of LPS from the OMV solution by addition of cold ethanol and NaCl, TFA (trifluoroacetic acid) hydrolysis for releasing fatty acids, their derivatization as phenacyl esters to allow quantification by RP-HPLC. A conversion factor taking into account fatty acid and LPS molecular weights, and the estimated 2:1 molar ratio between 3-OH-12:0 and LPS are then used to calculate the LPS content.

In order to ensure accuracy of the method, quantitative LPS precipitation from the OMV particles is critical. This cannot be evaluated by addition of the purified analyte 3-OH-12:0 since free fatty acids do not precipitate in the conditions used for LPS precipitation. Instead, purified commercial *Escherichia coli* LPS (a commercial rough strain from *E. coli* J5 Rc mutant) with a short chain of the lipopolysaccharide

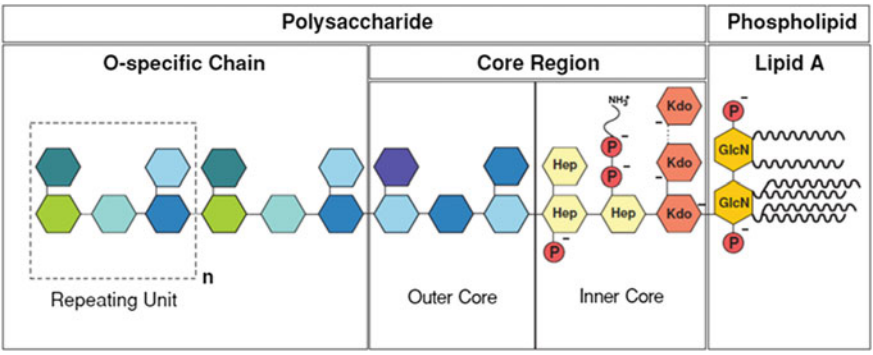


Fig. 6.16 *N. meningitidis* LPS structure: schematic representation of LPS, constituted by a polar oligosaccharide and nonpolar lipid portions; in addition by polar phosphates groups of *Lipid A* or other polar substituents that can decorate the core [Figure adapted from Alexander and Rietschel (2001) with permission]

(LPS), structurally related to *N. meningitidis* LPS was used successfully to demonstrate quantitative OMV precipitation.

OMV samples were spiked with purified *E. coli* LPS before precipitation; 3-OH-14:0 fatty acid, common to *N. meningitidis* and *E. coli* LPS was used for recovery evaluation as shown in Fig. 6.17.

Complete precipitation of LPS from OMV and accurate quantification was confirmed by GC-FID (Gas Chromatography with Flame Ionization Detector) in the precipitated OMV fraction by performing methanolysis on the precipitate followed by *n*-hexane extraction before GC analysis. Recoveries of 105 and 118 % with respect to the *E. coli* LPS spike were obtained.

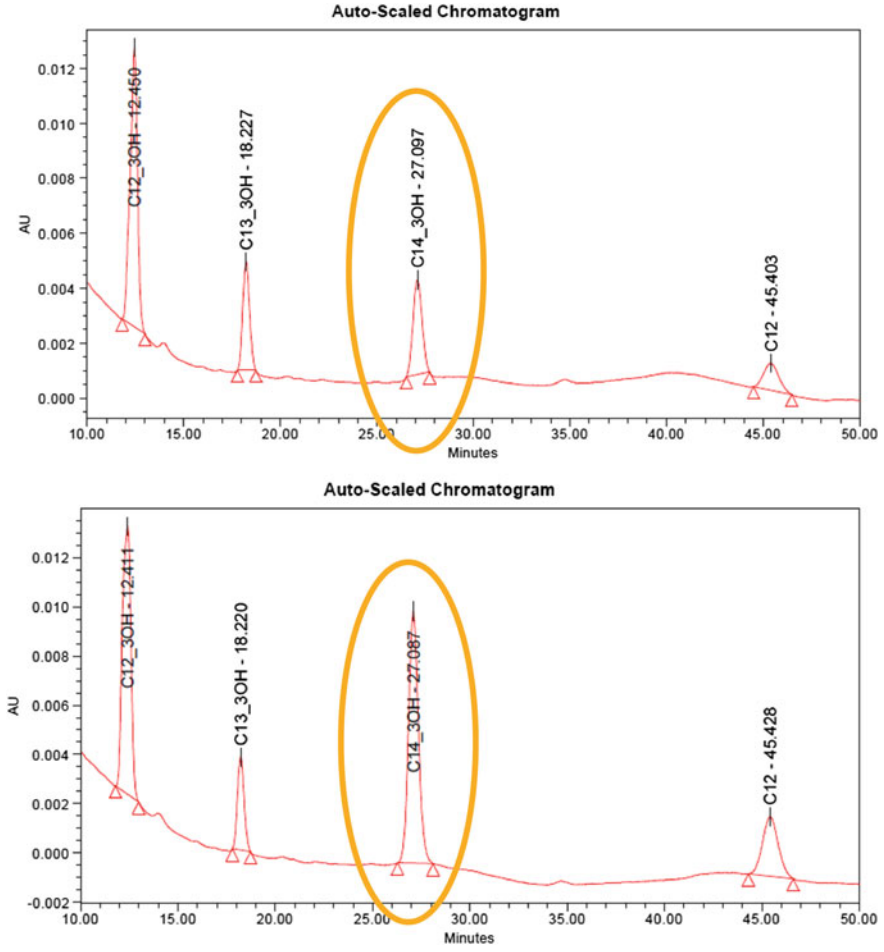


Fig. 6.17 Representative RP-HPLC chromatograms of OMV with (*bottom*) and without (*top*) *E. coli* LPS spike, respectively. The *peak* representing 3-OH-14:0 used for accuracy evaluation is indicated in the figure

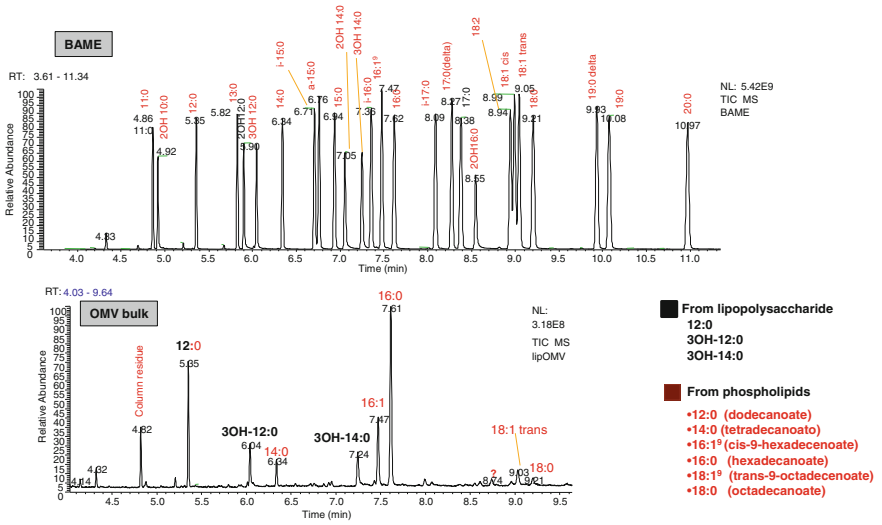


Fig. 6.18 Lipid pattern of OMV by GC-MS. GC profile of BAME standard (*top*) and GC profile of OMV bulk (*bottom*)

The GC analytical method was optimized to enable quantification of LPS in OMV without the need of the precipitation step. An additional advantage of this approach, besides the simplification of the analytical procedure, is the assessment of the complete lipid pattern of the vesicle.

Figure 6.18 shows a representative GC profile of OMV bulk with the optimized method without the precipitation step. Identification of the fatty acid peaks present in the sample was performed by comparison with a BAME (Bacterial acids methyl esters) standard analyzed under the same conditions and confirmed by MS/MS detection.

The analysis of fatty acids present in the OMV bulk shows the presence of short chains, mainly with 16, 14 and 12 carbons. The only unsaturated fatty acids are the 16:1 and 18:1. These findings are in agreement with literature data (Rahman et al. 2000) indicating as major phospholipids component of *N. meningitidis* the 16:0 acyl chain followed by the 16:1 and 14:0 fatty acids. The high abundance of 12:0 can be ascribed to it originating from both LPS and phospholipids.

6.3.4 OMV (MenB NZ) Other Components Characterization

Additional important component of the OMV particles are CPS (Capsular Poly-Saccharide) fragments deriving from the bacterial capsule. In fact, in the production process deoxycholate induces vesicle circularization as well as bacterial disruption and inactivation. Consequently, during vesicle formation part of the capsule is inserted in the OMV bilayer and not successively removed by purification.

MenB Capsular Polysaccharide is constituted by a polysialic chain α(2 → 8) N-acetyl neuraminic acid) of about 200 residues (about 80 kDa) in which the sialic acid (SA) is linked between position 2 and 8 (Fig. 6.19). This molecule retains structural homology to polysialylated components of human fetal and adult neural tissues resulting in a poor immunogen and precluding the development of an anti-MenB vaccine based on capsular polysaccharides. This is in contrast to MenC CPS, differing only in the SA linkage (between position 2 and 3), which is a highly efficacious vaccine (Khatami and Pollard 2010).

Although the theoretical risk that a MenB vaccine based on CPS might induce autoimmune disease in vaccinated persons was disqualified by several studies (Stein et al. 2006; Howitz et al. 2007), monitoring CPS presence in the OMV bulk intermediate remains important for product knowledge and process consistency reasons.

Presence of CPS in OMV bulk material was confirmed by DOT Blot using anti-CPS monoclonal antibodies and NMR. Quantification was performed by evaluation of CPS moieties by MEKC-UV (Micellar Electrokinetic Capillary Chromatography) and/or total SA quantification by HPAEC-PAD (High-Performance Anionic Exchange Chromatography—Pulsed Amperometric Detection) after chemical hydrolysis upon verification that SA contribution by sialylated LPS is negligible.

MEKC-UV can successfully separate small compounds as well as macromolecules, by use of a micelle–forming surfactant solution giving rise to separations into the fused silica capillary. The hydrophobic interactions between the analytes of interest and the micelles contribute to improve the separation selectivity, which is also based on the differential charge/radius ratio.

MenB CPS is partially degraded during the necessary lysis of the vesicles giving rise to a family of peaks with a specific electrophoretic mobility that can be baseline separated by MEKC and monitored by UV detection of the SA moieties. Although complex, such peak pattern allows a reliable CPS identification since a set of specific signals are associated to this relevant component. In Fig. 6.20, the Micellar CE profile (MEKC-UV detection) of an OMV sample after lysis (obtained by heating at 90 °C after SDS addition) is shown (OMV, middle). Comparing this

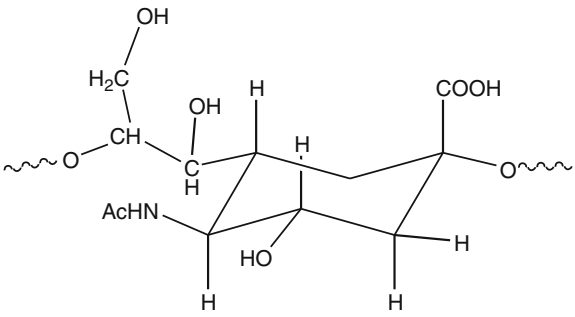


Fig. 6.19 Meningococcal group B CPS structure α(2 → 8) N-acetyl neuraminic acid)—a single moiety of SA is represented

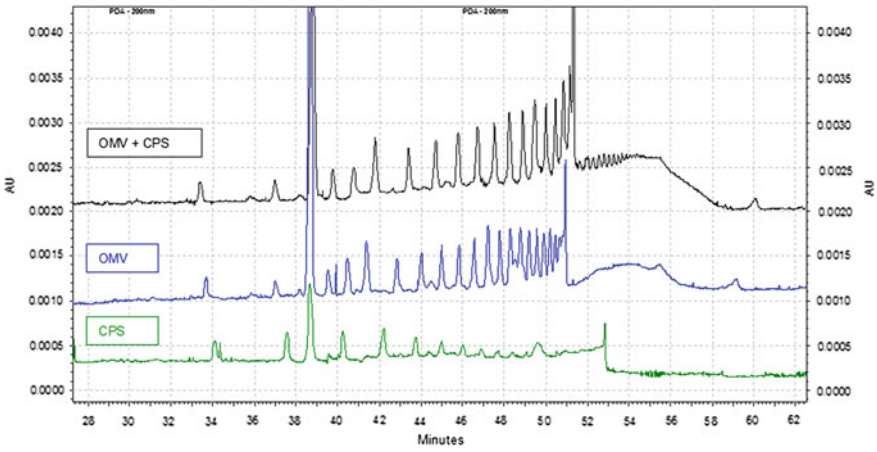


Fig. 6.20 MEKC-UV electropherograms of OMV (*middle trace*, *blue*), purified MenB CPS (*bottom trace*, *green*), and OMV + MenB CPS (*top trace*, *black*)

profile to that of purified CPS pretreated in the same way (CPS, bottom), it becomes clear that in the OMV sample, the polysaccharide component is present as a composite profile including several peaks corresponding to oligosaccharides of different length as demonstrated by an OMV sample spiked with CPS before lysis (OMV+ CPS, top).

Due to matrix complexity, quantitative CPS evaluation in OMV required building the standard curve directly on the sample by spiking of different standard amounts (standard additions method). In this case, purified commercial colominic acid (CA), presenting the same structure as MenB CPS, was used as quantification standard.

Figure 6.21 shows in the upper panel superimposed electropherograms of an OMV-bulk sample spiked with different CA standard amounts. Traces are normalized on the internal standard signal present in each run. The lower panel presents the related standard curve, built by plotting on the *x* axis of the graph the values of mg/ml of CA standard added to the sample, and on the *y*-axis, the value of the sum of the areas of all the peaks belonging to the CPS distribution. Using this calibration curve, the CPS content of the original OMV sample can be calculated extrapolating the concentration value at zero addition.

The CPS content determined using MEKC-UV and expressed as CPS/OMV total proteins ratio for more than 20 OMV lots resulted always lower than 5 %.

Alternatively, MenB CPS can be determined applying different strategy: acidic hydrolysis of CPS to SA units and quantification via HPAEC-PAD chromatography, using conditions suitable for monosaccharide separation.

Figure 6.22 presents the HPAC-PAD elution pattern of an OMV-bulk sample after hydrolysis. Apart from the SA peak, other monosaccharides released from chemical hydrolysis of the sample and present in the chromatogram were identified by use of the appropriate purified standards run under the same chromatographic

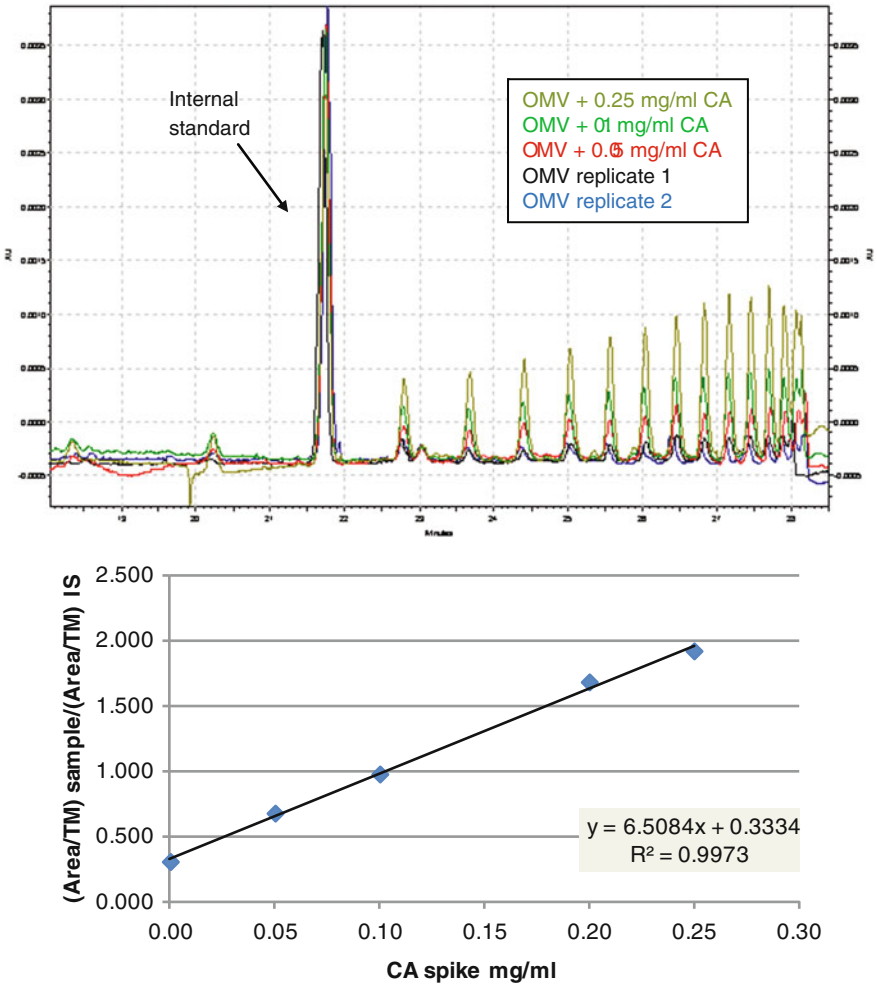


Fig. 6.21 *Upper panel* superimposed electropherograms of an OMV bulk sample spiked with different CA standard amounts; *lower panel* example of resulting standard curve

conditions: KDO, from the LPS inner core, glucose and fructose mainly as components of the sucrose added as stabilizer, and glucose from OMV glycosylations. Fucose was added as internal standard.

With the HPAEC-PAD quantification method, the CPS/protein ratio was determined to be in the range of 2–6 %, in line with the MECK-UV results.

Since an additional source of SA can be sialylated LPS in OMVs, the SA released by a chemical hydrolysis approach accounts for both CPS and LPS. Hydrolysis by enzymatic means, utilizing different neuraminidases, can alternatively break any SA linkage α(2 → 3, 6, 8, 9), or selectively only the α(2 → 3, 6),

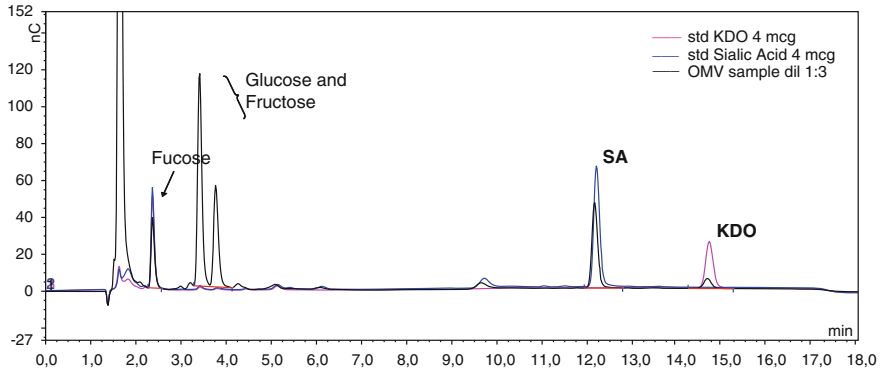


Fig. 6.22 HPAC-PAD elution profile of OMV after chemical hydrolysis

related to LPS, allowing to determine the CPS contribution of SA. The contribution to SA by LPS evaluated by the enzymatic approach was found to be negligible with respect to that deriving from CPS.

Potentially other components of the bacterium could be included in OMVs during the vesicle budding process. Further characterization was mainly focused on those molecules that could induce endotoxin-like actions, i.e., stimulating a complex intracellular signaling pathway that leads to the production of inflammatory cytokines, such as IL-6, IL-1, and TNF (McDonald et al. 2005).

One set of studies was aimed to verify the presence of the bacterial peptidoglycan (PGN) in OMV of *N. meningitidis* group B bulk samples. PGN is composed of carbohydrate chains of β-(1 → 4)-linked, *N*-acetylglucosamine and *N*-acetylmuramic acid, cross-linked by short peptide chains (Vollmer et al. 2008), as schematically reported in Fig. 6.23.

PGN presence was evaluated through the identification by GC of a specific and univocal component of PGN, monosaccharide N-acetyl-muramic acid, whose structure is presented in Fig. 6.23.

Monosaccharides from OMV bulk samples were analyzed by GC as acetylated O-methyl glycosides. Because of the presence of 3 % sucrose in the matrix, samples were ultrafiltered to reduce the sucrose content, then derivatized with methanolic HCl and a successive extraction with *n*-hexane allowed to remove the lipid components as FAME (Fatty Acid Methyl Esters).

The methanolic polar phase, containing the OMV monosaccharides as acetylated O-methyl-glycosides, was analyzed by GC with mass spectrometric detection equipped with a positive electron ionization mode source to allow univocal components identification through retention time comparison with standards and mass fragmentation pattern of the monosaccharides.

Figure 6.24 reports a representative GC-MS profile for OMV bulk in relation to its monosaccharide content. The acetylated O-methyl glycoside profile obtained confirmed the presence of monosaccharides belonging to the lipooligosaccharide

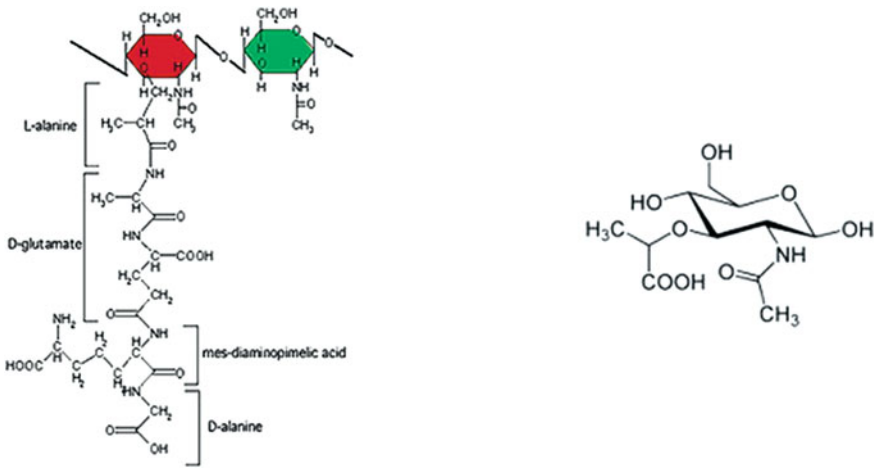


Fig. 6.23 Schematic representation of bacterial peptidoglycan, *left side*, and structure of NAM N-acetyl-muramic acid (2-N-acetyl-3-O-lactyl-glucosamine), *right side*

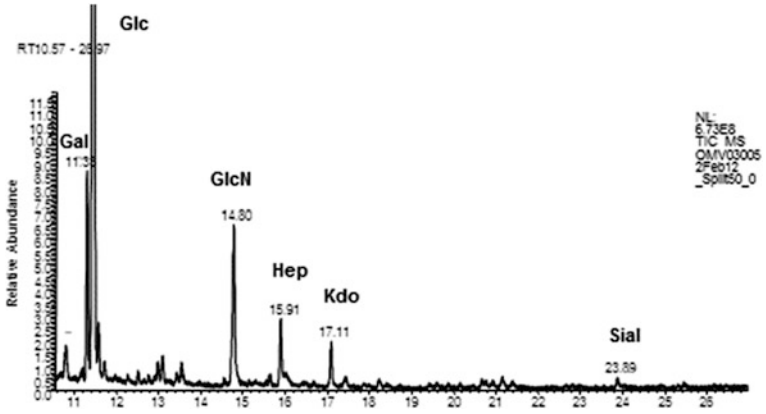


Fig. 6.24 GC-MS chromatogram (enlarged) of acetylated O-methyl glycosides of OMV bulk. Glucose comes from remaining traces of sucrose in the sample

structure: Gal, GlcN, Hep, Kdo, sialic acid. The latter can either derive from lipooligosaccharide or capsular polysaccharide (Pavliak et al. 1993) since it is a constituent of both structures. The predominance of glucose originates from sucrose (contained in 3 % as stabilizer) that was not entirely removed by ultrafiltration.

No traces of muramic acid (Mur) were detected in the saccharidic pattern of OMV. To confirm these results, Fig. 6.25 presents a comparison of the standard Mur elution (19.5 min, black trace) with the Mur spiked OMV sample (red trace), and the OMV sample without spike (blue trace). In the OMV sample without spike, no peaks corresponding to Mur were identified.

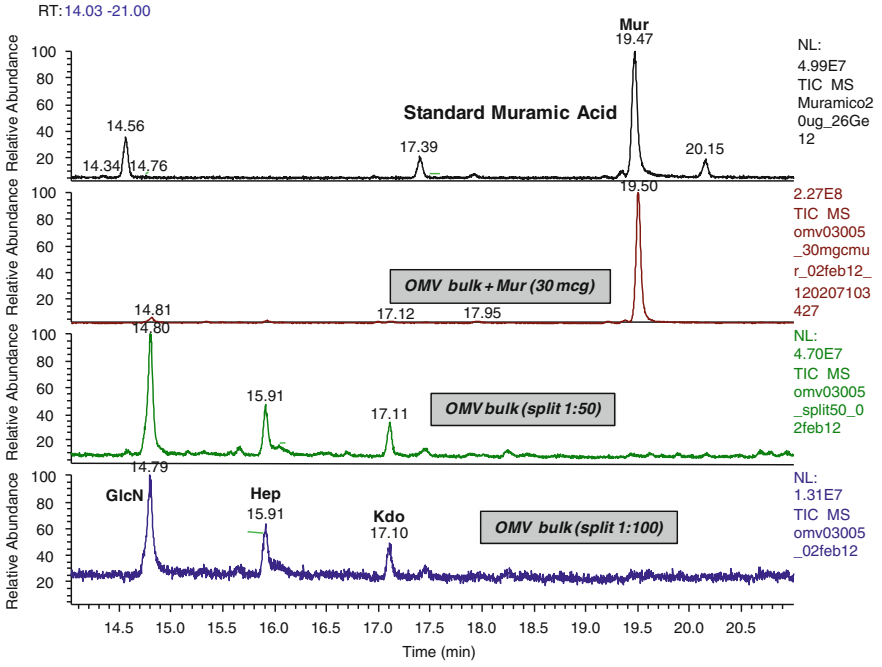


Fig. 6.25 Overlay of GC-MS chromatograms from OMV bulk w/o Mur spike, and Mur standard. The OMV sample without spike was analyzed with two different split arrangements

To increase sensitivity, a split of 1:50 was also used for OMV sample injection (green trace). The split allows a part of the sample injected by the syringe to be vaporized and escape through the split vent. A split ratio 1:100 (as normally used for the presented analyses) indicates that 100 parts are injected and 1 part goes to the column. A twofold quantity of sample is analyzed by split 1:50. The absence of Mur was confirmed also in this case.

The analysis was repeated on four lots. In all cases, no N-acetyl muramic acid was detected by the comparison of OMV sample w/o spike of the target Mur monosaccharide.

6.3.5 Overall Conclusions

The concentrated MenB OMV vaccine/vaccine intermediate is a stable colloidal suspension that consists of vesicles preserving the subcapsular cell surface composition of *N. meningitidis* serogroup B.

Due to the complex structure and presence of several antigenic components, the OMV particles present several analytical challenges. Here, we have described most of the classical and novel analytical methods required to characterize the OMV

particles at a structural and molecular level. Chemical composition and quantity of the single constituents are necessary but not sufficient for a complete characterization of OMV as a vaccine antigen. A comprehensive dimensional and morphologic characterization of the OMV particles can be obtained only by applying a number of analytical methods that target different properties of sizing and appearance. DLS, EM, and SE-HPLC represent complementary methodologies to detect vesicle degradation in forced degradation studies.

The OMV protein pattern is quantitatively dominated by OMPs like PorA and PorB porins. PorA is considered the immunodominant constituent of OMV in infants, but additional proteins may contribute to the immunogenic effect. The identity and relative amount of the main protein antigens in the OMV bulk are classically determined by the analysis of specific OMPs by SDS-PAGE with Coomassie Brilliant Blue staining and WB. In order to achieve a more comprehensive characterization of the OMV protein pattern mass spectrometric technologies are currently being developed. Mass spectrometry has been proven to be capable of simultaneously identifying and quantifying proteins out of complex mixtures and is ideally suitable for the OMV proteome.

LPS presence in OMV is monitored at bulk release both as total quantity and as bioavailable LPS. In addition to the physicochemical release test method by RP-HPLC specific for the MenB LPS quantification, an orthogonal GC-FID method has been developed to confirm LPS quantification results and to obtain a more comprehensive view of the OMV lipid pattern.

Presence of additional potential immunogenic OMV components was evaluated in addition to OMPs and LPS. The focus was on components that could have an impact on the safety and potency of the vaccine. The absence of significant quantities of PGN was verified. In addition, orthogonal methods for the monitoring of CPS were demonstrated to give consistent and reliable results.

6.4 Cholera Vaccine: Cholera Toxin B Subunit Derived Vaccine

6.4.1 Introduction

V. cholerae is a Gram-negative bacterium and the causative agent of cholera. Cholera is one of the dreaded epidemic and pandemic diseases. The disease has the unusual ability to spread rapidly to large numbers of people, to spread internationally and to kill a high proportion of those affected (WHO Cholera: global surveillance summary 2008; Bishop and Camilli 2011). Cholera is a rapidly dehydrating, watery diarrheal disease caused by intestinal infection with the bacterium *V. cholerae*, mainly of serogroup O1 that produces cholera toxins (CT). The division of *V. cholera* into O serogroups is based on its major surface antigen, the heat stable endotoxin. The endotoxin is a LPS, where the lipid portion of the

molecule is embedded in the cell wall and polysaccharide extends out from the surface of bacteria (Bishop and Camilli 2011). The classical biotype predominated up until the current pandemic, when the El Tor biotype surfaced and has since entirely replaced the classical biotype. Both the classical and O1 El Tor biotypes consist of two O1 serotypes: Ogawa and Inaba. A new serogroup, O139 was reported to be causing significant cholera-like disease in India and Bangladesh in 1992 (Ramamurthy et al. 1993; Cholera Working Group ICDDR, Bangladesh 1993; WHO 1991; 2000; Sack et al. 2004). Serogroup O139 appears to have arisen from O1 El Tor by the acquisition of a new LPS and capsule-encoding locus (Waldor and Mekalanos 1994). In fact, outside of the LPS locus, O139 shares the majority of traits with O1 El Tor. Although both serogroups O1 and O 139 can elicit serum antitoxin responses, infection with one serogroup has not been shown offer cross protection against the other (Albert et al. 1994). By contrast, the O1 El Tor and classical biotypes, despite having identical O-antigen loci, exhibit many genotypic and phenotypic differences.

V. cholerae survives passage through the stomach and colonizes the epithelial surface of the small intestine. *V. cholerae* uses adhesion factors, some of which may remain to be elucidated, that include: O1 LPS (Chitnis et al. 1982); GlcNAc-binding protein (GbpA) (Kirn et al. 2005); a protein (TcpF) secreted by the toxin coregulated pilus (TCP) biogenesis apparatus (Kirn and Taylor 2005); outer membrane protein OmpU (Sperandio et al. 1995); and CT, although this has only been implicated in an adult rabbit model (Pierce et al. 1985). TCP facilitates inter bacterial interactions that are important for colonization (Taylor et al. 1987; Herrington et al. 1988; Asaduzzaman et al. 2004). An effective cholera vaccine could prevent colonization by inducing the production of antibodies that directly neutralize the function of key colonization factors and/or facilitate phagocytosis and killing through bacterial opsonization (Bishop and Camilli 2011).

6.4.2 Cholera Vaccines

The following oral cholera vaccines are licensed and available without and with rCTB:

1. Killed Whole Cell (WC) of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus recombinant CT B (rCTB*) subunit vaccines—Dukoral™.
2. Killed WC (Modified WC-only) of *V. cholerae* (serogroups O1 classical and E1-Tor; possibly O139) and no clinical evaluation to date. This vaccine is formulated without rCTB vaccines—Shanchol™ or mORCVAX.
3. Killed Whole Cell-only of *V. cholerae* (serogroups O1 classical and E1-Tor; possibly O139) and no clinical evaluation to date—ORC-Vax™.

In this chapter section, we focus on CT derived B subunit protein (CTB) containing cholera vaccines and the vaccine analytical characterization and testing strategy for drug substance (bulk) and drug product (final product) release, stability,

and rationale for inclusion of recombinant rCTB based on guidelines for the production and control of inactivated oral cholera vaccines (WHO requirements as adopted in 2001; Annex 3, Guidelines for the production and control of inactivated oral cholera vaccines, WHO Technical Report, Series No. 924, 2004).

More details on licensed and in-use whole-cell inactivated and live-attenuated cholera vaccines can be found in Chap. 5 in this book.

6.4.3 *Dukoral™*

Dukoral was developed in Sweden and first licensed in 1991 and is currently produced by Crucell. It is licensed in >60 countries, primarily as a vaccine for travelers to cholera-endemic areas. However, it has also been used in crisis situations in Indonesia, Sudan, and Uganda, and in a demonstration project in an endemic area of Mozambique. Dukoral is a monovalent vaccine based on formalin and heat-killed WCs of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus rCTB subunit (see Package Insert and also Chap. 5 of this book). The B subunit of CT was originally produced chemically (WC-BS) but is now produced by recombinant technology (WC-rBS). B subunit and recombinant B subunit are identical in terms of immune response (Jertborn et al. 1992; Sanchez and Holmgren 1989). To protect the CT B subunit from being destroyed by gastric acid, the vaccine should be given with a bicarbonate buffer (Clemens et al. 1986). The vaccine is provided in 3-ml vials together with the bicarbonate buffer (effervescent granules in sachets). Vaccine and buffer are mixed in 150 ml of water for persons aged >5 years and in 75 ml of water for children aged 2–5 years (Crucell Product Package Insert and Chap. 5).

6.4.4 *Rational for Inclusion of Cholera Toxin B Subunit (CTB) in the Vaccine*

Cholera toxin is an oligomeric protein complex secreted by the bacterium *V. cholerae* and is the pathogenic agent responsible for the symptoms of cholera. The holotoxin, like many other bacterial toxins, is built up from two subunits A and B. The A-subunit, itself built up by two peptides A1 and A2, exerts the toxic enzymatic activity of the toxin. The B-subunit, in the form of a homopentamer, binds the toxin heterohexamer to GM1 ganglioside receptors on mammalian cell surfaces and facilitates entrance of the A subunit into the cell but has no toxic effect in itself. The A subunit bears the ADP-ribosyl-transferase activity, which deregulates the G protein causing activation of adenylate cyclase. The B subunit alone is considered nontoxic. In vitro, CT B subunit (CTB) is an efficient mucosal adjuvant and carrier molecule for the generation of mucosal antibody responses and/or induction of

systemic T-cell tolerance to linked antigens. Due to the ubiquitous occurrence of the GM1 ganglioside receptor on eukaryotic cell membranes, CTB can be used in a wide variety of model systems. CTB is also used for tract tracing in neurological research, taking advantage of its high affinity to GM1 gangliosides and retrograde transport (Tayot et al. 1981; Spangler 1992; Merritt et al. 1994, 1998).

The vaccine contains whole-cell bacteria to create an antibacterial response and the B subunit of CT to create an antitoxin response. A majority of the antibodies against CT, obtained either after natural disease or immunization, is directed against the B-subunit. Also, because most part of the A-subunit is embedded within the ring-formed homopentameric structure of the B-subunit (see Fig. 6.1), antibodies against the A subunit, even if they were induced, would have little effect on the neutralization of the toxin. Using the B-subunit alone instead of the whole CT molecule in the oral cholera vaccine will thus not noticeably reduce the neutralizing immune response (EMA 2005). The B subunit exists as a homopentamer surrounding the toxic A subunit (CTA) (Merritt et al. 1994, 1998; Dakterzada et al. 2012). A response to the B subunit will also neutralize the toxic effect of the CTA (Merritt et al. 1998). This is the rationale for inclusion of CTB in the vaccine. The current vaccine (Dukoral™) formulation contains recombinant XT B subunit (rCTB) (Fig. 6.26).

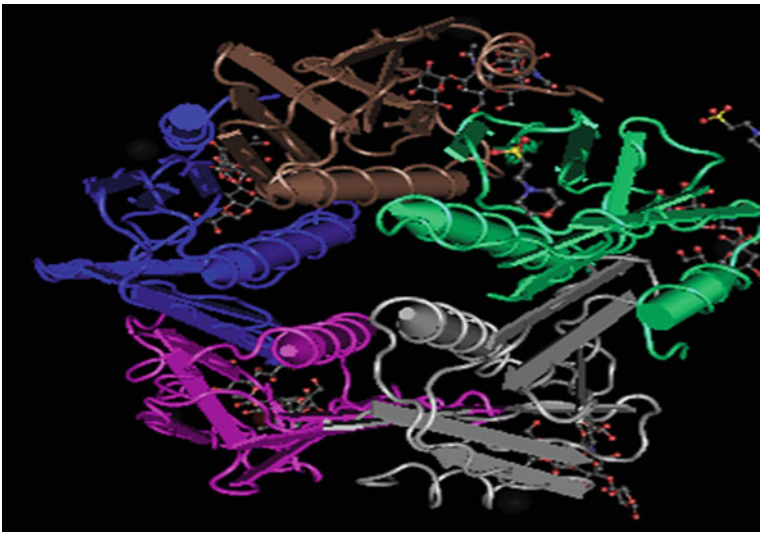


Fig. 6.26 X-ray diffraction structure of Cholera Toxin B-pentamer complexed with GM1 Pentasaccharide ganglioside receptors on mammalian cell surfaces and facilitates entrance of the A subunit into the cell (The 1.25 Å resolution). Figure adopted from reference Merritt et al. (1998). Printed with permission of NCBI NIH http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=8325

6.4.5 Cholera Vaccine Product Development

The vaccine is formulated as an oral suspension. The antigens are aseptically mixed with PBS and filled in 3.0 ml doses (EMEA 2005, Crucell Product Package Insert). This dose of the vaccine was chosen based on knowledge about intestinal immunity, indicating that the oral route is superior to the parenteral for stimulating an immune response in the gut (Jerborn et al. 1993). The only additive PBS was chosen to control the pH and salt concentration, in order to improve the stability of the antigens. Regardless of the fact that the vaccine is intended for oral administration and it is formulated as a sterile product (see Table 6.11 for details).

A bicarbonate buffer is included in the product and is taken with the vaccine in order to neutralize the stomach acid that otherwise destroys rCTB. The phosphate-buffered saline (PBS) consist/ml of Sodium dihydrogen phosphate monohydrate 0.576 mg Disodium phosphate dihydrate 3.13 mg, Sodium chloride 8.5 mg and water for injections add 1 ml (see Product Package Insert, Crucell) (Table 6.12).

Each ingredient is tested and released as per the standard methods described in EP and these analytical methods can be found in the Pharmacopeia.

6.4.6 Drug Substances (Final Bulks)

The whole-cell bulks are produced using standard manufacturing procedures (cGMP). A routine in-process and release test for this has been introduced. The bacterial bulks are produced from established seed-lot systems that are controlled by standard microbiological and biochemical methods generally used in vaccine production WHO 1992, Annex 1 (WHO Technical Report, Series No. 822); WHO 2004 Annex 3 (WHO Technical Report, Series No. 924).

Table 6.11 The complete composition of the WC of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa) plus recombinant cholera toxin B (rCTB) subunit vaccines is given

Ingredient	Reference	Amount	Function
Recombinant CTB		1 mg	Active ingredient
Vibrio cholerae O1 Inaba classical biotype, heat inactivated	Ph. Eur.	25×10^9 bacteria	Active ingredient
Vibrio cholerae O1Inaba El Tor biotype, formalin inactivated		25×10^9 bacteria	Active ingredient
Vibrio cholera O1Ogawa classical biotype heat inactivated		25×10^9 bacteria	Active ingredient
Vibrio cholerae O1Ogawa classical biotype, formalin inactivated		25×10^9 bacteria	Active ingredient
Phosphate-buffered saline pH 7.2–7.4		Ad 3 ml	Buffer

Table 6.12 The buffer sachet contains the following ingredients as specified in European Pharmacopeia

Ingredient	Reference	Amount	Function
Sodium hydrogen	Ph. Eur.	3. 6 g	Effervescentagent
Carbonate			
Citric acid anhydrous	Ph. Eur.	1.45 gm	Effervescent agent
Raspberry flavor	Monograph	70 mg	Aroma
Saccharin sodium	Ph. Eur.	30 mg	Sweeting agent
Sodium carbonate	Ph. Eur.	400 mg	Effervescent agent
Sodium citrate	Ph. Eur.	6 mg	Effervescent agent
Water, purified	Ph. Eur.	Disappears during manufacture	Granulating liquid

The active substances (bulk vaccine) are

1. rCTB subunit and
2. Four whole-cell bulks;

2a. *V. cholerae* O1 Inaba, classical biotype, heat inactivated,
2b. *V. cholerae* O1 Inaba, El Tor biotype, formalin inactivated
2c. *V. cholerae* O1 Ogawa, classical biotype heat inactivated and
2d. *V. cholerae* O1 Ogawa, classical biotype, formalin inactivated.

6.4.7 Vaccine Product and Analytical Development

6.4.7.1 RCTB Bulk (Drug Substance)

Initially during the development of vaccine production native CTB was used (the initial clinical studies) (EMEA 2005; Clemens et al. 1986; Peltola et al. 1991), and the current vaccine contains a recombinant CTB (rCTB) (EMEA 2005; Scarpella et al. 1995). Both molecules have been extensively characterized and shown to be comparable and identical, except for six short peptide native CTB extensions at the N-terminal in rCTB (EMEA 2005; WHO 2004). The rCTB is a protein of 102 amino acids containing one disulphide bridge (cys 9-cys 86). It is produced in *V. cholerae* strain 213 of serotype Inaba, biotype classical with a deleted CTA gene.

6.4.7.2 Characterization of rDNA-Derived B-Subunit (RCTB)

Rigorous characterization of the rDNA-derived B-subunit product should be undertaken using a variety of analytical techniques exploiting several different properties of the molecule, including size, charge, and amino acid composition.

Techniques suitable for such purposes include SDS-PAGE, size exclusion, and reverse-phase chromatography. Sufficient sequence information should be obtained by direct sequencing and by peptide mapping, or another appropriate molecular techniques. For example, mass spectrometry, in comparison with natural CTB. The identity of the product should be confirmed by at least partial N-terminal and C-terminal amino acid sequencing. Several lots of the product should be as fully characterized as possible (Annex 3, WHO 2004).

6.4.7.3 Biophysical Characterization of RCTB

Mass Spectrometry Analysis

Five or six molecular species with different molecular mass corresponding to different N-termini were found in MS analysis (EMEA 2005). The findings are consistent with what was found in N-terminal analysis where six different N-termini were detected, all of them extensions of the native CTB up to seven amino acids. No truncated forms compared to native CTB were found. The results are due to the construct of the vector and expression cassette and the variants found were the expected, based on the construct and forms in between (EMEA 2005).

RP-HPLC Analysis

After long time storage of the rCTB under refrigerated conditions and shorter under RT, there is a tendency of a shoulder of the peak found in the RP-HPLC which is explained to be related to the presence of low proteolytic enzyme activity, probably due to a type I signal peptidase. In no case has a sequence shorter than the native CTB been seen and the protease activity has been shown to decrease in later manufacturing batches. In view of the route of administration, the risk associated with the peptidase activity in the formulation is negligible when administered to humans and of no significance to the safety of the product (EMEA 2005).

MS have been used to determine the molecular weight and functional tests (GM1 binding, binding to monoclonal antibodies) have been performed. The DNA sequence has been confirmed and it is deemed unlikely that a protein emanating from the correct DNA sequence, with the molecular weight expected from the N- and C-terminal analyses and reacting comparably with native CTB in functional assays would differ from the expected structure (EMEA 2005).

Analytical methods and release specifications for rCTB bulk

rCTB bulk analytical methods and release specifications are shown in Table 6.13.

rCTB bulk stability indicating methods and release specifications are shown in Table 6.14.

Table 6.13 Examples of rCTB (as a component of recombinant cholera toxin B subunit) bulk release specifications

Critical quality attributes	Analytical method	Release specification	Rationale
Physical appearance	Visual inspection	Clear, colorless to weakly yellow solution. Some particles may occur	This test evaluates physical appearance of the bulk solution and should be free of aggregates and extraneous particles
pH	Potentiometry	7.0–7.6	The test measures pH of the whole-cell bulk suspension
Identification	Ouchterlony immunoelectrophoresis	Immunological identity with rCTB and CTB	The test measures immunological identity with rCTB and CTB by specific B subunit antibodies
Sterility	Membrane filtration (Ph. Eur.)	Sterile	The test measures sterility of the rCTB drug substance, required before vaccine formulation
Purity	RP-HPLC	<10 % unrelated proteins	The test evaluates the total % of unrelated proteins in the rCTB bulk by reverse-phase chromatography
Purity	SDS-PAGE	Not more than 2 bands visible; one major at 12 kD and one minor if present at 23 kD	The test measures by electrophoresis the presence of antigen bands. One major band representing rCTB and a minor band representing high molecular weight. It also evaluates purity of the bulk
Purity	SE-HPLC	Area of pentamer peak >90 % of integrated area	Size exclusion chromatography determines area of B subunit pentamer peak of rCTB and % of integrated area
Antigen concentration	Mancini	>1 mg rCTB/ml	The test measures antigen concentration as measured by single radial immunodiffusion
Protein content	Kjeldahl	>1 mg protein/ml	The test measures protein nitrogen and

(continued)

Table 6.13 (continued)

Critical quality attributes	Analytical method	Release specification	Rationale
			estimates protein content in the bulk
Antigenic purity	Antigen content/protein content	NLT 0.8 mg rCTB/mg protein	The test measures absorbance at 280 and 310 nm to determine rCTB purity. It evaluates the ratio of rCTB and protein content in the bulk

Table 6.14 rCTB stability indicating methods and release specifications

Critical quality attributes	Analytical method	Release specification
Physical appearance	Visual inspection	Clear, colorless to weakly yellow solution. Some particles may occur
pH	Potentiometry	7.0–7.6
Sterility	Membrane filtration (Ph. Eur.)	Sterile
Purity	RP-HPLC	<10 % unrelated proteins
Purity	SDS-PAGE	Not more than 2 bands visible; one major at 12 kD and one minor if present at 23 kD
Purity	SE-HPLC	Area of pentamer peak >90 % of integrated area
Antigen concentration	Mancini	>1 mg rCTB/ml
Protein nitrogen	Kjeldahl	>1 mg protein/ml
Antigen content/protein content	Absorbance at 280 and 310 nm by spectrophotometry	NLT 0.8 mg rCTB/mg protein

rCTB bulks are stored for a period of 36 months at 2–8 °C and it is substantiated by stability data. A shoulder is seen in the main peak in RP-HPLC chromatograms of samples stored for 6 months at 25 °C. Upon even further storage, this shoulder may even turn into a distinct peak and could also be detected in samples stored at 5 °C. This phenomenon was not observed in samples stored at −70 °C. It was shown that this was the result of cleavage of the longer N-terminal extra amino acids present in rCTB into shorter extensions, due to the presence of minute amounts of the signal peptidase I, responsible for cleavage of the signal peptide at the N-terminal of the rCTB molecule. However, no cleavage product shorter than native CTB has been found (EMEA 2005).

Whole-cell monovalent bacterial bulk (Drug Substance) analytical methods, release and stability specifications are shown in Table 6.15.

Table 6.15 Stability indicating release specifications for monovalent bacterial bulks

Critical quality attributes	Analytical method	Release specification
Physical appearance	Visual inspection	Beige opalescent suspension
Sterility	Ph. Eur. direct inoculation	Sterile
Homogeneity	Visual	Homogeneous suspension. No visible aggregation
Innocuity	Mouse weight-gain assay	No decrease in weight, no signs of morbidity
pH	Potentiometry	6.7–7.6
O1-LPS content	Inhibition ELISA	Tested and reported

Drug Substance Stability Indicating Methods (Whole-Cell bulks)

Whole-cell monovalent bacterial bulk (Drug Substance) stability indicating methods, and release specifications are shown in Table 6.16.

Whole-cell bulks (heat and formalin inactivated) are stored for a period of 3 years under refrigerated conditions and it is substantiated by stability data. The testing meets the EP and the WHO requirements (WHO 1992, Annex 1 (WHO Technical Report, Series No. 822); WHO 2004 Annex 3 (WHO Technical Report, Series No. 924). The other ingredient of the preparation is a PBS which constituents and testing meet the EP requirements (Ph. Eur.).

Table 6.16 Release and shelf life specifications for final bulk lot

Test attribute	Test method	Specification
Physical appearance	Visual control	Beige opalescent suspension
PH	Ph. Eur.	6.5–7.4
Homogeneity	Visual control	Homogeneous suspension
Purity	Gram staining	
Antigen concentration		Only gram-negative rods
O1-LPS	Inhibition ELISA	No visible aggregation
rCTB	Mancini	≥750 ELU/dose
Residual formaldehyde	Ph. Eur.	0.8–1.2 mg/dose
Sterility	Direct inoculation	<6.7 mM
		No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
		Identity with B subunit (rCTB/CTB)
	Ouchterlony	

Drug Product Formulation

The vaccine is formulated with the monovalent cholera bulks (see Table 6.11). As the bacteria are inactivated, live count can for this reason not be performed. The amount added is instead added based on the bacterial content prior to inactivation. The PBS buffer, the monovalent bulks and the rCTB bulk is aseptically mixed and stirred to homogeneity. Each vial is sealed with a rubber stopper and a screw cap with a safety ring (EMEA 2005).

The vaccine production process has been appropriately validated and is substantiated by batch analysis data, which conform to the preset acceptance criteria for including the final lot and final bulk specifications. Homogeneity of the bulk was maintained throughout the filling process, as demonstrated by measurements of the optical density at 600 nm of samples withdrawn during and after filling. Environmental monitoring and media fills also showed the aseptic nature of the process. Thus, the production process for the vaccine is shown to be suitable and to consistently yield a product of the desired quality (EMEA 2005).

Final bulk lot of vaccine release analytical methods and their specifications are shown in Table 6.16.

Final bulk lot of vaccine stability indicating analytical methods and their specifications are shown in Table 6.17.

Whole-cell (killed) rCTB finished product release analytical methods and their specifications are shown in Table 6.18.

As per WHO guidelines for the production and control of inactivated oral cholera vaccines (Annex 3, WHO 2004), analytical methods should be appropriately validated.

Table 6.17 Release and shelf life specifications for final lot

Test attribute	Test method	Specification
Sterility	Ph. Eur. direct inoculation	No microbial growth
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
	Ouchterlony	Identity with B subunit (rCTB/CTB)

Table 6.18 Release and shelf life specifications for WCK-rCTB finished product

Test attribute	Test method	Specification
Identity	Slide agglutination	Agglutination with Inaba and Ogawa specific antibodies
Control of labeling	Visual inspection	Labeling in accordance with specifications

6.4.8 Potency/Immunogenicity

At present, there is no animal potency or immunogenicity assay that can be recommended for use as a reliable indicator of the protective efficacy of inactivated oral cholera vaccine in humans or for the detection of subpotent batches. There is as yet no internationally accepted direct method for measuring the potencies of such oral products that guarantees that protective immunity will be elicited in the target population. At present, there is no animal model that can meaningfully be used to measure or predict the potency of these vaccines in humans (Annex 3, WHO 2004). Tables 6.7, 6.8 and 6.9 shows release and shelf life analytical methods for final bulk lots.

6.4.9 General Toxicity Assay

The mouse weight-gain assay in the testing of the cholera whole-cell bulks is aimed at indicating general toxicity as extra precaution in addition to the abnormal toxicity test according to Ph. Eur. This is due to the lack of a meaningful animal model for toxicity testing of oral killed whole-cell cholera vaccine. The assay has, however, not been validated for the purpose of detecting residual CT in the routine testing of the bulks or at the finished product stage which is required according to the WHO requirements. Therefore, the manufacturer has introduced a properly validated GM1 ELISA for routine testing of bulks for the detection of residual CT (EMEA 2005; Annex 3, WHO 2004).

6.4.10 Conclusion

CT is a multifunctional protein that is quite remarkable in many respects. CT was initially thought to be another enterotoxic protein that caused the life-threatening symptoms of cholera, however, numerous studies showed that CT possessed many unique features. Besides its unique structure, mode of intracellular trafficking, and ADP-ribosyltransferase activity. CTB possesses a multifaceted character with regard to functionality. The major contribution of this unique toxin has been in the field of Immunology, functioning as an effective immunogen, adjuvant, or an immunomodulator and holding promise in the area of therapeutics against various types of autoimmune diseases. Moreover, this toxin has a remarkable property for downregulating inflammatory reactions. The molecular mechanisms of its action could open avenues toward development and design of new anti-inflammatory agents for modulating various immune disorders in the foreseeable future (Merritt et al. 1998).

The currently licensed WCK-CTB vaccine has a higher efficacy in children <6 years of age (see Chap. 5 in this book). This vaccine is also considered disadvantageous in terms of high cost and need for cold chain distribution, and leaving room for an improved cholera vaccine for use in developing countries (Lopez et al. 2008; Cumberland 2009). In contrast, WCK without CTB vaccine produced affordably in Vietnam and technology transferred to Shantha Biotechnics (Shanchol, Sanofi Pasteur India) to produce and market internationally (Thiem et al. 2006; Anh at el. 2007; Mahalanabis et al. 2008). This vaccine is similar to Dukoral, except that the formalin-killed classical Ogawa strain Cairo 50 was replaced with strain 569B, in order to increase the amount of the putatively protective TcpA antigen, which is more efficiently expressed by strain 569B (Trach et al. 1997). Two doses of the Vietnam vaccine were shown to be safe, immunogenic, and 66 % protective 8–10 months after immunization for children 1–5 years of age and for older vaccines (Trach et al. 1997).

Though an affordable oral cholera vaccine (WCK without rCTB) has been developed by collaborative efforts from vaccine manufacturers (Vietnam, Shantha Biotechnics, India) and WHO for use in endemic regions, the current challenge for improvement of cholera vaccines during the coming years is the most effective protection of children in developing countries. Children are the most at risk from cholera due to a lack of preexisting immunity (Benenson et al. 1968a, b, c; Mosley et al. 1968; Deen et al. 2008), and a poor vibriocidal response has been observed for children to *V. cholera* vaccines or natural infection (Deen et al. 2008).

There are number of studies that are in progress to develop cholera vaccines with better characteristics of cross-serogroup protection, protection of children, and longer term protective memory (Bishop and Camilli 2011). A number of protein antigen candidates have been investigated using LPS-protein conjugates, outer membrane proteins (OMP), and adsorbed with LPS (Sengupta et al. 1992). Prior studies have shown the presence of anti-El Tor TcpA antibodies in pooled human serum and anti-TcpA seroconversion for 93 % of patients in Bangladesh (Hang et al. 2003; Larocque et al. 2008) and anti-TcpA responses have now been correlated with protection. In vivo-induced antigen technology has also been used to find immunogenic *V. cholerae* proteins expressed in vivo, including the type IV pilus proteins TcpA and PilA (Hang et al. 2003). The *V. cholerae* hemolysin HlyA generates a particularly robust and long-term memory T-cell response, making it a potentially useful immunogen that has not yet been extensively investigated (Weil et al. 2009). Proteomics of stool bacteria, although not yet carried out in a quantitative manner, may reveal insights into antigens expressed in the 'hyperinfectious' state that may also yield candidate protein antigens for inclusion in future cholera vaccines (Larocque et al. 2008). The WHO has issued an updated recommendation that states that vaccination should be used to help control of endemic and epidemic cholera. The availability of an affordable and a safe, feasibly delivered, effective oral vaccine that can be used in resource-limited regions, together with a new pipeline candidate vaccines that may be available in single-dose regimens and long-term protection in children and adults in the future to help fight cholera (WHO 2010; Shin et al. 2011).

Recent insights into correlates of protection, epidemiology and pathogenesis, as well as well characterization of biological vaccine with advance analytical methods, quality and consistent manufacturing may help us design improved vaccines.

6.5 Anthrax Protective Antigen

6.5.1 Introduction to Biodefense Vaccines

Recently, a weapons of mass destruction advisory panel reported to the United States congress that biological agents and not nuclear weapons represent the most significant world threat (A report of the National Biodefense Science Board 2010). Vaccines intended for prophylaxis against potential biological warfare threats (*biothreats*) require special care and procedures because their effectiveness cannot be tested in human challenge studies. They also entail added safety risk and precautions due to handling of the dangerous infectious microbes they target, essential to confirming efficacy in animal models that predict effectiveness in humans. Alternatively, it is difficult to conduct human efficacy clinical trials for new or existing medical countermeasure (MCM) against natural zoonotic infection, such as tularemia, plague, or viral equine encephalitis, because such outbreaks are unpredictable and entail geographical challenges. Instead, licensure in the United States for these MCMs will only be possible using the "Animal Rule" of the FDA, which allows for a demonstration of efficacy in one or more well-characterized animal models which are demonstrated to be relevant to both the disease and its proposed prophylaxis or treatment in humans (Wolfe et al. 2013).

6.5.2 Animal Rule

Vaccine development and ultimate licensure of a Biodefense (BD), countermeasure vaccine is different than that followed for non-BD vaccines. The FDA's Animal Rule was promulgated a decade ago to address the dilemma of not being able to conduct human efficacy trials for biothreats, and its application is based on demonstration in at least two mammal species with evidence of relevance to humans in disease pathology and physiologic/immunological mechanisms of protection (21 CFR 601, 21 CFR 314, FDA 2002 and Also see recent comment periods Federal Register 2009, 2014). This does not mean that studies in two animals is sufficient for a BLA, and may require more, depending on current understanding of the disease and treatment. Thus, testing under the Animal Rule serves as a surrogate for human clinical efficacy studies. Effectiveness must be tested in relevant models and using live, virulent agent. For biothreats of highest military/homeland security concern, the treatment must be protective against organism delivered via the aerosol

mode of exposure. The Animal Rule applies to any new drug designed against biothreats, including vaccines being developed for general use prophylaxis or postexposure prophylaxis, and therapeutics. To clarify some misconceptions, the FDA Animal Rule is not a fast track or accelerated route to licensure, and its purpose is for development of a product for human use, not animal protection. New BD vaccine products still encounter the conventional challenges of a new biological product development, including documentation of methods suitable to define product composition, in process control, release, stability, formulation, safety, and administration. Due to the requirement for demonstrating efficacy against virulent agent, a layer of complexity is added to the standard requirements and for development and validation of analytical methods for any new drug entity. In particular, nonclinical animal studies for potency require that scientists, technicians and operators to maintain top secret security clearance and work within specially designed, licensed, and monitored biological containment facilities. Within these constraints, a candidate must have robust institution sponsorship and programmatic funding to navigate a long and expensive path to a narrow market. Such costs and risks are a principal disincentive to the development of BD vaccine products by industry (Lang and Wood 1999). In response, government agencies have provided the means and funding to help offset these risks and help drive innovation in an effort to supply stockpiles needed for emergency response to biothreats (Matheny et al. 2007).

6.5.3 Anthrax Background

One of several microbial candidates believed to pose great risk as a potential biological weapons threat is the bacterium *B. anthracis*, the cause of the infection anthrax (Wistreich 2001). Anthrax is of particular concern because the infection can occur via inhalation and the etiological agent (spores) is resistant to extreme environmental conditions and so can be weaponized. Awareness, responsiveness, and development of medical countermeasures begun during the Gulf War of the early 1990s (Desert Storm) accelerated after the September 2001 adulteration of the US mail with Anthrax spores. Since then, the United States government has initiated a program to increase awareness, and has established a strategic plan and system for the detection and response to bioterrorist attacks (Wistreich 2001). Humans contract anthrax almost exclusively from contact with, ingestion of, or inhalation of *B. anthracis* spores. Cutaneous anthrax results from a break in the skin and has a mortality rate of about 20 % in untreated cases. Incubation is usually 2–3 days, although it can occur within 12 h and as late as 2 weeks. A small papule appears, followed by a surrounding ring of vesicles about 24 h later. The lesions ulcerate and become black and edematous. In pulmonary anthrax, inhaled spores are carried by macrophages from the lungs to adjacent lymph nodes. The spores germinate, multiply, and cause septicemia. Primary inflammation of the lungs

(pneumonia) may not be detectable (Leppla 2002). Clinical presentation progresses much faster (1–7 days) and mortality is high without immediate antibiotic treatment or prior prophylaxis.

6.5.4 Evolution of the Anthrax Vaccine, Anthrax Vaccine Adsorbed

The existing or *first generation* anthrax vaccine, called Anthrax Vaccine Adsorbed (AVA), originated with studies performed in the 1950s and was first licensed for use in humans in 1970. AVA is produced from culture filtrates of an avirulent, nonencapsulated mutant of the *B. anthracis* Vollum Strain known as V770-NP1-R. AVA, which does not contain *B. anthracis* cells or spores, is a mix of cellular products and contains all three toxic components of lethal factor (LF), edema factor (EF), and protective antigen (PA). However, PA is the principal protein ingredient and primary immune-protective component of AVA (Morb and Mort Weekly Report 2000). The vaccine is now manufactured by Emergent BioSolutions, Inc., USA under the label of BioThrax® AVA (BioThrax® Package Insert—Anthrax Vaccine Adsorbed). To date, more than 11.6 million doses of BioThrax® (Anthrax Vaccine Adsorbed) have been administered to more than 2.9 million individuals. BioThrax® is indicated for the active immunization of individuals between 18 and 65 years of age at high risk of exposure to anthrax. BioThrax® is not licensed for use in a postexposure setting. The safety and efficacy of BioThrax® have not been established in pregnant women, nursing mothers, pediatric populations, or geriatric populations (BioThrax® Package Insert—Anthrax Vaccine Adsorbed).

BioThrax® is a sterile, milky-white suspension for intramuscular injections made from cell-free filtrates of microaerophilic cultures of an avirulent, nonencapsulated strain of *B. anthracis*. The production cultures are grown in a chemically defined protein-free medium consisting of a mixture of amino acids, vitamins, inorganic salts and sugars. The final product, prepared from the sterile filtrate culture fluid contains protein, including the PA protein, released during the growth period, and contains no dead or live bacteria. The final product is formulated to contain 1.2 mg/mL aluminum, added as aluminum hydroxide in 0.85 % sodium chloride. The authors note that elemental aluminum varies among anthrax vaccines, ranging 0.6–2.4 mg/mL in vaccines described below. The final product is formulated to contain 25 μg/mL benzethonium chloride and 100 μg/mL formaldehyde, and added as preservatives (FDA 2012). The Final Drug Product also contains amino acids, vitamins, inorganic salts and sugars (CDC 2012). Some early lots of AVA appeared to contain small amounts of LF and lesser amounts of EF, as determined by induction of antibody responses in the animals, although this has not been reported in human vaccines. No detectable EF was found by Western blotting analysis. ELISA studies found LF to be present in the range of 10–30 ng/mL of fermentation filtrate before aluminum hydroxide adsorption (Joellenbeck et al. 2002). Mouse

macrophage cytotoxicity assay showed that LF is present in a biologically inactive form. Although it is clear that PA by itself is an effective immunogen, it is unknown whether the small amounts of LF or EF that may be present in some lots of the vaccine contribute to protective efficacy (Plotkin et al. 2008).

An FDA approved potency assay for BioThrax® measures survival of vaccinated guinea pigs that are challenged with ~500 LD_{50} of Vollum 1B strain spores injected via intradermal route on the side of the abdomen opposite the vaccination site. Assay details can be found in Gu et al. (2007).

The vaccine is stored at 2–8 °C and is stable for 3 years after a successful potency test results. This vaccine is administered under the skin (subcutaneous, s.c.), rather than directly into the muscle (intramuscular, i.m.). Since subcutaneous administration may be partly responsible for the high number of observed local side effects "(temporary soreness, redness, swelling, itching, and lumps at the injection site in 30 % of male recipients and 60 % of female recipients)," the CDC and collaborators are conducting a human clinical trial of BioThrax® to compare reactogenicity and immunogenicity via the s.c. and i.m. routes of administration. Following the study, the sponsor will present the entire results of the trial to FDA for consideration in elimination of additional doses from the licensed BioThrax® schedule. The sponsor will also supplement clinical data with results from parallel nonhuman primate (NHP) challenge studies plus additional research on immunologic correlates of protection (CDC).

6.5.5 Other Anthrax Licensed Vaccines

The Center for Applied Microbiological Research (Porton Down Salisbury, Wiltshire, UK) developed a similar Anthrax vaccine precipitated by the sterile cell-free culture filtrate of a derivative of the attenuated, unencapsulated Stern stain 34F2 with aluminum potassium sulfate (Hambleton et al. 1984). This vaccine was first administered to humans in the early 1950s and licensed in 1979 (Plotkin et al. 2008). The vaccine contains thimerosal as a preservative. The method to test thimerosal is discussed elsewhere in the chapter (Shrivastaw and Singh 1995a, b). The UK vaccine is administered intramuscular in a regimen of three 0.5 mL doses at 0, 3, and 6 weeks, with a booster dose 6 months after the third dose. Subsequent booster doses are given annually (Turnbull 1991).

A vaccine consisting of a suspension of live spores (similar to Stern strains), named STI-1 for the Sanitary-Technical Institution, has been used for humans in the Soviet Union and its subsequent independent republics since 1953. This vaccine, manufactured by Tblisi Scientific Research Institute of Vaccines and serums (Tblisi, Georgia), the Institute of Microbiology [Kirov (Viatka), Russia Federation], is given by scarification through a 10- to 20-mcL drop of vaccine containing 1.3 to 4×10^8 spores or subcutaneously. The initial dose is followed by a second dose 21 days later, with yearly booster (Plotkin et al. 2008). The Lanzhou Institute of

Biological Products (Lanzhou, Gansu, People's Republic of China) produced in the 1960s another live spore human vaccine given by scarification. This vaccine is based on avirulent strain A16R and a single dose contains 1.6 to 2.4 × 10^8 colony-forming units. A single booster dose is given 6–12 months after the first vaccination (Plotkin et al. 2008).

In the USA, the principal purchasers of the vaccine are the Department of Defense and the Department of Health and Human Services. Ten million doses of AVA have been purchased for the US Strategic National Stockpile for use in the event of a mass bioterrorist attack, used primarily for immunization of troops and defense contractors. However, due to the lengths of time to reach full immunity (18 months for the primary series of 3–6 doses), known reactogenicity, a record of adverse events, and governmental funding for discovery research and product development, there has been renewed interest and effort for the development of an improved anthrax vaccine.

It has been suggested that other antigens in addition to PA would confer improved protection, including those from the spore surface, the vegetative cell capsule and S-layer, as well as LF and ET toxin components (Cote et al. 2012; Kaur and Bhatnagar 2011; Friedlander and Little 2009). These approaches are still early in development. However, as supported by an abundance of basic information plus preclinical and clinical data, the next vaccine after AVA (second generation) will likely be a liquid formulation of rPA combined with an aluminum salt adjuvant. The remainder of this section focuses on the analysis of human anthrax vaccines, including both recombinant PA (rPA) subunit vaccines, as the leading second-generation candidate product form under development, and AVA (BioThrax) for which solutions to efficacy and potency have been pioneered. The current approach from funding agencies and regulatory bodies is that the new rPA vaccines are noninferior to BioThrax.

6.5.6 Pathophysiology and the PA Subunit Approach

Anthrax disease is caused by the gram-positive spore-forming bacterium *B. anthracis*. It is the only member of the genus *Bacillus* capable of causing lethal epidemic disease in humans and other mammals, though closely related *B. cereus* and *B. thuringiensis* species cause human gastroenteritis outbreaks and nosocomial pseudoepidemics (Al-Abir et al. 2011; Jackson et al. 1995). The anthrax toxin is a binary exotoxin comprised from three protein modules: PA functions as a transporter after biding to either lethal factor, forming lethal toxin (LeTx), or to edema factor, forming edema factor (EdTx). Once internalized, LF causes macrophage lysis, immune-system suppression, and death (Agrawal 2004; Friedlander 1986). As a calmodulin-dependent adenylate cyclase, EF stimulates production of the secondary messenger 3′-5′-cyclic adenosine monophosphate (cAMP), contributing to the establishment of infection and anthrax pathophysiology (Liu et al. 2013). Being central to both LeTx and EdTx holotoxins, PA is so named because by itself

it can induce antibodies that protect against both LeTx and EdTx and confer protective immunity against the disease.

PA is a 735 amino acid protein (82,684 kDa), also known as PA83. PA83 binds to a cell surface receptor, where it is cleaved by a furin-like protease, to yield an active 63 kDa fragment (PA63) and an N-terminal 20 kDa fragment (PA20) of unknown function (Hammamieh et al. 2008). Cleavage is essential for toxin action, as PA harboring mutations in the furin cleavage site is completely nontoxic to murine macrophages and devoid of pathogenic effects in vivo in sensitive mice when reconstituted with LF (Brey 2005). Current understanding models effector-bound PA63 holotoxin as an octomer (e.g., PA_8LF_4), which is endocytosed and then translocates effector (e.g., LF) into the acidified endosome (Feld et al. 2010). Although the key targets LT and ET causing host lethality are still unknown, it is believed that pathophysiology results from their coordinately damage to two distinct vital systems composed of myeloid cells and hepatocytes (Liu et al. 2013; Cote et al. 2011). As PA is essential to the function of both toxins and anthrax pathophysiology, PA is the proposed primary immunogen for an improved human anthrax vaccine against the biothreat posed by *B. anthracis* spores.

The subunit PA anthrax vaccine has been shown in numerous animal studies to confer protective immunity against lethal aerosol challenge (Little et al. 2006, 2007; Williamson et al. 2005; Laird et al. 2004). A variety of adjuvants have been shown to be effective for inducing protective immunity of PA formulations in animal spore challenge studies, including aluminum salts, Saponin Monophsophoryl lipid A, and spore antigens (Ivins et al. 1998; Berthold et al. 2005; Cote et al. 2012; Peachman et al. 2012). Nevertheless, the aluminum hydroxide salts of alum (aluminum hydroxide gel) or Alhydrogel® (aluminum oxyhydroxide gel) are the adjuvants used in the currently licensed vaccine and the leading second-generation anthrax vaccine candidate products. While the choice of these formulations is based on ample prior testing and evidence, the deposition of a protein antigen active ingredient onto an aluminum salt presents challenges for the design and use of stability methods, as is discussed further below.

6.5.7 Recombinant PA (RPA)

The first rPA was expressed in, and purified from, the supernatant of a nonsporogenic, avirulent *B. anthracis* strain (Ivins et al. 1995, 1998; Farchaus et al. 1998). Substantial effort was given to reducing fragments observed as a major source of impurities (Rhie et al. 2005; Jendrek et al. 2003; Ramirez et al. 2002; Miller et al. 1998). Other popular derivations express and produce rPA in *E. coli* (Brown et al. 2010; Laird et al. 2004; Gwinn et al. 2006; Williamson et al. 2005), as pioneered by demonstration of unusually pure rPA protein from inclusion bodies (Gupta et al. 1999). In one of these processes, soluble rPA is expressed in relatively high amounts in the periplasm of *E. coli* from shake flasks and bioreactors and purified using Q-Sepharose-HP and hydroxyapatite chromatography, and routinely found to

be 96–98 % pure. Yields of purified PA varied depending on the method of production; however, medium cell density fermentations resulted in approximately 370 mg/L of highly pure biologically active PA protein. These results exhibit the ability to generate gram quantities of PA from *E. coli*.

Following is a discussion of the analytical approaches used in the development of rPA anthrax vaccines. Some of these methods have translated for use as release assays.

6.5.8 Analysis of the Recombinant Anthrax Protective Antigen

6.5.8.1 Animal Survival Models and Protective Immunity

Protective immunity is accessed via animal survival studies against an aerosol challenge of *B. anthracis* spores. A number of animal models have been used including mice, guinea pig, rabbit, and up to NHP models. An FDA advisory meeting report has indicted that both the NHP and the New Zealand white (NZW) rabbit are considered animal models of choice to generate pivotal animal survival data for assessing the efficacy of a PA-based vaccine (FDA 2010). Animals are vaccinated and then challenged with a targeted dose of LD_{50} spores from the Ames isolate of *B. anthracis* and monitored for survival. The LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores (Pitt et al. 2001). Since survival data alone is no longer sufficient, a primary goal of such studies is to define antibody response in a given animal model for developing a serological correlate of protective immunity, as described next. Concentration of protective antibody can be demonstrated as a correlate of protection if an increase in the amount of inoculated vaccine immunogen produces increased survival after challenge and increased antibody response. A correlate of protective immunity against anthrax has been identified for NZW rabbits immunized with a recombinant PA (rPA) vaccine formulated with aluminum hydroxide adjuvant (Alhydrogel) (Little et al. 2004a, b; Pitt et al. 2001). Due to the presence of PA as principal immune component, animal models and potency tests for AVA (BioThrax) have served as the benchmark for development and regulatory oversight of similar requirements for rPA subunit vaccines. Human protection obviously cannot be test; the approach is to compare human serology to a comparative, *Human Equivalent*, response in a model animal. This also applies to the notion of potency.

6.5.8.2 Potency Assays

Due to considerations of animal welfare, assay variability, plus the high cost and security requirements for handling biological threat agent such as viable anthrax

spores, the FDA and NIH have issued goals to replace assays of lethal animal challenge. As a result, immunogenicity assays are developed as potency tests and serological surrogate of protection. Two potency assays for anthrax vaccines have centered on measuring the activity or concentration of specific antibodies, based either on quantitative anti-rPA IgG ELISA or on macrophage lysis tests known as toxin neutralizing antibody assay (TNA). Both have been compared and serological correlates of protection for each have been established in rabbits for a recombinant anthrax vaccine (Parreiras et al. 2009; Little et al. 2004a, b; Pitt et al. 2001). An FDA effort to justify either the ELISA or TNA approach for potency measurement has indicated that the ELISA may not be as reliable (Brady et al. 2010). A TNA has been validated that measures the functional ability of antisera to specifically protect J774A.1 cells against *B. anthracis* LeTx cytotoxicity using human and rabbit antisera produced against the AVA vaccine (Hering et al. 2004). An important additional concern for potency testing is the maintenance of recombinant LF protein (rLF) and macrophage cell lines as critical reagents for TNA-based assays.

6.5.8.3 Biophysical Methods for Lot Release

Although specific lot release strategies are proprietary for rPA products in development, the general approach follows FDA guidelines for API release and DS release, including tests for identity, strength, purity, and potency of API, residual concentrations (antibiotics, endotoxin) strength of excipients, preservatives, stabilizers, and adjuvants.

The purity of the PA product has been characterized by reversed-phase high-pressure liquid chromatography (Farchaus et al. 1998), sodium dodecyl sulfate (SDS)-capillary electrophoresis, capillary isoelectric focusing, native gel electrophoresis, and SDS-polyacrylamide gel electrophoresis (PAGE). Methods for rPA identity, quality, strength, and structural characterization have included PAGE, Ion exchange chromatography (IEX), isoelectric focusing IEF/cIEF, RP-HPLC, HP-SEC, peptide mapping (LC/MS), circular dichroism (CD), differential scanning calorimetry (DSC), intrinsic fluorescence (Wagner et al. 2012; Soliakov et al. 2012), and light scattering.

6.5.8.4 Stability

While a standard ensemble of release and stability tests applies to rPA for insuring consistency in purity, strength, and quality, cumulative evidence has indicated that deamidation is the principle stability issue confronting rPA as API and formulated DP (Verma et al. 2013; D'Souza et al. 2013; Powell et al. 2007; Ribot et al. 2006; Zomber et al. 2005). Figure 6.27 illustrates correlation between the progression of charged isoforms, loss of biological activity, and increased deamidation at specific sites (Powell et al. 2007). Fragmentation, oxidation, and aggregation are also associated with rPA degradation though to lesser extents than deamidation.

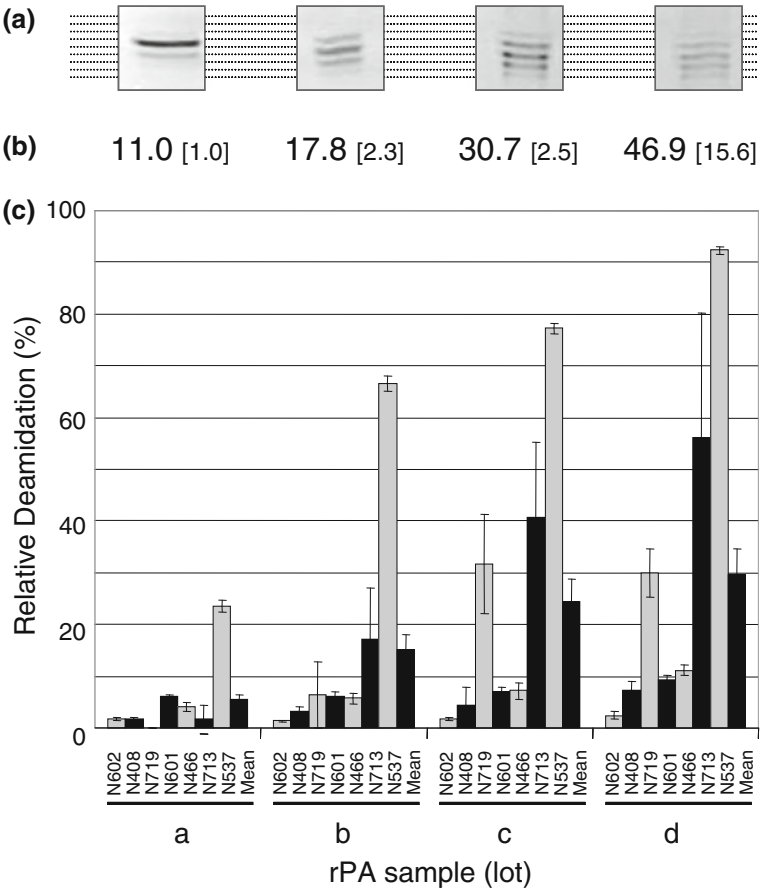


Fig. 6.27 Multiple-site deamidation and decreased biological activity correlate with increased isoform complexity of different rPA preparations. Comparison of isoform complexity and biological activity with percent deamidation at multiple Asn sites in different rPA preparations. **A** Native PhastGels of whole PA protein preparations as designated below. Horizontal guidelines aid in comparing vertical positions of protein isoforms between gels. **B** Cytotoxic activities of same PA preparations given in EC50 (ng/mL) with standard deviation in brackets. **C** Relative deamidation for seven Asn residues in each protein preparation (abscissa) expressed as percent deamidated (ordinate). Samples, *vertically* aligned with gel images in A, comprise: *a* List Biologicals; *b* R&D rPA lot 121096a; *c* R&D rPA lot 121096b; *d* rPA lot 022795b. *Residues* (1) N602; (2) N408; (3) N719; (4) N601; (5) N466; (6) N713; (7) N537; *M* mean. The figure is adopted from Powell et al. (2007) and reproduced with permission from John Wiley and Son

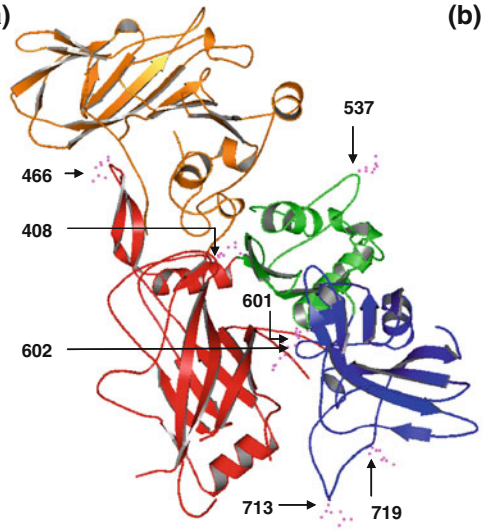
Fragmentation has largely been resolved with preparations of higher purity, including expression in *E. coli* and improved purification schemes, as noted above. Structural properties and quality attributes of rPA in these respects have been measured by a variety of methods which require supplementary preanalytical techniques to desorb protein from the adjuvant matrix in formulated product, as discussed below.

6.5.8.5 Quality

As discussed, charge heterogeneity is a principal concern for rPA release and stability. Charge isoforms in the anthrax toxin proteins were noted since their first isolation and this phenomenon is now known to be caused by deamidation at specific asparagines (Powell et al. 2007). Of the PAGE methods employed, the native PhastGel (GE Healthcare) provides a rapid and simple analysis for screening rPA charge isoforms (Ribot et al. 2006; Powell et al. 2007). Standard and two-dimensional PAGE provide better separation for analysis of whole protein and isoforms (Powell et al. 2007; Zomber et al. 2005). While anion exchange chromatography (IEX) has been used to isolate isoforms for structural and functional study (Ribot et al. 2006), isoelectric focusing (IEF) in gel and capillary format (cIEF) have been useful in monitoring rPA charge heterogeneity (Ribot et al. 2006; Powell et al. 2007; Zomber et al. 2005). Peptide mapping and amino acid sequencing by mass spectrometry (LC/MS and LC-MS/MS) enable the direct connection between charge heterogeneity and deamidation, as noted above (Verma et al. 2013; D'Souza et al. 2013; Powell et al. 2007; Zomber et al. 2005). Figure 6.2 shows a ribbon structure depiction of rPA and the most labile asparagines (Powell et al. 2007). An important general finding from this work, applicable to all protein products intended for use in humans, is the demonstration that computer algorithms for predicting deamidation are not yet sufficiently accurate to eliminate deamidation as potential route of product degradation. Such models are built on peptide data, and proteins deamidate differentially as has been shown by empirical evidence (D'Souza et al. 2013; Powell et al. 2007; Zomber et al. 2005). For example, in rPA, AsnN537 showed the highest percent of deamidation at all stages of purification tested, although its predicted rate of deamidation ranked 10th by, and alternatively, other asparagines of higher predicted rates were unmodified (Fig. 6.28).

Direct correlation between isoform complexity and percent deamidation has been observed such that each decreased with purity and increased with protein aging or forced stress (Powell et al. 2007; D'Souza et al. 2013). rPA with more isoforms and greater deamidation displayed lower activities for furin cleavage, heptamerization, and holotoxin formation (Powell et al. 2007) and has been confirmed in other rPA products both as pure protein (Zomber et al. 2005) or bound to adjuvant (D'Souza et al. 2013), including observation that ASN 713 and 719 deamidation rapidly when rPA is adjuvanted on Alhydrogel® (D'Souza et al. 2013). To further explore whether deamidation of rPA affects vaccine immunogenicity, recombinant "genetically deamidated" forms of rPA using site-directed mutagenesis that replaced six deamidation-prone asparagine residues with aspartate, glutamine, or alanine. While the structure and biological function of the six-Asp mutant rPA was not significantly altered relative to the wild-type rPA protein product, its immunogenicity as an aluminum-adjuvanted vaccine was significantly lower than the wild-type rPA vaccine formulation by TNA (Verma et al. 2013). This confirmed that specific deamidation of rPA adversely affects the immunogenicity of rPA, and suggests that concurrent deamidation six sites may be a threshold for such effect.

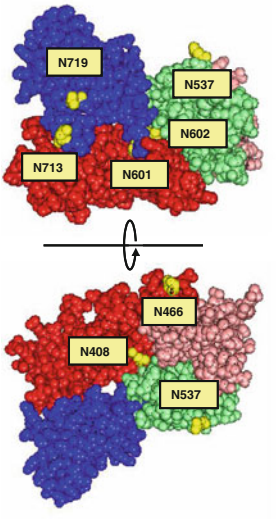
(a)



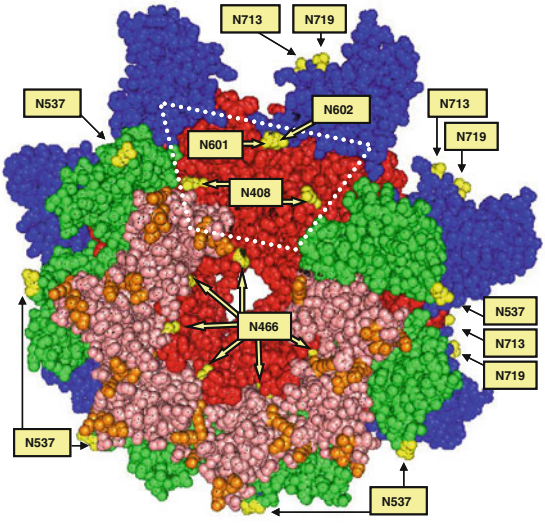
(b)

Asn Site	$t_{1/2}$ (days)	Rank Order		
		P	F	T
713	24	1st	2nd	2nd
162	38	2nd	0	0
719	49	3rd	5th	3rd
306	53	4th	0	0
010	57	5th	0	0
102	67	6th	0	0
006	81	7th	0	0
281	117	8th	0	0
466	133	9th	3th	4th
537	219	10th	1st	1st
408	4363	37th	6th	6th
601	48150	57th	4th	5th
602	2,594,300	68th	7th	7th

(c)



(d)



◀ **Fig. 6.28** Comparison of computational and observed deamidation in rPA. **a** Table comparing rank order of observed Asn deamidation to that predicted by rate at www.deamidation.org using the protein database identifier for PA (1ACC). Abbreviations as follows: Asn site, amino acid coordinate of Asn as defined in Fig. 6.2. t1/2, half-life in days of deamidation calculated from the theoretical coefficient of deamidation; rank order for propensity of deamidation as listed by: *P* predicted rate calculated by the Robinson algorithm; *F* observed percentage for fresh PA protein; *T* observed percentage for treated PA protein; 0 no deamidation observed; **b** Projection of seven observed sites of deamidation onto the three-dimensional structure of PA. *Arrows mark* deamidated Asp with numeric designation by coordinate number, as defined in Fig. 6.5; *pink dots* projection of original asparginyl side group. Color scheme as follows: *domain 1* (*orange*, residues 1–258) contains two calcium-binding sites, the cleavage site for furin class proteases, and the binding site for EF and lethal LF. *Domain 2* (*red*, 259–487) forms most of the channel and protein–protein interactions. *Domain 3* (*green*, 488–595) may play a role in oligomerization. *Domain 4* (*blue*, 596–735) is responsible for binding the cell surface receptor; **c** following page, Projection of seven observed Asn deamidation sites onto a space-filling model of the X-ray structure of monomeric PA, and rotated 1808 around its long axis for full viewing. Color scheme as for panel B except domain 1 is *pink*; **d** projection of seven observed Asn deamidation sites onto the modeled structure of PA heptameric prepore (PA63)7, central pore axis angled slightly *down* and *left*, and one LF-binding domain (*pink*) removed (*dotted trapezoid*), to expose buried sites and position others for viewing. Deamidation at residue N537 is highest among all samples tested, while deamidation at residues N408 and N466 statistically correlate with loss of PA biological activity. Same coloration as in panel C except LF/EF factor binding sites are shown in *orange*. The figure is adopted from Powell et al. (2007) and reproduced with permission from John Wiley and Son

6.5.9 Adjuvanted Formulated Vaccine

As a formulated drug product, rPA is combined with an adjuvant since it is not protective by itself, and aluminum salts such as Alhydrogel® are used in current development of second-generation anthrax vaccines. In order to apply structural assays developed on soluble rPA, the methods must be modified, first to remove (desorb) protein from the adjuvant and then to adjust acceptance criteria. Desorption methods must strip protein from the aluminum depot, in sufficient amount and without altering quality, and then leave it in a matrix suitable for analysis. Assay specifications and acceptance need to be reestablished since some alteration occurs as protein is bound to and then removed from aluminum salts.

Bound rPA can be desorbed from Alhydrogel by 18-h exposure to 25 mM phosphate ion to yield test material that confirms to rPA standard by SDS-PAGE and HP-SEC (Jendrek et al. 2003). Recovery drops from 75 to 12 % in the presence of 10 mM EDTA. Analysis since then has shown that the native structure of rPA, which binds two moles of 2 mol of calcium in Domain 1 per mole of protein, is not disrupted by adjuvanting and that Alhydrogel does not bind residual calcium (Soliakov et al. 2012). Other successful methods have been developed for desorption, including the use of chaotropic agents or detergents for subsequent analysis by SDS-PAGE, RP-HPLC, cIEF, and LC-MS.

To circumvent the complications of desorbing protein prior to analysis, in situ methods have been developed to characterize rPA structure as bound to Alhydrogel®. There is a concern that structural and functional properties of rPA may alter

after long-term adsorption to aluminum adjuvants, leading to instability of quality and efficacy (Wagner et al. 2012). Several studies describe dynamic changes in rPA structure and immunogenicity while it is adsorbed to Alhydrogel® (Wagner et al. 2012; Solaikov et al. 2012; Hu et al. 2012; Ganessan et al. 2012; Watkinson et al. 2013), and deamidation appears to increase with contact of rPA to Alhydrogel® (D'Souza et al. 2013). As yet, there is no direct connection between changes in secondary structure and loss of protection against a lethal challenge. Nevertheless, aluminum adjuvants including Alhydrogel® clearly increase the stability of thawed rPA protein in vitro and in vivo (Watkinson et al. 2013).

6.6 Conclusions

Recombinant bacterial subunit vaccines are 1. Purified surface and secreted proteins, 2. Genetically Detoxified virulence factors, 3. Mutated chemical proteins, and 4. Polysaccharide-protein complexes. These are made by manipulating bacterial pathogen's reverse vaccinology and structural vaccinology and cloning and expression in heterologous host and thereby expressing and purifying these recombinant bacterial subunit vaccines. These subunit vaccines offer few major advantages and disadvantages (Unnikrishnan et al. 2012), which can be summarized as follows:

Advantages	Disadvantages
• Well-characterized products	• Manufacturing process highly complex and demanding
• Increased safety and efficacy	• Usually need proper adjuvants
• Only the useful and more potent antigens can be included in the vaccine	• Subjected to post-translational modification and not guaranteed in a heterologous system

Subunit vaccines, containing specific antigens or their respective genes from pathogenic organisms, are regarded as one of the safest, although their efficacy is lower than that observed for vaccines consisting of whole, attenuated cells. To increase efficacy, one strategy would be to obtain a universal vaccine against many isolates of specific bacterial species is to include more than one antigen into the vaccine prototype as noted above. And the other way to enhance and/or modulate the host immune response is to deliver a vaccine with an appropriate adjuvant. The tremendous progress of proteomics during the past few years has contributed to every successive stage of antigen characterization (Adamczyk-Poplawska et al. 2011).

In summary, recombinant DNA technology has enabled the rationale design of vaccines against complex human diseases, including of subunit vaccines as described in this chapter. Despite excellent evidence of success of recombinant vaccines in preclinical and clinical studies, few recombinant vaccines are licensed for human use. This can however be attributed to the intense regulatory process

revolving around recombinant vaccine production. In the case of medical countermeasures against biological threats such as anthrax, there are additional rules and regulations including adherence to biosecurity licensing and oversight plus demonstration of proposed human efficacy via the Animal Rule. Improved adjuvant technologies, new approaches like systems biology, proteomics and genomics, and rapidly advancing technologies for enhanced manipulation of humoral, cellular, and innate immunity will all play a role in driving the next generation of protein subunit vaccines (Unnikrishnan et al. 2012). Finally, with the advent of the first synthetic bacterium, applications of synthetic biology in vaccine design will be the most rational approach in the years to come.

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