Annex 2

REQUIREMENTS FOR MENINGOCOCCAL POLYSACCHARIDE VACCINE

(Requirements for Biological Substances No. 23)

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INTRODUCTION

There have been numerous attempts to develop vaccines against meningococcal meningitis employing whole-cell suspensions or autolysates. Unfortunately, however, the data from trials using these vaccines were often difficult to interpret because the trials were not adequately controlled.

Over the last few years considerable progress has been made in the development of vaccines containing purified capsular polysaccharides of Group A and Group C meningococci. Controlled field trials of
the Group A vaccine in Egypt, Finland and the Sudan have demonstrated excellent protection against the clinical disease, and similar studies in the USA with the Group C vaccine have shown it to be 90% effective. The protection is group-specific. As these vaccines are already in use in public health practice in several countries their standardization is necessary.

GENERAL CONSIDERATIONS

Meningococcal polysaccharide vaccines differ substantially from commonly used whole-cell bacterial vaccines and the requirements therefore differ in many particulars from those applied to other vaccines. The most important difference is that these products are made from highly purified polysaccharides that are partially chemically defined so that they can be substantially characterized by their composition and molecular weight.

The evidence suggests that the antibody responses of animals to the purified polysaccharides differ from those observed in man. This factor necessitates an approach different from that applied to the biological standardization of other bacterial vaccines in common use. With meningococcal vaccines it is important that the initial batches from a new manufacturer and even batches prepared by an existing manufacturer using a new process be evaluated in man for immunogenicity by monitoring antibody responses.

Unfortunately the lack of susceptibility of experimental animals to meningococcal infections also makes it impossible to estimate the efficacy of these vaccines for man on the basis of studies in animals. Efficacy can be evaluated at present only by clinical studies. The field trials concluded to date have provided a considerable body of data. However, there is still a lack of definitive data on the efficacy of Group C vaccines in infants and young children. It would therefore be desirable to keep appropriate groups of vaccinated infants and young children under surveillance to obtain such data.

Group A polysaccharide consists of repeating units of \(N,O\)-diacetylmannosamine phosphate linked with 1→6-phosphodiester bonds, and these components, together with calcium ions and residual moisture, account for over 90% of the dry weight of the preparation. Unfortunately this polysaccharide is intrinsically unstable and readily depolymerizes at ambient temperature, but the rate of degradation becomes negligible at temperatures of \(-20^\circ\text{C}\) or lower. The molecular weight
is thus not readily identifiable, because the molecular size distribution is polydispersed, with components ranging from less than 50 000 to well over 200 000. The distribution of the molecular weight is best estimated by gel filtration on Sepharose 4B, and this procedure has been included as a requirement. It is known that preparations degraded by a prolonged exposure to ambient temperatures are significantly less immunogenic; however, the requirements concerning molecular size are such that they are met relatively easily in production yet allow a margin of safety for changes that may occur under non-ideal but practical conditions.

The Group C polysaccharide is a polymer of \(N,O\)-diacetyleneuraminic acid, and these components, together with calcium ions and residual moisture, account for over 90% of the weight of the material. Unlike Group A polysaccharide this polymer is not susceptible to degradation at ambient temperatures but in all other respects the properties of the two polysaccharides are similar.

Another unusual feature is that purification of the polysaccharides is carried out under chemically clean but not aseptic conditions (because the latter would be prohibitively difficult), the final product being rendered bacteriologically sterile by membrane filtration.

Chemical tests are not carried out on the final bulk but rather on aliquots of lots of purified polysaccharide. If these meet all the requirements they may be pooled to form a final bulk of any desired size. Moreover, any lot of purified polysaccharide that fails to meet any of the chemical requirements may be re-treated and if it is then able to meet the requirements it may be included in a final bulk.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. Detailed descriptions of technical methods that may be used are given in Part C of these requirements.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning meningococcal vaccine, it is recommended that a clause be included that would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated
below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory", as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A:
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be *Vaccinum meningitidis cerebrospinalis* followed in parentheses by the serogroup specificity, thus: polysaccharide Group A and/or polysaccharide Group C. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

*Vaccinum meningitidis cerebrospinalis* shall consist of purified Group A and/or Group C meningococcal polysaccharide(s). The polysaccharides shall be prepared from strains of *Neisseria meningitidis* that have satisfied the requirements of Part A, section 3.1.1, and purified by methods outlined in Part A, sections 3.3.3 and 3.4. The final preparation shall satisfy all the requirements formulated below.

1.3 Terminology

*Parent seed lot*. A quantity of living *Neisseria meningitidis* organisms of a specific strain processed together and of uniform composition. A parent seed lot may be maintained in the dried form or frozen at temperatures below −45°C.

*Working seed lot*. A quantity of living *Neisseria meningitidis* organisms derived from the parent seed lot by selecting a colony, growing the organisms and maintaining them in aliquots in the frozen state at −45°C or below.
**Single harvest.** A sediment obtained on the same day by centrifugation of one culture irrespective of the volume of hexadecyl trimethyl-ammonium bromide\(^3\) that has been added to precipitate the group-specific polysaccharide.

**Lot of purified polysaccharide.** The material obtained after final purification. The lot may be derived from a single harvest or from several single harvests.

**Final bulk.** A pool of lots of purified polysaccharide that have been dissolved and filtered through membranes under aseptic conditions. The final bulk is contained in a single vessel from which the final containers are filled.

**Filling lot (final lot).** A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or drying. A filling lot must, therefore, have been aseptically filtered, filled, and dried together.

2. **GENERAL MANUFACTURING REQUIREMENTS**

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)\(^4\) shall apply.

Staff assigned to the production of meningococcal vaccines should be vaccinated against the particular serogroup that is under production.

3. **PRODUCTION CONTROL**

3.1 **Control of source of material**

3.1.1 **Strains of Neisseria meningitidis**

The strains of *Neisseria meningitidis* Group A and the strains of *N. meningitidis* Group C used for preparing polysaccharide shall be approved by the national licensing authority. They shall also have been shown to be capable of producing polysaccharide known to be safe and effective in man.

The following strains have been shown to be suitable: for Group A polysaccharide—A1, M1027; for Group C polysaccharide—C71, C2241.

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\(^3\) The international nonproprietary name for this substance is cetrimonium bromide.

The parent seed lot used shall be identified by a record of its history, including the source from which it was obtained and particulars of all tests made periodically for the verification of strain characters. The cultures shall have the following characteristics: (1) stained smears made from a culture shall be typical of N. meningitidis; (2) the organism shall grow at 37°C but not at 25°C; (3) the culture shall ferment glucose and maltose; (4) the colonies on agar shall be oxidase-positive; and (5) a suspension of the culture shall be agglutinated specifically with an appropriate grouping serum.

3.1.2 Seed lot system

The production of meningococcal vaccine shall be based on a seed lot system. Cultures of the working seed lot shall have the same characteristics as cultures of the strains from which the parent seed lot was derived (Part A, section 3.1.1). The preparation of seed lots shall be in compliance with the requirements of Part A, section 3.2.

A suitable menstruum for drying or preserving a parent or working seed lot in the frozen state is one containing 5% monosodium glutamate and 5% bovine plasma; in some countries skinned milk is used. Mueller-Hinton agar medium may be used for the preparation of working seed lots.

3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) shall apply.

Particular efforts are necessary to maintain the culture tanks and all other equipment scrupulously clean. In particular, efforts should be made to avoid cross-contamination of the polysaccharides.

3.2.1 Culture medium for vaccine production

The fluid culture medium used for vaccine production shall be free from ingredients that will form a precipitate on addition of hexadeyl trimethylammonium bromide to a final concentration of 1 g/l. It shall be free also from substances that may give rise to sensitization in man if the ingredients appear in the final vaccine.

3.2.2 Method of production

The growth of the cultures shall take place at 35.5°C ± 1.5°C. A working seed lot shall be plated on solid medium and after 12–18 hours of incubation the culture shall be used as an inoculum for liquid precultures. As these precultures reach the stationary phase they shall be used as an inoculum for the production tanks, and the production cultures shall be harvested during the late logarithmic or early stationary phase.

The moment of harvest of the production culture should be determined keeping two considerations in mind. Late harvest tends to improve the final yield of polysaccharide but its average molecular size will be lower. Most often the optimum time for harvest has been 10–12 hours after inoculation.

3.3 Control of production culture

3.3.1 Freedom from contamination

Samples of the culture taken before killing shall be tested for bacterial contamination by microscopic examination of Gram-stained smears and by inoculation into appropriate media. Several microscopic fields shall be examined at high magnification such that at least 10,000 organisms have been inspected. If any contaminating bacteria are found the culture or any product derived from it shall be discarded.

3.3.2 Treatment of production culture

As soon as freedom from contamination is established by examination of stained smears purification shall be undertaken.

The culture may be harvested directly or after killing the organisms by heating at 50°C for a period of 10 minutes. Two methods exist; either hexadecyl trimethylammonium bromide is added to the culture to a final concentration of 1 g/l, or the culture is first clarified by centrifugation after which hexadecyl trimethylammonium bromide is added to the supernate.

3.3.3 Purification of polysaccharide

All steps in the purification procedure shall be carried out in clean glass or plastic ware. Only plastic ware compatible with the solvents in use during the particular step of the procedure shall be used. Reagent grade chemicals or equivalent shall be employed, and aqueous solutions of calcium chloride, sodium acetate, or other salts shall be filtered to remove particulate matter present in these materials. All purification steps shall be carried out in a cold room at a temperature of 8°C ± 5°C with chilled reagents. The precipitated detergent-polysaccharide complex shall be collected as rapidly as possible by centrifugation and, unless preliminary purification is carried out immediately, the sediments shall be stored frozen at −20°C or lower.

3.3.3.1 Preliminary purification of meningococcal polysaccharide. The precipitate of hexadecyl trimethylammonium bromide and polysaccharide shall be partially purified, the material resulting from this preliminary purification being referred to as intermediate product. This product shall be stored at −20°C or below. The method of purification shall be approved by the national control authority.

Suitable methods for preliminary purification are shown in the Appendix to these Requirements.

3.3.3.2 Final purification of meningococcal polysaccharide. The intermediate product, which contains approximately 50% by weight of the desired polysaccharide (the remainder being principally protein and lipopolysaccharide with endotoxic activity), shall be subjected to further purification. The method of further purification shall be approved by the national control authority.

Suitable methods for further purification are shown in the Appendix to these Requirements.

After final purification the material is referred to as the lot of purified polysaccharide. The final dry product shall be handled with sterile precautions and stored at −20°C or lower.

If tests carried out on the lot of purified meningococcal polysaccharide indicate that nucleic acid, protein, or endotoxic contamination is present, the purification procedure applicable to the particular contaminant may be repeated, followed by the procedure for drying the polysaccharide.
3.4 Chemical requirements for lots of purified polysaccharide

All chemical analyses of all lots shall be based on the dry weight of polysaccharide in the salt form. The moisture content of the purified polysaccharide shall be determined by thermogravimetric analysis at 50°C. In laboratories lacking facilities for thermogravimetric analysis, the moisture content may be determined by the Karl Fischer method or by drying in vacuo over P₂O₅ at 37°C until a constant weight is reached. These data shall be used for the calculation of the dry weight.

3.4.1 Protein content

Each lot of purified polysaccharide shall contain less than 10 mg of protein per gram of polysaccharide as determined by the method of Lowry et al.¹ using bovine plasma albumin as a reference.

3.4.2 Nucleic acid content

Each lot of purified polysaccharide shall contain less than 10 mg of nucleic acid per gram of polysaccharide as determined by spectroscopy, assuming that the internal transmission density of a 10 g/l solution contained in a cell 1 cm wide at 260 nm is 200.

3.4.3 O-acetyl content

The O-acetyl content of each lot of purified polysaccharide shall be equal to or greater than 2 mmol/g of polysaccharide for Group A and 1.5 mmol/g of polysaccharide for Group C. The O-acetyl content shall be determined by the method of Hestrin.²

3.4.4 Phosphorus content

Each lot of Group A polysaccharide shall contain at least 80 mg/g of phosphorus.

3.4.5 Sialic acid content

The sialic acid content of the purified polysaccharide, calculated as free N-acetylmuramid acid (molecular weight 309), shall be not less than 800 mg/g of the dry weight of the isolated product as determined

by the method of Svennerholm,\textsuperscript{1} using the reference sialic acid, \textit{N}-acetyleneuraminic acid, as the reference preparation.

3.4.6 Molecular size

The molecular size of each lot of purified polysaccharide shall be estimated by gel filtration using Sepharose 4B. Chromatography shall be carried out in a solvent having a concentration of 0.2 ionic strength. The molecular weight shall be determined by measuring the distribution constant \(K_D\) of the polysaccharide at the main peak of the elution curve. The \(K_D\) value must be no greater than 0.40.\textsuperscript{2}

There are clinical data indicating that vaccines with a \(K_D\) value as high as 0.45 are immunogenic for man. As there is a general relationship between immunogenicity and molecular weight, however, material with the lower \(K_D\) value of 0.40 is considered preferable. Attempts should be made to produce material with still lower \(K_D\) values, and when such vaccines are being produced regularly these regulations may be amended to require a reduction in \(K_D\) value below the currently specified upper limit of 0.40.

3.5 Preparation of final bulk

The final bulk shall be prepared either from a single lot of purified polysaccharide or from several pooled lots. The polysaccharide shall be dissolved under aseptic conditions in a sterile solution containing mannitol or other suitable materials as the menstruum for freeze-drying and shall be sterilized by membrane filtration. The solvent used shall be free from pyrogenic substances.

Membranes with a pore size of 0.22 \(\mu\)m have been found satisfactory.

3.5.1 Sterility test on the final bulk

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).\textsuperscript{3}

\textsuperscript{1} Svennerholm, L. Biochimica et biophysica acta, 24: 604 (1957).
\textsuperscript{2} The technology of meningococcal polysaccharide production is such that it is not easy to obtain agreement on an absolute figure for molecular size. In some countries it has been suggested that the \(K_D\) value should be less than or equal to 0.40 and that at least 65\% of the material should elute with a \(K_D\) value less than or equal to 0.50.
3.5.2 Test for serological specificity

The final bulk of meningococcal Group A polysaccharide and the final bulk of meningococcal Group C polysaccharide used for the combined vaccine shall be tested for serological specificity and identity by the haemagglutination inhibition assay procedure. The meningococcal Group A final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group A meningococcal antigen in the presence of Group A antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group C antiserum and erythrocytes sensitized with Group C meningococcal antigen. The meningococcal Group C final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group C meningococcal antigen in the presence of Group C antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group A antiserum and erythrocytes sensitized with Group A meningococcal antigen.

4. FILLING AND DRYING

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)\(^1\) shall apply. The meningococcal vaccine shall be freeze-dried. The Group A and the combined Group A and Group C vaccines shall be stored at a temperature of \(-20\)°C or lower whereas the Group C vaccine shall be stored at a temperature of 5°C or lower.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be performed on at least one labelled container from each filling lot. The presence of the group-specific antigen(s) shall be confirmed by tests described in Part A, section 3.5.2.

5.2 Test of contamination by heterologous polysaccharide

The monovalent vaccine shall be tested for the presence of heterologous polysaccharide by the test described in Part A, section 3.5.2.

5.3 Concentration of polysaccharide

At least one final container shall be checked to determine that it contains the stated amount of polysaccharide. It shall be shown that Group A vaccine contains 75 mg of phosphorus per gram of polysaccharide and that Group C vaccine contains 750 mg of N-acetylneuraminic acid per gram of polysaccharide.¹

5.4 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).²

5.5 Innocuity tests

5.5.1 Pyrogenicity test

Each filling lot shall be tested for pyrogenicity by the intravenous injection of rabbits. Three or more healthy rabbits that have not been injected previously shall be used. The vaccine, reconstituted in the form in which it is to be used, shall be diluted further in pyrogen-free physiological saline so that each rabbit shall receive, by injection into the ear vein, the following doses of dry weight polysaccharide per kilogram of rabbit weight:

- Group A vaccine, 0.0025 μg
- Group C vaccine, 0.0025 μg
- combined Groups A and C vaccine, 0.0050 μg

In each instance the specified dosage level of polysaccharide for each rabbit shall be suspended in 1 ml of physiological saline per kilogram of rabbit weight. The criteria for passing the test shall be those specified in the International Pharmacopoeia.³

¹ The content of phosphorus and N-acetylneuraminic acid in the final containers is inevitably somewhat lower than the content of these substances in the vaccine lots, as given in sections 3.4.4 and 3.4.5 of this annex.
5.5.2 Tests for abnormal toxicity

5.5.2.1 Guinea pig toxicity. No fewer than five guineapigs weighing approximately 350 g each shall be injected intraperitoneally with 500 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.5.2.2 Mouse toxicity. No fewer than five mice weighing approximately 18 g each shall be injected intraperitoneally with 100 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.6 Estimation of molecular size

The molecular size of the polysaccharide in at least one final container from each filing lot shall be determined by Sepharose 4B gel filtration as outlined in Part A, section 3.4.6. The polysaccharide shall elute with a distribution constant, $K_D$, of 0.40 or less.¹

5.7 Test for residual moisture

A test shall be performed on at least one container from each filing lot to determine the amount of moisture in the product. The filing lot shall pass the test if the moisture content is not more than 3%. The method used for the determination of the moisture content shall be approved by the national control authority.

5.8 Storage

The Group A vaccine shall be stored at a temperature of −20°C or lower. The Group C vaccine shall be stored at a temperature of 5°C or lower.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)² shall apply.

¹ See footnote on p. 59.
7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \(^1\) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \(^1\) shall apply, with the addition of the following.

The label on the container shall show:

- the number of micrograms of polysaccharide(s) in the human dose
- the volume and nature of the reconstituting fluid.

Furthermore, the label on the container or the label on the carton enclosing several containers, or the leaflet accompanying the container, shall contain the following additional information:

- a statement that after the dried vaccine has been reconstituted it should be used within 8 hours;
- a statement that the Group A vaccine shall be stored at \(-20^\circ\mathrm{C}\) or lower;
- a statement that the Group C vaccine shall be stored at \(5^\circ\mathrm{C}\) or lower;
- a statement that a combined Group A and Group C vaccine shall be stored at \(-20^\circ\mathrm{C}\) or lower.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \(^1\) shall apply.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to these requirements until the expiry date as stated on the label. The Group A vaccine shall be stored at \(-20^\circ\text{C}\) or lower; the Group C vaccine shall be stored at \(5^\circ\text{C}\) or lower.

10.2 Expiry date

The expiry date for dried vaccines shall be not more than 5 years from the date of harvest or not more than 18 months from the date of issue. The manufacturer shall provide data to confirm the stability of the product.

PART B:

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \(^1\) shall apply.

In addition, the national control authority shall give directions to manufacturers concerning the most suitable strains from which polysaccharide vaccine shall be produced.

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfills all requirements set forth in Part A of the present document.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements.

The certificate shall also state the date of the last satisfactory test for molecular weight, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of meningococcal polysaccharide vaccine between countries.

3. REACTIVITY AND IMMUNOGENICITY OF VACCINE IN MAN

The national control authorities shall satisfy themselves that adequate control of meningococcal vaccine has been achieved by arranging for studies in man\(^1\) to be made on the first series of production lots and thereafter at regular intervals on some of the vaccine lots. Such studies shall be repeated if any changes in production methods are made.

The national control authorities shall ensure that the studies are done in an adequate number of subjects to obtain statistically valid data on reactivity and immunogenicity.

The following methods of demonstrating safety and efficacy may be used.

Healthy adult human subjects should be immunized in the manner prescribed for the product and their temperatures measured between 4 and 6 hours, and again between 24 and 48 hours, after injection. Such temperatures, as well as all adverse reactions during the first 48-hour period should be reported.

Samples of sera from each subject taken immediately prior to injection and again at 2-4 weeks after immunization should be assayed for bactericidal antibodies. The bactericidal assay should be performed with paired sera from each subject in serial twofold dilutions against the A1 strain for Group A and/or the C-11 strain for Group C or equivalent strains of Neisseria meningitidis. The antibody titre should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. The antibody titres of the sera from at least 90% of the subjects should show a fourfold or greater rise after immunization. If the sera from less than 90% but greater than 80% of the subjects show such a rise, one re-test of the product may be allowed, but in such a case the sera from at least 90% of all subjects in the two tests combined should show a fourfold or greater antibody increase.

\(^1\) The persons should be volunteers.
DETAILS OF SOME METHODS AND TESTS USED IN THE MANUFACTURE AND CONTROL OF MENINGOCOCCAL POLYSACCHARIDE VACCINES

These methods and tests are given as a guide to control laboratories.

1. Suitable methods for preliminary purification

A satisfactory method of preliminary purification is as follows. The quantities suggested assume that the sediment from 100 litres of culture is available. The sediment, which may come from a single harvest or may be a pool of several single harvests, is smoothly suspended in 1 litre of distilled water. One litre of 2 mol/l calcium chloride is added to dissociate the detergent-polysaccharide complex and the suspension is stirred for one hour. In order to precipitate the nucleic acids and the bulk of soluble protein, absolute ethanol or 95% ethanol is added to a final ethanol concentration of 250 ml/l. After one hour of standing the precipitate is sedimented by centrifugation at approximately 20 000 g for 20 minutes and the perfectly clear supernatant is retained. Unless this solution is free from particulate matter it is probable that the final product will contain an unacceptable level of nucleic acid contamination. Ethanol is added to this solution to a final concentration of 750 ml/l for the Group C polysaccharide and 800 ml/l for the Group A polysaccharide. After agglomeration of the precipitate is complete (usually within one hour), the precipitated polysaccharide is collected by centrifugation at approximately 3000 g for 10 minutes. The sediment is washed at least twice with about 1 litre of absolute ethanol (each time) to remove hexadecyl trimethylammonium bromide and calcium chloride. It is essential to use absolute ethanol because unacceptable losses of polysaccharide will occur if 95% ethanol is employed. The sediment is washed twice with approximately 1 litre of acetone and dried in vacuum.

2. Suitable methods for further purification

Two methods for further purification have been used. The first is based on the method of Sevag and consists of homogenizing in a blender an aqueous solution of the intermediate product (about 20 g/l) and a mixture of chloroform and d-butanol (in the ratio of 3:1). After centrifugation for 10 minutes at approximately 10 000 g, the clear aqueous supernatant is decanted and the homogenization of this layer repeated until a negligible interfacial precipitate forms on centrifugation.

The other method depends on cold-phenol extraction. The intermediate product is dissolved in 0.1 saturated neutral sodium acetate to give a concentration of intermediate product of 20 g/l. It is then extracted three times with twice its volume of cold phenol (100 g crystalline phenol dissolved in 40 ml of 0.1 saturated neutral sodium acetate). The extraction consists of vigorous shaking by hand for approximately 30 seconds. To minimize losses the three phenol layers are sequentially extracted with twice their volume of distilled water and added to the first aqueous supernatant. Phase separation is achieved by centrifugation for 15 minutes at 35 000 g. The polysaccharide solution is dialysed for 24 hours against cold 0.1 mol/l calcium chloride.

* Sevag, M. G. Biochemische Zeitschrift, 273: 419 (1934).
The solution is centrifuged for 3 hours at 100,000 g to sediment endotoxic lipopolysaccharide. Ethanol is added to the supernatant to a final concentration of 750 ml/l. The precipitated polysaccharide is collected by centrifugation, washed twice with absolute ethanol and twice with acetone. It is then dried in vacuum.

For the production of the Group A polysaccharide the cold-plenol procedure is to be preferred because the yield and molecular size are much higher. This procedure can also be used for the production of Group C polysaccharide, but frequently endotoxic activity is not readily removed by ultra-centrifugation unless one or more cycles of homogenization with a mixture of chloroform and butanol have been carried out.

3. Molecular sizing of capsular polysaccharide antigens by Sepharose 4B column chromatography

Reagents

- Sepharose 4B gel
- 0.2 mol/l ammonium acetate, pH 7.0, as eluant
- 14C-labelled sodium acetate
- Blue dextran 2000
- Scintillation liquid

Reagents for chemical assay of polysaccharides

Equipment

- Column (1.5 x 90 cm)
- Automatic fraction collector and test-tubes
- Gel and eluant reservoir
- Liquid scintillation counter and counting vials
- Refractometer or other appropriate monitoring device and recorder (optional)

Procedures

1. Washing of the Sepharose 4B gel:

(a) To approximately 200 ml of packed Sepharose 4B gel add 400 ml of 0.2 mol/l ammonium acetate. Stir the gel thoroughly and let it sediment for about 1 hour. Remove the supernatant fluid together with the fine particles suspended in it.

(b) Repeat the above procedure until there are no fine particles in the supernatant.

(c) Decant all the supernatant and add 200 ml of fresh 0.2 mol/l ammonium acetate to the gel. Decanter the gel suspension in a vacuum.

2. Packing of the column:

(a) Mount the column vertically and connect it to the gel and eluant reservoir.

(b) Fill the column with 0.2 mol/l ammonium acetate to a depth of about 30 cm.

(c) Pour the washed Sepharose 4B slurry into the reservoir slowly to avoid trapping air bubbles and adjust the height of the surface of the slurry above

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1 Advice on the availability of suitable products may be obtained from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.
the column outlet to about 70-75 cm. Open the column outlet and let the gel sediment at that operating pressure for about 16 hours; the flow rate of the column is approximately 15-20 ml/h.

(c) When the column is packed to a height of about 87 cm, equilibrate the gel bed with two to three column volumes of eluant. The operating pressure should always be equal to or less than that at which the column was packed. Mark the gel bed level.

(3) Calibration of the column:

(a) Void volume (V):(i) The void volume is determined with blue dextran dissolved in 0.2 mol/l ammonium acetate to a concentration of 2 g/l.
(ii) Drain the excess eluant from the column until the eluant level is equal to the gel bed level; close the column outlet.
(iii) Carefully layer 1 ml of blue dextran on top of the gel bed and allow the sample to drain into the gel bed by opening the column outlet.
(iv) When the sample level is equal to the gel bed level, fill the column with eluant and connect it to the eluant reservoir. Start collecting the fractions (approximately 2 ml per fraction) with an automatic fractionator.
(Simultaneously, the eluant may be monitored for blue dextran with a monitoring device and a recorder.)
(v) The internal transmission density (optical density) of each fraction is read at 260 nm and plotted against the number of fractions. V can be determined as the volume of eluant collected up to the position of the maximum of the first blue dextran peak in the elution diagram. Determination of the position of the apex of the elution peak may be done by extending upwards the two straight lines on the slopes. The interception of the two slopes is taken as the apex of the elution peak.

(b) Total bed volume (V): (i) The total bed volume is determined with ^1^C-labelled sodium acetate. The column is loaded with 1 ml of the sodium acetate solution containing 0.4 μg of radioactivity.
(ii) Fractions of about 2 ml each are collected and a 0.5 ml portion from each fraction is transferred into separate scintillation vials containing 10 ml of scintillation liquid. The vials are counted in a liquid scintillation counter.
(iii) The radioactivity, expressed as counts per minute, is plotted against the respective fraction numbers. The total bed volume is the volume of eluant collected up to the position of the maximum of the ^1^C sodium acetate peak in the elution diagram. Symmetrical peaks in the elution profiles are indicative of the acceptability of the column.

(4) Vaccine measurement:

(a) Preparations of meningococcal polysaccharide vaccine Group A or Group C or Groups A and C combined in the final containers are reconstituted with water to a concentration of 2.5 g/l of antigen for Group A or Group C or
5.0 g/l of antigens for Groups A and C combined. The vaccine pool should contain at least 2.2 ml. No more than 10 mg of polysaccharide should be used.

(b) One millilitre of the vaccine is used to charge the column, the column is eluted with a volume of 0.2 mol/l ammonium acetate using not less than 1.5 times the total bed volume of the column, and the eluate is collected.

c) The quantity of phosphorus in fractions of Group A vaccine and the quantity of sialic acid in fractions of Group C vaccine are determined. The readings are plotted against the number of fractions. Alternatively, the eluate may be monitored with a refractometer or an absorbptiometer and a recorder. However, the profile thus obtained by the monitoring device should be compared with a profile of the same type of vaccine as determined by chemical assay of fractions eluted under identical chromatographic conditions. Immunochemical analyses of the polysaccharides are not sufficiently quantitative to be reliable.

(d) The elution volume $V_e$ is measured at the main peak of the polysaccharide elution curve, and the distribution constant is calculated from the equation:

$$K_d = \frac{V_e - V_0}{V_1 - V_0}$$

using the values of $V_0$ and $V_1$ determined for the column. The $K_d$ value of the polysaccharide must be no greater than 0.40.¹

4. Determination of the O-acetyl group in meningococcal polysaccharides Group A and Group C

The methods described in this section are based on the work of Hestrin² and Kabat & Mayer.³

**Instrumentation**

Spectrophotometer for the ultraviolet and visible regions of the spectrum.

**Mechanism**

O-acetyl groups react with hydroxylamine in alkali to form hydroxamic acid. The hydroxamic acid formed is measured by the formation of a coloured complex with Fe²⁺ in acid solution.

**Reagents**

1. 2 mol/l hydroxylamine hydrochloride. Store in the cold.
2. 3.5 mol/l NaOH.
3. Concentrated HCl (specific gravity 1.18), diluted with twice its volume of water.
4. 0.37 mol/l solution of FeCl₃·6H₂O in 0.1 mol/l HCl.
5. 0.001 mol/l sodium acetate, pH 4.5.
6. Reference solution of acetylcholine chloride (molecular weight 181.7).

¹ See footnote on p. 59.
Method of preparing reference solution

The reference solution should contain 150 mg acetylcholine chloride per vial. To each vial 10 ml of 0.001 mol/l sodium acetate (pH 4.5) are added with a volumetric pipette. One millilitre of this solution is transferred to a test tube and mixed with 4 ml of 0.001 mol/l sodium acetate (pH 4.5), using a 5 ml volumetric flask if available. From this solution 0.1, 0.2, 0.3, 0.4, and 0.5 ml quantities are transferred accurately in duplicate to 10 test tubes, and 0.9, 0.8, 0.7, 0.6 and 0.5 ml of water are added to make the volume in each tube 1 ml. These reference tubes contain respectively 1.66, 3.32, 5.0, 6.64 and 8.30 micromoles of O-acetyl per millilitre. The duplicate at each concentration is used as a reference blank. The approximate internal transmission densities of these solutions at 540 nm in the visible range of the spectrophotometer are 0.2, 0.5, 0.8, 1.0 and 1.3. The readings are not taken at the maximum of absorption but rather on the shoulder of the peak. Further details are given by Hestrin.\(^1\)

The reaction is carried out at room temperature.

A reference blank is run for each standard prepared as indicated above. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

Procedure

(1) Prepare the sample for analysis by making up a 0.1% solution of the polysaccharide (no salt) in water. For each test use 1 ml of this solution, which contains 1 mg of polysaccharide.

(2) To 1 ml of the sample and reference preparations add 2 ml of a freshly prepared (within 3 hours) mixture of equal parts of reagents (1) and (2) (2 mol/l hydroxylamine, and 3.5 mol/l sodium hydroxide) and mix.

(3) After 4 minutes at room temperature, add 1 ml of reagent (3), HCl, to bring the pH to about 1.2 ± 0.2, and mix.

(4) Add 1 ml of reagent (4), ferric chloride, and mix.

(5) Swirl the contents of the test tubes rapidly during the addition of each of the above reagents.

(6) Both a reference blank (one blank for each concentration of the reference) and a sample blank (1 ml of 0.1% polysaccharide solution) must be included. In Hestrin's method the blank determination is called the test for non-specific colour. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

(7) Read the internal transmission density of the purple-brown colour at 540 nm promptly.

(8) Subtract the sample blank readings from those of the samples and subtract the reference blank readings from those of the reference preparations.

(9) Plot a curve of internal transmission density versus molarity of O-acetyl. The values of internal transmission density of the samples on the reference curve correspond to micromoles of O-acetyl per milligram of polysaccharide (since the test was conducted on 1 ml of the polysaccharide solution, which contains 1 mg of polysaccharide).

The weight of polysaccharide used in the 1 g/l polysaccharide solution is corrected for its moisture content\(^2\) and the final result is expressed as "millimoles of O-acetyl per gram of dry polysaccharide".

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2. See section 3.4 of Annex 2.
Example: Moisture content = 10%. Thus 1 mg of "wet" polysaccharide comprises 0.1 mg of water and 0.9 mg of "dry" polysaccharide. If, for every milligram of "wet" polysaccharide there are 1.95 μmol of O-acetyl, this figure is equivalent to:

\[
\begin{align*}
1.95 \text{ μmol} & \text{ O-acetyl} \\
\text{1 mg "wet" polysaccharide} & \\
= & \\
1.95 \text{ μmol} & \text{ O-acetyl} \\
0.9 \text{ mg "dry" polysaccharide} & \\
= & \\
2.16 \text{ μmol} & \text{ O-acetyl per milligram of "dry" polysaccharide.}
\end{align*}
\]

5. Procedures for haemagglutination-inhibition assay for meningococcal polysaccharide vaccine

Sensitization of erythrocytes

Sensitized erythrocytes are prepared according to the method of Artenstein et al. with the following modifications:

1. Dulbecco's phosphate-buffered saline, pH 7.4, with calcium and magnesium salts is used not only as a diluent but for washing cells as well.
2. Sheep erythrocytes (washed three times) are used.
3. Antisera are heated at 56°C for 30 minutes and adsorbed on to packed normal sheep erythrocytes before use.
4. Sensitizing antigens (Group A or Group C polysaccharides) are used at a concentration of 20 mg/ml.

Titration of antisera

Sera are titrated in triplicate using microtitre U-plates.

1. 0.025 ml of buffered saline is added to each well.
2. 0.025 ml of antiserum is added to the first well of each row and dilutions are made with 0.025 ml loops down the rows of the wells.
3. 0.025 ml of buffer is added to each well after the antiserum has been diluted.
4. 0.05 ml of sensitized cells is added to each well. The plates are incubated for 1-2 hours at room temperature for Group C antigen-coated cells and at 4°C for Group A antigen-coated cells. The cell control consists of 0.05 ml of sensitized cells and 0.05 ml of normal serum or saline.

The highest serum dilution giving a definitive haemagglutination pattern is considered as having one unit of activity. The preceding well is considered to have two units of activity and the next preceding well four units, etc.

A titration of antiserum on an appropriate range of dilutions is included with each haemagglutination inhibition test.

Haemagglutination inhibition

1. The test vaccine to be used as inhibitor is reconstituted with diluent so that its final concentration in the first well will be at least 100 mg/ml. It should be noted that, since the dilution factor for the test vaccine in the first well is 1:8, the initial vaccine concentration should be at least 800 mg/ml.

2. 0.025 ml diluent is added to each well.
3. 0.025 ml test vaccine is added to the first well of each row. Using 0.025 ml
   loops, make serial twofold dilutions.
4. The antiserum titrated as described above (see section on titration of antisera)
   is diluted in tubes to cover a range of at least 1, 2, 4, and 8 units. A 0.025 ml quantity
   of each antiserum dilution is added to each well of the appropriate rows of vaccine
   dilutions. The microtiter plate is gently swirled to ensure the mixing of the material in each
   well and is incubated at 37°C for 30 minutes.
5. 0.05 ml of the appropriate sensitized cells is added to each well.

The cell control consists of two drops (0.05 ml total) of diluent in place of the test
vaccine and antiserum. A known inhibitor should be included in each experiment as
antigen control. Incubation is continued for 1–2 hours at room temperature for
Group C antigen-coated cells and at 4°C for Group A antigen-coated cells.

A positive test for inhibition is defined as a reduction of the titre of the titrated
antiserum by two twofold dilution steps or more by any vaccine dilution with a final
concentration in the well of 100 mg/l or less.

6. Bactericidal antibody assay

Materials 1

Tissue culture trays, 20 × 30 cm, 96 cups, 2 ml per cup, with sterile covers
Sterile automatic syringes, 1 and 2 ml
Disposable serological pipettes, 1 ml, sterile glass plugged
Nephelometer flasks with side-arm, 300 ml capacity
Nephelometer
Sterile pipettes and test-tubes
Liquid medium: Mueller-Hinton broth or soybean-casein digest broth
Solid medium: soybean-casein digest broth with 1.2% agarose
Normal horse serum, heat-inactivated
Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium
0.85% saline, sterile
Complement from baby rabbits (3–4 weeks old), or freshly collected baby rabbit
(approximately 3 weeks old) serum stored at −80°C. Complement should be
pre-screened for the absence of anti-meningococcal activity.

Procedure

1. Serum samples:
   (a) All test sera are diluted 1 : 2 in DPBS and inactivated at 56°C for
       30 minutes. A reference is included as positive control.
   (b) Serial twofold dilutions of antisera are made by placing 0.2 ml DPBS in
       each cup of the tray, using a sterile 1-ml automatic syringe, and adding

1 Advice on the availability of suitable products may be obtained from Biologica1s, World Health Organization, 1211 Geneva 27, Switzerland.
0.2 ml of inactivated test serum to the first well, using a sterile disposable 1-ml pipette aspirated by a 1-ml automatic syringe connected to it by a piece of rubber tubing (taking care not to fill the syringe). The serum is mixed, and 0.2 ml of the mixed serum is transferred to the next well using the same pipette. This dilution process is continued successively through the rest of the wells, the 0.2 ml taken from the last well being discarded.

2. **Bacteria**:
   
   (a) A lyophilized meningococcal culture or a recently transferred culture is inoculated on to the solid medium and incubated overnight at 37°C in air containing 3% CO₂. A 25-ml quantity of broth is added aseptically to a sephelometer flask and a 5-ml quantity to a sterile culture tube. Approximately ½ loopful of the overnight culture is transferred to the 5 ml of broth in the culture tube. After mixing thoroughly, the culture is poured into the flask and incubated at 37°C on a shaker for approximately 5 hours. Turbidity is read at 530 nm and the number of cells (colony forming units) per millilitre is determined from a standard curve. The culture is diluted in saline to a concentration of 500 cells/ml.

   (b) 0.2 ml of the cell suspension is added to each well, using a sterile 1-ml automatic syringe.

3. **Complement**:
   
   (a) 0.2 ml of pre-screened complement is added to each well with a 1-ml automatic syringe.

   (b) The following controls are included.

   (i) Cell control: 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml heat-inactivated complement.

   (ii) Complement control: 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml of active complement.

   (iii) Serum control: 0.2 ml serum, 0.1 ml bacteria, 0.1 ml DPBS.

4. **Incubation**:
   
   (a) The plates are covered and incubated at 37°C for 30 minutes in 5% CO₂.

   (b) Sterile solid medium is prepared afresh each time in the following manner. Agar gel is heated to 120°C and sterilized in an autoclave. When it has cooled to approximately 45°C, normal horse serum (heat-inactivated) is added to a concentration of 10 ml/l. One millilitre of the medium is added to each well, using a warm 2-ml automatic syringe. The trays are then gently swirled and the medium is allowed to set at room temperature. The trays are covered and incubated at 37°C for 18–24 hours in 5% CO₂.

5. **Reading of results**:
   
   The growth in the complement control is used as growth control, which should be the same or only slightly less than that of the cell control. The end-point of each serum titration is the last well that gives 50 % or greater killing as compared with the cell control. Some sera, especially high-titered samples, may have a prozone. Sera showing a killing effect in the serum control should be titrated without complement to determine the extent of this killing effect.
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