Annex 1

Requirements for Vi polysaccharide typhoid vaccine (Requirements for Biological Substances No. 48)

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Introduction

Typhoid fever is still a common disease, causing significant morbidity and mortality in countries that have not yet achieved satisfactory control of drinking-water, food and sewage disposal (1, 2). Control of typhoid fever may be aided by immunization, but widespread protection by this method has not been achieved for various reasons, including the limitations and insufficient use of available vaccines (3, 4).

Typhoid fever is a septicaemic disease caused by a capsulated bacterium, Salmonella typhi (S. typhi), whose capsular polysaccharide is called the Vi polysaccharide. The success of immunization with purified capsular polysaccharides against invasive diseases caused by other capsulated bacteria such as Neisseria meningitidis groups A and C, pneumococci and Haemophilus influenzae type b provided background for the development of a Vi capsular polysaccharide vaccine for the prevention of typhoid fever.
Two controlled, double-blind, randomized field trials of the Vi polysaccharide typhoid vaccine (5, 6) have demonstrated protection against typhoid fever in areas with high endemicity.

The Vi polysaccharide elicits serum antibodies in mice (7) and in adult humans (5, 6). In clinical studies 25 µg or 50 µg of the Vi polysaccharide elicited a fourfold or greater rise in the level of serum antibodies in 95% of adults from France and the United States. The vaccine was less immunogenic in children and adults from Nepal (5) and the eastern Transvaal, South Africa (6), where a fourfold or greater rise in serum levels of anti-Vi antibodies was observed in only about 75% of the recipients.

The serum antibody responses to individual polysaccharides in multivalent vaccines consisting of four meningococcal polysaccharides (A, C, Y and W-135) or 23 pneumococcal polysaccharides (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) are the same as those elicited by each polysaccharide administered alone (8). Manufacturers wishing to combine such multivalent polysaccharide vaccines with the S. typhi Vi polysaccharide should demonstrate, during the validation procedure required by the licensing process, that the serum antibody response to each polysaccharide component is not adversely affected by the presence of the other components. Furthermore, such multivalent products should meet the appropriate manufacturing and control requirements for each bacterial component.

As the Vi vaccine is already licensed and being used in several countries, it is appropriate to issue international Requirements for the manufacture of this product.

**General considerations**

Development of vaccines for typhoid fever has been hindered because the causative agent, S. *typhi*, infects and is pathogenic in humans only. Vaccines composed of whole, inactivated S. *typhi* have been available since the turn of the century. Their development followed the principle set forth by Pasteur, i.e. isolate, inactivate and then inject the pathogenic agent.

In the 1950s and 1960s, WHO sponsored trials of cellular vaccines manufactured according to protocols developed at the Walter Reed Army Institute of Research, USA. S. *typhi* was inactivated by one of two procedures: (1) heat and then treatment with phenol (L vaccines); or (2) treatment with acetone (K vaccines). In direct comparisons, both vaccines conferred protection in clinical trials, but the K-type was more effective (9). Later it was shown that potency assay, by mucin-enhanced intraperitoneal challenge in mice, could reliably predict the effectiveness of whole-cell vaccines (10, 11). Subsequently, experimental data showed that the K-type vaccine contained more Vi polysaccharide than the L-type, and that the resistance to challenge was mediated by the induction of
anti-Vi antibodies (7, II). A vaccine containing the Vi polysaccharide was prepared for clinical evaluation. Because methods of purification at that time were not sufficient to remove contaminating lipopolysaccharide, which produced unwanted side-reactions, an investigation lot of Vi polysaccharide was prepared by heating in 1.0 mol/l acetic acid at 100 °C for 24 hours (9). The resultant product was less pyrogenic, reacted with Vi polysaccharide antiserum and elicited anti-Vi antibodies in mice and adult humans. It did not, however, confer protection on adult volunteers challenged with S. typhi. This method of preparation was later shown to remove O-acetyl and N-acetyl moieties and to reduce the molecular mass of the Vi antigen (II).

In 1985, two randomized, double-blind controlled clinical trials were conducted (5, 6) on a Vi vaccine manufactured by a method similar to that used for meningococcal polysaccharide vaccine. A single injection of the Vi or the control vaccine was administered. The first trial was conducted in Nepal in five villages outside Kathmandu, with participants aged from 5 to 44 years. The control group received the pneumococcal 23-valent vaccine. The protection rate for typhoid was about 70% for the two years of surveillance. The second randomized and controlled trial was conducted in eastern Transvaal, South Africa, with participants aged from 5 to 16 years. The control vaccine was the meningococcal groups A and C polysaccharide. A protection rate of about 60% for typhoid was shown for the ensuing 21 months. In both trials, an attack rate of typhoid fever of about 1% per year in the controls was recorded during the study. Following injection of the Vi vaccine, local reactions were slight and there were no serious systemic reactions such as high fever.

In terms of vaccine efficacy, these results are similar to those obtained with whole-cell inactivated vaccines and live Ty21a vaccines. Direct comparisons of efficacy in large-scale field trials have not been made.

The Vi polysaccharide is a linear homopolymer of (1→4)-linked N-acetyl-α-D-galactosaminuronic acid, acetylated at O-3 (I2). It is present on almost all strains of S. typhi isolated from the blood of patients (I3). Non-capsulated variants of S. typhi may be detected in stool cultures and from blood isolates after several passages of the strain in the laboratory. The Vi polysaccharide may also be detected in some strains of S. paratyphi C, S. dublin and Citrobacter freundii (I4). Because of its chemical structure, the Vi polysaccharide vaccine is not normally susceptible to depolymerization; its integrity is maintained when the polysaccharide is kept at 25 °C for several months as a powder or in solution at pH 6.0–7.8.

As a consequence of the polysaccharide nature of the Vi antigen, the requirements for vaccine production and control contained in this document have many similarities with those included in the Requirements for Biological Substances No. 23 (Requirements for Meningococcal Polysaccharide Vaccine) (I5, I6).

National control authorities should satisfy themselves that a Vi vaccine is
suitable for clinical use by reviewing safety and immunogenicity studies on
the first production lots manufactured in accordance with the present
Requirements. Such studies should be conducted whenever significant
changes in production methods occur. To obtain statistically valid data on
reactivity and immunogenicity, national control authorities should ensure
that an adequate number of subjects are studied.

Each of the following sections constitutes a recommendation. The parts of
each section printed in normal type have been written in the form of
requirements, so that, if a health administration so desires, they may be
adopted as they stand as definitive national requirements. The parts of
each section printed in small type are comments or recommendations for
guidance. Detailed descriptions of technical methods that may be used for
the manufacture and control of Vi polysaccharide typhoid vaccine are
given in the Appendix.

Should individual countries wish to adopt these Requirements as the basis
for their national regulations concerning Vi capsular polysaccharide
typhoid vaccine, it is recommended that modifications be made only on
condition that the modified requirements ensure at least an equal degree of
safety and potency of the product. It is desirable that the World Health
Organization should be informed of any such changes.

**Part A. Manufacturing requirements**

**A.1 Definitions**

**A.1.1 International and proper name**

The international and proper name of the vaccine shall be “Vi
polysaccharide typhoid vaccine”, translated into the language of the
country of use. The use of this name shall be limited to vaccines that satisfy
the requirements formulated below.

**A.1.2 Descriptive definition**

Vi polysaccharide typhoid vaccine shall consist of purified Vi
polysaccharide. The polysaccharide shall be prepared from a strain of
*Salmonella typhi* that satisfies the requirements of section A.3.1.1, and shall
be purified by the methods outlined in section A.3.3 of these
Requirements. The Vi polysaccharide typhoid vaccine shall satisfy all the
requirements formulated in sections A.3-A.5 (inclusive).

**A.1.3 Reference materials**

The Vi polysaccharide typhoid vaccine is controlled by examination of
physical and chemical characteristics. An international reference material
is therefore not needed. In-house standards are required for determining
identity and comparing immunochemical activity, and should be stored
desiccated at a temperature below −20°C to prevent degradation.
A.1.4 **Terminology**

**Master seed lot**: A bacterial suspension of living *S. typhi* organisms, stored as aliquots derived from a strain that has been processed as a single lot and has a uniform composition. The master seed lot is used for preparing working seed lots. It shall be maintained in the freeze-dried form or at a temperature not higher than −45 °C.

**Working seed lot**: Living *S. typhi* organisms derived from the master seed lot by growing the organisms and maintaining them in aliquots in the freeze-dried form or at a temperature not higher than −45 °C.

**Single harvest**: The material obtained from one batch of cultures that have been inoculated with an inoculum derived from the working seed lot, harvested and processed together.

**Purified Vi polysaccharide lot**: The material obtained after final purification. The lot of purified Vi polysaccharide may be derived from a single harvest or a pool of single harvests processed together.

**Final bulk**: The homogeneous material derived from a purified Vi polysaccharide lot or pool and present in a single container from which the final containers are filled. The final bulk may be used to produce one or more final lots.

**Final lot**: A collection of sealed final containers that have been filled from a single container in a single continuous working session, are uniform in their contents, and are homogeneous with respect to the risks of contamination during filling and, if appropriate, freeze-drying.

**Stability**: The capacity of Vi polysaccharide typhoid vaccine to remain unmodified during storage under specified conditions, so that its composition, characteristics and immunological properties do not change. The stability of the vaccine is checked by recording the molecular size and O-acetyl content of the polysaccharide from at least three final lots (each derived from a different final bulk) stored at the recommended temperature for a specified period of time (see section A.6). This information is used to ensure that the product meets the validity-period specification (see section A.9).

**Validity period and expiry date**: The validity period is the maximum time permitted by the national control authority for storage and use of the product after the final containers have been filled. The expiry date is set after stability studies have been performed on the vaccine (see section A.6); it is normally the date after which it is no longer possible to guarantee that the vaccine meets each of the manufacturing and control requirements.

A.2 **General manufacturing requirements**

The requirements of Good Manufacturing Practices for Pharmaceutical Products (17) and Good Manufacturing Practices for Biological Products
shall apply to establishments manufacturing Vi polysaccharide typhoid vaccine. Other biological products, in particular other polysaccharide vaccines, shall not be manufactured at the same time in the same area. If other polysaccharide vaccines are manufactured at other times in the same area, the rooms and equipment shall be cleaned and decontaminated by methods shown to be effective in removing polysaccharides whenever production is switched to a different vaccine. The Vi polysaccharide shall be purified under clean, but not necessarily sterile, conditions.

Details of the standard operating procedures adopted by the manufacturer for the preparation and testing of Vi polysaccharide typhoid vaccine, with appropriate validation of each production step, shall be submitted for approval to the national control authority. Data on stability at different stages of production shall be obtained so that “intermediate” products are stored under adequate conditions.

Proposals for modifications, if any, of the manufacturing or control methods shall also be submitted for approval to the national control authority.

Personnel in the production and control facilities shall be adequately trained and protected, including by immunization, against accidental infection with S. typhi.

A.3 Production control

A.3.1 Control of source of materials

A.3.1.1 Strains of S. typhi
The strain of S. typhi used for production of the Vi polysaccharide shall be approved by the national control authority. The strain shall have been shown to be capable of producing the Vi polysaccharide.

S. typhi Ty2 has been shown to be a suitable strain.

The cultures shall have the following characteristics: (1) stained smears made from a culture shall be typical of S. typhi; (2) the cultures shall utilize glucose without production of gas; (3) the colonies on agar shall be oxidase-negative; (4) a suspension of a culture shall be agglutinated specifically with an appropriate anti-Vi antiserum or colonies shall form haloes on an antiserum-containing agar plate (19).

A.3.1.2 Seed lot system
The production of Vi polysaccharide typhoid vaccine shall be based on a seed lot system. The master seed lot used shall be identified by a record of its history, including the source from which it was obtained, and by its biochemical and serological characteristics. Cultures derived from the working seed lot shall have the same characteristics as cultures of the strain from which the master seed lot was derived. Seed lots shall comply with the requirements of section A.3.2.
Ultrafiltered tryptic soya-bean medium may be used in the preparation of working seed lots. A medium containing 50 g/l monosodium glutamate and 50 g/l bovine plasma albumin is suitable for drying or preserving master or working seed lots in the frozen state. If bovine albumin is used it should come from herds certified free from bovine spongiform encephalopathy and bovine leukosis. In some countries bovine albumin is replaced by skimmed milk.

A.3.1.3 Culture media for vaccine production

The fluid culture medium used for vaccine production shall be free from ingredients that form a precipitate upon addition of hexadecyltrimethylammonium bromide at the concentration used by the manufacturer. It shall also be free from adventitious substances that may give rise to sensitization in humans, such as blood-group substances, unless such substances are removed by the purification process.

A semi-synthetic medium (15, 16, 20, 21) supplemented with 5 g/l dialysate of yeast extract may be used. The basal medium may be autoclaved but the supplement, consisting of yeast extract dialysate, magnesium sulfate and glucose, should be sterilized by filtration.

A.3.2 Single harvests

The growth of *S. typhi* shall be shown to be consistent by monitoring the growth rate of the bacteria, the pH of the culture and the yield of Vi polysaccharide.

Cultures will normally grow satisfactorily between 35°C and 37°C. The working seed lot may be plated on solid medium, and after 12–18 hours of incubation the culture may be used as an inoculum for liquid precultures. As the precultures reach the stationary phase, they may be used as an inoculum for the production tanks, and the production cultures may be harvested during the early stationary phase.

A.3.2.1 Control of bacterial purity

Samples of the culture taken before inactivation shall be tested for 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 10000 organisms are examined. If any contaminants are found, the culture and any product derived from it shall be discarded.

A.3.3 Purification of Vi polysaccharide

After inactivation with formalin, the organisms are separated from the culture and the Vi polysaccharide is precipitated from the culture fluid by hexadecyltrimethylammonium bromide.

All steps in the purification shall be carried out in containers compatible with the solvents in use. Analytical-grade reagents, or their equivalent, shall be employed, and aqueous solutions of salts shall be sterilized, for example by filtration through a membrane of pore size not greater than 0.22 μm. All purification steps shall be carried out in the cold with chilled
reagents, except for phenol treatment, which is conducted at room
temperature. Either the time intervals between purification steps shall be
kept to the minimum, or the products shall be kept frozen at or below
-20°C between steps. After addition of hexadecyltrimethylammonium bromide, the precipitate shall be collected.

The Vi polysaccharide shall be purified from its complex with hexadecyltrimethylammonium bromide. The material resulting from this
preliminary purification is referred to as the “intermediate product”.

The dry product obtained after final purification constitutes a purified Vi
polysaccharide lot. It shall be handled with sterile precautions and stored
at a temperature below 8°C.

The cold phenol method (27), as described in the Requirements for Biological
Substances No. 23 (Requirements for Meningococcal Polysaccharide Vaccine)
(15, 16), has been found to be suitable.

The method of purification and the limits on residual chemicals in the
purification process shall be approved by the national control authority.

The following tests shall be applied to each purified Vi polysaccharide lot;
measurements shall be based on the dry weight of the polysaccharide in its
salt form.

A.3.3.1 Moisture content
The moisture content of the purified polysaccharide shall be determined.

Thermogravimetric analysis at 100°C is generally used. In laboratories lacking
facilities for thermogravimetric analysis, the moisture content may be
determined by the Karl Fischer method, or by drying the polysaccharide under
vacuum over phosphorus pentoxide at 37°C until a constant weight is reached.

A.3.3.2 Protein content
Each purified Vi polysaccharide lot shall contain less than 10 mg of protein
per gram of polysaccharide as determined by the method of Lowry et al.
(22) using bovine plasma albumin as a reference.

A.3.3.3 Nucleic acid content
Each purified Vi polysaccharide lot shall contain less than 20 mg of nucleic
acid per gram of polysaccharide as determined by ultraviolet
spectroscopy, on the assumption that, at a wavelength of 260 nm, the
absorbance of a 1 g/l solution of nucleic acid contained in a cell 1 cm wide
is 20 (15, 16).

A.3.3.4 O-acetyl content
The O-acetyl content of each purified Vi polysaccharide lot shall be not
less than 2.0 mmol/g of Vi polysaccharide as determined by a method
based on that described by Hestrin (23) (see Appendix).

A.3.3.5 Molecular size
The molecular size of each purified Vi polysaccharide lot shall be
estimated by gel filtration (15, 16).
Sepharose CL-4B, with 0.2 mol/l sodium chloride as eluent, is suitable for this purpose.

At least 50% of the Vi polysaccharide from the column shall elute before a distribution constant ($K_D$) of 0.25 is reached (see Appendix). The concentration of Vi polysaccharide in eluted fractions is measured by the method of Hestrin (23).

As there is a relationship between immunogenicity and molecular size, manufacturers are encouraged to produce Vi polysaccharide with even lower $K_D$ values (higher molecular masses) (15, 16, 24).

A.3.3.6 Identity
The identity of the Vi polysaccharide lot with an in-house standard Vi polysaccharide conforming to the purity requirements of sections A.3.3.2-3.3.5 shall be established by immunoprecipitation.

Hyperimmune serum prepared by multiple intravenous injections of Citrobacter freundii, a non-pathogenic organism that has a Vi polysaccharide identical to that of S. typhi, has proved reliable for serological assays.

A.3.4 Final bulk
The final bulk shall be prepared from either a single lot of purified Vi polysaccharide or several pooled lots. If multi-dose vials are used, a preservative shall be added. The amount of preservative in the final bulk shall have been shown to have no deleterious effect upon the polysaccharide or upon other vaccine components with which the Vi polysaccharide may be combined. The preservative and its concentration shall be approved by the national control authority.

The final bulk shall be dissolved under aseptic conditions in a solution free of pyrogenic substances and shall be sterilized by filtration through a membrane.

Membranes with a pore size of 0.22 μm have been found to be satisfactory. The osmolality of the final product shall be such that vaccines to be injected are isotonic with serum.

A.3.4.1 Sterility
The final bulk shall be tested for bacterial and mycotic 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) (25), or by a method approved by the national control authority. If a preservative has been added to the product, appropriate measures shall be taken to prevent any interference by the preservative in the sterility test.

A.3.4.2 Vi polysaccharide concentration
The final bulk may be assayed for its polysaccharide content by a quantitative immunochemical test approved by the national control authority.
A.3.4.3 Identity
The identity of the final bulk shall be established by a serological assay based on immunoprecipitation, as described in section A.3.3.6.

A.4 Filling and containers
The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (28) shall apply.

A.5 Control tests on final lot

The following tests shall be carried out on each final lot of Vi polysaccharide typhoid vaccine.

A.5.1 Identity
An identity test shall be performed by a serological method (see section A.3.3.6) on at least one labelled container.

A.5.2 Vi polysaccharide content

At least three final containers, taken at random, shall be assayed for Vi polysaccharide content by a quantitative immunochemical test in comparison with a reference material conforming to the purity requirements of sections A.3.3.2 and A.3.3.5 and approved by the national control authority. The national control authority shall approve the extent of deviation from the target value that is acceptable for each of the three estimates of Vi polysaccharide content.

The target value is 25 µg of Vi polysaccharide per single human dose (5, 6). In one country, the maximum authorized deviation of each estimate is 30% from the target value.

A.5.3 Sterility

Each final lot shall be tested for sterility as specified in section A.3.4.1.

A.5.4 Pyrogenicity

Each final lot shall be tested for pyrogenicity by intravenous injection into rabbits. The national control authority shall define acceptable pyrogen levels.

The test shall be conducted as specified in The International Pharmacopoeia (26) or in the national pharmacopoeia.

A test that has been found suitable for the current vaccine involves injection into the ear vein of rabbits of 1 ml per kg of body weight of a dilution of vaccine containing 25 ng per ml.

A.5.5 Abnormal toxicity

Each final lot shall be tested for abnormal toxicity by the intraperitoneal injection of one human dose into each of five mice (weighing 17–22 g)
and at least one human dose into each of two guinea-pigs (weighing 250-350 g). The tests shall be approved by the national control authority. The final product shall be considered satisfactory if the animals survive for at least seven days without weight loss.

A.5.6 Preservative content

If preservative has been added to the bulk, the preservative content of each final lot shall be determined. The method used shall be approved by the national control authority.

A.5.7 Residual moisture

If the vaccine is freeze-dried, the moisture content of the dried material shall be determined as indicated in section A.3.3.1. The method used shall be approved by the national control authority.

The test shall be performed on 1 vial per 1000 up to a maximum of 10 vials, but on not less than 5 vials, taken at random from throughout the final lot. The average residual moisture shall be not greater than 2.5%, and no vial shall be found to have a residual moisture content of 3% or greater.

A.5.8 pH

The pH of each final lot shall be determined.

A pH of 7 ± 0.5 measured at ambient temperature has been found to be compatible with vaccine stability.

A.5.9 Inspection of final containers

Every container in each final lot shall be inspected, and those showing abnormalities, such as improper sealing, lack of integrity and, if applicable, clumping or the presence of particles, shall be discarded.

A.6 Stability studies

Stability studies are needed for determining the expiry date of the Vi polysaccharide typhoid vaccine. They shall be based on tests for O-acetyl content and molecular size (see sections A.3.3.4 and A.3.3.5) and shall be conducted on at least three final lots, each derived from a different purified Vi polysaccharide lot, stored under the conditions to be recommended in the leaflet of instructions to the user. In the molecular-size assay, at least 50% of the Vi polysaccharide from the column shall elute before a K_D of 0.25 is reached. The tests for stability shall be approved by the national control authority.

Liquid Vi polysaccharide vaccine may require concentration before the molecular-size assay is performed.

Whenever significant changes are made in the production of Vi polysaccharide typhoid vaccine, further stability studies shall be conducted to determine the proposed validity period of the product.
A new validity period may be approved by the national control authority on the basis of the results of validated accelerated-degradation tests.

A suitable method for conducting accelerated-degradation tests on certain biological products has been described by Kirkwood (27).

A.7 Records

The requirements of Good Manufacturing Practices for Biological Products (18, pages 27-28) shall apply.

A.8 Samples

The requirements of Good Manufacturing Practices for Biological Products (18, page 29, paragraph 9.5) shall apply.

A.9 Labelling and package insert

The requirements of Good Manufacturing Practices for Biological Products (18, pages 26-27) shall apply, with the addition of the following:

The statements concerning storage temperature and expiry date shall be based on experimental evidence of stability obtained as specified in section A.6, and shall be submitted for approval to the national control authority.

The expiry date on the label shall be based upon experimental data but shall in any case not be more than three years from the date the final containers are filled.

Storage at a temperature of 2–8°C has been found to be suitable by one manufacturer.

The label on the carton or the leaflet accompanying the container shall indicate:

– the amount of Vi polysaccharide in each single human dose;
– the volume and nature of the reconstituting fluid, if applicable.

Furthermore, the leaflet accompanying the container shall contain:

– a statement that, once a freeze-dried product has been reconstituted or a multi-dose container of liquid product opened, the vaccine should be used within a working day;
– a statement of the temperature at which the Vi polysaccharide typhoid vaccine should be stored.

A.10 Distribution and shipping

The requirements of Good Manufacturing Practices for Biological Products (18) shall apply.
Part B. National control requirements

B.1 General

The Guidelines for National Authorities on Quality Assurance for Biological Products (28) shall apply.

The national control authority shall approve:

- the strain(s) of _S. typhi_ from which the Vi polysaccharide typhoid vaccine is produced;
- the method of manufacture;
- the methods of purification;
- the preservative and its concentration, if applicable;
- the Vi polysaccharide content of each single human dose and the method of assay; and
- the tests for stability, the validity period and the expiry date.

B.2 Release and certification

A vaccine lot shall be released only if it fulfils the national requirements and/or Part A of the present Requirements. A summary protocol for vaccine production and testing, signed by the responsible official of the manufacturing establishment, shall be prepared and submitted to the national control authority in support of a request for release of vaccine for use.

At the request of the manufacturing establishment, the national control authority shall provide a certificate that states whether the vaccine meets all national requirements and/or Part A of the present Requirements. The certificate shall also state the number under which the lot was released by the national controller, and the number appearing on the labels of the final containers.

The purpose of the certificate is to facilitate the exchange of Vi polysaccharide typhoid vaccine between countries. The certificate may be prepared along the lines of those required for the release of vaccines acquired by United Nations agencies (29).

Authors

The first draft of these Requirements was prepared by Dr J. Robbins, Chief, Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA. It was modified by Dr P. Sizaret, Scientist, and Dr D. Magrath, Chief, Biologicals, WHO, Geneva, Switzerland, and then reviewed and further modified by the following persons who attended a WHO informal consultation in Geneva in July 1991:

Mr P. Crooy, Director, Scientific Affairs, SmithKline Beecham Biologicals, Rixensart, Belgium
Dr C. Frasch, Chief, Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA (Rapporteur)
Dr V. Grachev, Scientist, Biologicals, World Health Organization, Geneva, Switzerland
Dr A. Isibasi, Mexican Institute of Social Security, Mexican Health Foundation, Mexico
Dr B. Ivanoff, Microbiology and Immunology Support Services, World Health Organization, Geneva, Switzerland
Dr B. Lindberg, Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden
Dr D. Magrath, Chief, Biologicals, World Health Organization, Geneva, Switzerland
Dr J. Petres, Institute of Immunology, Zagreb, Yugoslavia
Dr J. Robbins, Chief, Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA (Chairman)
Dr D. Schulz, Pasteur Mérieux Sera and Vaccines, Marcy l’Etoile, France
Dr P. Szarek, Scientist, World Health Organization, Geneva, Switzerland (Secretary)
Dr A. Suer, Département des Vaccins bactériens et antiseptiques, National Health Laboratory, Montpellier, France
Dr B. Szewczyk, Department of Biochemistry, University of Gdansk, Gdansk, Poland
Dr R. S. W. Tsang, Department of Microbiology, Queen Mary Hospital Compound, University of Hong Kong, Hong Kong
Dr Wang Li-ya, National Vaccine and Serum Institute, Beijing, China.

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References


Appendix

Examples of suitable methods and tests for standardizing Vi polysaccharide typhoid vaccines

1. Determination of the O-acetyl group in the Vi polysaccharide
The method described in this section is based on that described by Hestrin (1).

Instrumentation
A spectrophotometer for the visible region of the spectrum is required.

Mechanism
O-Acetyl groups react with hydroxylamine in alkali to form hydroxamic acid. The hydroxamic acid formed is measured by the formation of a purple-brown complex with Fe^{3+} in acid solution.

Reagents
The following reagents are needed:
1. Hydroxylamine hydrochloride, 2 mol/l, which should be stored in the cold.
2. Sodium hydroxide (NaOH), 3.5 mol/l.
3. Concentrated hydrochloric acid (HCl), relative density 1.18, diluted with twice its volume of water.
4. Iron(III) chloride hexahydrate (FeCl₃ · 6H₂O), 0.37 mol/l, in 0.1 mol/l hydrochloric acid.
5. Sodium acetate, 0.001 mol/l, pH 4.5.
6. A standard solution of acetylcholine chloride (relative molecular mass 181.7).
7. Vi polysaccharide solution.

Preparation of standard dilutions and blanks
To prepare the standard solution of acetylcholine chloride (reagent 6), dissolve 150 mg of acetylcholine chloride in 10.0 ml of 0.001 mol/l sodium acetate. Transfer 1 ml of this solution to a test-tube and mix it with 9 ml of 0.001 mol/l sodium acetate. From this solution, accurately transfer 0.1, 0.2, 0.3, 0.4 and 0.5 ml quantities in duplicate to 10 test-tubes, and add respectively 0.9, 0.8, 0.7, 0.6 and 0.5 ml of 0.001 mol/l sodium acetate to make the volume in each tube 1.0 ml. The O-acetyl concentrations in these standard tubes are respectively 0.83, 1.66, 2.48, 3.32 and 4.15 µmol/ml. The duplicate at each concentration is used as a standard blank.

\[\text{Draft prepared by Dr. C. Frasch, Chief, Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA.}\]
After addition of reagents, as detailed in the procedure below, the absorbances of the standard solutions at 540 nm are about 0.1, 0.25, 0.4, 0.5 and 0.65. The precise wavelength at which the readings should be made is the wavelength at which the net absorbance between 520 and 540 nm (i.e. that of the standard minus that of the corresponding blank) is the highest.

**Test procedure**

The test procedure is carried out at room temperature. The O-acetyl content of the Vi polysaccharide sample is determined by reference to the standard dilutions of acetylcholine chloride. All samples, standard dilutions and blanks are tested in duplicate.

1. Prepare the sample for analysis by making up a 1.0 g/l solution of the polysaccharide in water. For each duplicate test polysaccharide use 1 ml of this solution.
2. To 1 ml of the polysaccharide sample and to each of the standard dilutions add 2 ml of a freshly prepared (within three hours) mixture of equal parts of reagents 1 and 2 and mix.
3. After exactly four minutes at room temperature, add 1 ml of reagent 3 to bring the pH to 1.2 ± 0.2 and mix.
4. Add 1 ml of reagent 4 and mix.
5. Swirl the contents of the test-tubes rapidly during the addition of each of the above reagents.
6. For the blank solutions (one blank for each concentration of the standard and a sample blank of 1 g/l Vi polysaccharide solution) carry out step 3 before step 2, i.e. add the hydrochloric acid before the NaOH-hydroxylamine solution. In Hestrin's method the blank determination is called the “test for non-specific colour”.
7. Promptly read the absorbance of the solution in each tube at the appropriate wavelength.
8. Subtract the sample blank reading from that of the test sample and subtract the standard blank readings from those of the standard dilutions.
9. For the standard dilutions, plot a curve of absorbance against O-acetyl concentration in μmol/ml. From the values of absorbance obtained for the sample, the corresponding number of micromoles of O-acetyl per milligram of Vi polysaccharide can then be read off the reference curve (since the test was conducted on 1 ml of the Vi polysaccharide solution, which contains 1 mg of Vi polysaccharide).

The weight of Vi polysaccharide used in the 1 g/l polysaccharide solution is corrected for its moisture content and the final result is expressed in “millimoles of O-acetyl per gram of dry polysaccharide”.

*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 micromoles of O-acetyl, then:

\[
\frac{1.95 \, \mu\text{mol} \text{ O-acetyl}}{1 \, \text{mg “wet” polysaccharide}} = \frac{1.95 \, \mu\text{mol} \text{ O-acetyl}}{0.9 \, \text{mg “dry” polysaccharide}} = 2.17 \, \text{mmol O-acetyl per gram of “dry” polysaccharide.}
\]

2. **Molecular sizing of Vi polysaccharide by Sepharose CL-4B gel permeation**

The method used for molecular sizing is based on the protocol described in the Requirements for Meningococcal Polysaccharide Vaccine (2, 3).

**Reagents**

The following reagents are needed:
- Sepharose CL-4B gel
- sodium chloride, 0.2 mol/l, as eluent
- sodium azide
- Blue Dextran 2000
- reagents for O-acetyl determination by the method of Hestrin (1).

**Equipment**

The following equipment is required:
- column (1.5 x 90 cm)
- automatic fraction collector and test-tubes
- gel and eluent reservoir
- refractometer or other appropriate monitoring device and recorder (optional).

**Procedure**

*Preparation of the gel-filtration column*

Prepare the column according to the recommendations of the supplier.

*Calibration of the column*

The void volume \( V_0 \) is determined with Blue Dextran and the total volume \( V_t \) with sodium azide. Dissolve 200 mg of Blue Dextran in 100 ml of 0.2 mol/l sodium chloride solution containing 500 mg of sodium azide. Apply 1 ml of this solution to the column and collect fractions of about 2 ml each. Determine \( V_0 \) and \( V_t \) either by refractometry or by ultraviolet absorption at 206 nm or 260 nm.

*Assay of the purified Vi polysaccharide*

Dissolve 5 mg of Vi polysaccharide in 1 ml of eluent. Apply this solution to the column and collect 2-ml fractions. Make two pools of fractions:
- pool I: fractions eluted before a distribution constant \( (K_D) \) of 0.25 is reached,
- pool II: fractions eluted after a \( K_D \) of 0.25 is reached.

Quantify the Vi polysaccharide in each pool by determining the O-acetyl content by the method of Hestrin (1).

At least 50% of the recovered Vi polysaccharide shall be eluted before a \( K_D \) of 0.25 is reached.

References

