

Annex 8

REQUIREMENTS FOR HEPATITIS B VACCINE PREPARED FROM PLASMA

(Requirements for Biological Substances No. 31)
(Revised 1987)

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INTRODUCTION

Viral hepatitis is a major public health problem occurring endemically in all parts of the world. There is substantial evidence that hepatitis B may progress to chronic liver disease, including chronic persistent hepatitis, chronic active (aggressive) hepatitis, and cirrhosis. About 80% of hepatocellular carcinomas are ascribed to chronic infection with hepatitis B virus. In some regions of the world, delta virus coinfection and superinfection have been associated with a high morbidity and mortality in hepatitis-B-positive individuals.

Hepatitis B virus (HBV) has been identified as a 42-nm particle (known as the Dane particle) containing double-stranded DNA. At least three antigenic components are produced during infection with hepatitis B virus as a result of replication of the virus in hepatocytes. These components are hepatitis B surface antigen (HBsAg), the core antigen (HBcAg), and the *e* antigen (HBeAg). Infection also produces high titres of anti-HBcIgM. The surface antigen is most frequently found as 20–22 nm spherical particles (sometimes slightly larger or smaller) and as tubular forms, and possesses common determinants *a* and generally at least two mutually exclusive subdeterminants *d* or *y* and *w* or *r*. Other subspecificities have also been recognized.

The protein moiety of hepatitis B surface antigen particles consists of a polypeptide of relative molecular mass 23 000, existing in glycosylated and non-glycosylated forms. Minor components are also present which contain amino acid sequences referred to as pre-S1 and pre-S2 as well as this polypeptide. The importance of these additional sequences in immunization is under investigation.

Hepatitis B virus has not yet been cultivated in cell culture and small laboratory animals are not susceptible to infection. The infection can be transmitted to certain of the apes, of which the chimpanzee is the only available susceptible animal model.

Transmission of hepatitis B infection from carrier mothers to their babies can occur during the perinatal period and among children in the first years of life and is an important factor determining the prevalence of the virus infection in some regions. Such transmission can be interrupted by the use of vaccine.

Because of the urgent need for a hepatitis B vaccine, particularly for groups that are at increased risk of acquiring infection (1), WHO Requirements were formulated in 1980 (2) and revised in 1984 (3). Since it has been shown that the separated viral coat proteins, containing hepatitis B surface antigen, lead to the production of protective antibody, it is possible to use purified, non-infectious 22-nm spherical hepatitis B surface antigen particles, or subunits derived from the surface antigen, as vaccines. However, the preparation of such vaccines for use in man from human viral antigens not grown in cell culture, but obtained from the plasma of infected persons—namely, from persistent carriers of hepatitis B antigens—demands special consideration in the tests applied to the production and quality control of the vaccines. Still more important, it has now been shown that human blood and plasma may harbour

a number of infectious agents in addition to hepatitis B virus. Particular attention, therefore, must be given to the selection of the donors of the plasma, the process of separation of the antigen, and the inactivation procedures to ensure that all potential infectious agents that may still be present after the purification of the antigen have been inactivated.

The development of vaccines

A number of laboratories have prepared vaccines using HBsAg purified from plasma obtained from antigenaemic carriers of hepatitis B. Vaccines of varying degrees of purity and technological complexity have been prepared and some have been tested in humans.

Source plasma could contain infectious agents that possess a wide range of physico-chemical and biological characteristics and various degrees of susceptibility or resistance to different modes of inactivation. Consequently, to ensure as far as possible the inactivation of a wide range of infectious agents, it is desirable that, in addition to separation and purification, a procedure or procedures that will inactivate all infectious agents that may be present in human blood should be applied during the vaccine manufacturing process.

Whatever the procedure used, it is universally accepted that the vaccines must be safe (i.e., free from demonstrable virus and other microbial agents), potent (i.e., capable of eliciting antibody against the virus in animals and in man by the administration of a standardized dose of antigen) and efficacious (i.e., protective against the disease).

There have been a number of significant findings in the production of hepatitis B vaccine since the WHO Requirements were first formulated in 1980. In choosing methods of purification and inactivation, it is important to appreciate that hepatitis B surface antigen is a relatively stable glycoprotein that can withstand fairly harsh treatment. This permits the production of an essentially pure vaccine for which assurances as regards safety can be given. The consistency of vaccine production, including in particular uniformity of composition and potency of the final product and effectiveness of virus inactivation procedures applied during manufacture, is most readily achieved for highly purified materials. Consequently it is desirable that the manufacturing procedure

adopted enables a high degree of purification of HBsAg to be reliably achieved.

Hepatitis B vaccines prepared to date have been adjuvanted, and all are assayed for their ability to stimulate anti-HBs in small laboratory animals. An immunogenicity test performed in mice has been developed for quantification of the immunizing potency of batches of vaccine, and an International Reference Reagent has been established for this purpose.

Controlled studies in chimpanzees have shown the efficacy of several vaccines in preventing hepatitis B following challenge with human hepatitis B virus. Cross-protection studies carried out in chimpanzees have shown that subtypes are not of major importance in vaccine composition. In one country, the use of the vaccine in the staff of a renal dialysis unit has shown considerable protection against *ay* infection by *ad* vaccine. The reverse is also true because of the common *a* components.

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.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning hepatitis B vaccine, it is recommended that a clause be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. 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 final vaccine is manufactured.

PART A

MANUFACTURING REQUIREMENTS

1. Definitions

1.1 International name and proper name

The international name shall be *Vaccinum hepatitis B ex plasma humanum*. 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 hepatitis B ex plasma humanum is a preparation of purified hepatitis B surface antigen (HBsAg) that has been treated to inactivate HBV and other viruses known to be present in human blood. The preparation shall satisfy all the requirements formulated below.

1.3 International reference materials

For the assessment of immunogenicity of vaccines, an international reference reagent exists in the form of an adjuvanted vaccine, for injection into animals (see Part A, section 5.6). This preparation is in the custody of the National Institute for Biological Products and Control, Potters Bar, England.

For the assay of antigenic content by techniques such as radioimmunoassay, ELISA, or single radial immunodiffusion, a purified international reference reagent without adjuvant is required (see Part A, section 3.6.2).

For the measurement of antibody to hepatitis B vaccines, an international reference preparation is available. This preparation is calibrated in terms of anti-HBs. The International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (fractionated human plasma, freeze-dried). This preparation is in the custody of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands.

1.4 Terminology

The following definitions are given for the purposes of these Requirements only.

Whole blood (sometimes referred to as blood): the blood collected in an anticoagulant solution.

Plasma: the liquid part of blood collected in a receptacle containing an anticoagulant.

HBV: hepatitis B virus. A 42-nm enveloped virus, originally known as the Dane particle.

HBsAg: hepatitis B surface antigen, the complex of antigens associated with the virus envelope and subviral forms (22-nm spherical and tubular particles). Native HBsAg is coded for by envelope gene sequences S plus pre-S of HBV DNA.

HBcAg: hepatitis B core antigen. The hepatitis B antigen found within the core of the virus.

HBeAg: the *e* antigen has now been identified as a cryptic HBc antigen.

Anti-HBs: antibody to hepatitis B surface antigen.

Anti-HBc: antibody to hepatitis B core antigen.

Anti-HBe: antibodies to the *e* antigen(s).

Single donor plasma: plasma obtained from a single donation of whole blood or obtained by plasmapheresis.

Plasma pools: pools of single-donation plasmas that have been shown to be satisfactory before pooling.

Purified HBsAg batch: purified HBsAg prepared from one or more plasma pools by suitable procedures that inactivate HBV and any other viruses that may be present in human blood.

Final aqueous bulk: the final bulk before the addition of an adjuvant.

Final bulk: the finished biological material prepared from one or more batches of purified HBsAg that have been treated to inactivate HBV and any other viruses that may be present in human blood from which the final containers are filled.

Final lot: a collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or preparation of the finished vaccine. A final lot must therefore consist of finished material dispensed into containers in one working session from a single final bulk.

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 to establishments manufacturing hepatitis B vaccine, with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine. Completely separate areas shall be used for the separation and inactivation steps. All separation and inactivation steps shall be carried out in closed systems and closely monitored.

The production of hepatitis B vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy and not carriers of hepatitis B.

Steps should be taken to ensure that all such persons in the production areas are immune to hepatitis B as shown by the presence of anti-HBs, either as a result of natural infection or by immunization.

Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 13) regarding the training and experience of persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

3. Production Control

The general production precautions formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 15) shall apply to the manufacture of hepatitis B vaccine.

3.1 The collection of blood and plasma

3.1.1 *The selection of donors*

Source materials for further processing are obtained from donations of blood or plasma. The medical criteria for accepting donors—criteria relating to the safety, purity, potency, and efficacy of the final products—must be the same for donors of whole blood (see Part A, section 3.1.2) components or blood components collected by plasmapheresis (6, Annex 1, p. 38), except that the donors must be antigenaemic and need not meet the exclusions relating to hepatitis. Only plasma from donors who are seronegative in appropriate tests for human immunodeficiency virus (HIV) shall be used (5).

In some countries separate areas or special times are set aside for the collection of plasma known to be HBsAg positive.

In some countries donors with a high HBsAg content but negative for HBeAg are selected for the donation of plasma.

Records should be kept of the identity of the donors for the identification of each batch of vaccine.

3.1.2 *Donors of whole blood*

The physical fitness of a donor shall be determined by a licensed physician or a person under the direct supervision of a licensed physician. Donors shall be asymptomatic persons of either sex between the ages of 18 and 65 years, except that the findings in liver function tests may exceed normal limits provided that the values obtained are stable.

When plasma is collected from regions or populations with a high prevalence of hepatitis delta virus infection, screening of individual donors for evidence of chronic delta virus infection should be considered.

3.1.3 *Medical history*

General. Before each donation questions shall be asked to determine that the donor is asymptomatic and has not suffered, or is not suffering, from any serious illness—e.g., malignant disease, diabetes, epilepsy, hypertension, renal disease, malaria.

Any donor who appears to be suffering from symptoms of acute or chronic disease, or who is receiving oral or parenteral medication, with the exception of vitamins or oral contraceptives, may not be

accepted for donation unless approved by a physician. The values obtained in liver function tests may exceed normal limits provided that the values obtained are stable.

Any donor who appears to be under the influence of alcohol or any drug or who does not appear to be providing reliable answers to medical history questions shall not be accepted.

National health authorities shall develop policies designed to prevent the transmission of other infectious diseases based on the prevalence of these diseases in the donor population and the susceptibility of recipients to the same diseases.

Minor surgery. Donors shall not have a history of tooth extraction or other minor surgery during a period of 72 hours prior to donation.

Pregnancy. Pregnant women shall be excluded from blood donation. In general, mothers shall also be excluded for the period of lactation and for at least 6 months after full-term delivery.

Immunization. Symptom-free donors who have recently been immunized may be accepted with the following exceptions:

- those receiving attenuated vaccines for measles (rubeola), mumps, yellow fever, or poliomyelitis shall be excluded until 2 weeks after the last immunization or injection;
- those receiving attenuated rubella (German measles) vaccine shall be excluded until 8 weeks after the last injection;
- those receiving rabies (therapeutic) vaccine or rabies immunoglobulin shall be excluded until 1 year after the last injection;
- those receiving passive immunization using animal serum products shall be excluded until 4 weeks after the last injection.

3.1.4 *Physical examination*

Donors shall have a weight, blood pressure, pulse rate, and temperature within normal limits. Donors with any measurements outside the established normal limits of weight, blood pressure, and pulse rate may be accepted only if approved by the responsible licensed physician.

The following recommendations may be useful for guidance:

- (1) Blood pressure. Systolic blood pressure between 12 and 24 kPa (90 and 180 mmHg); diastolic blood pressure between 6.7 and 13.3 kPa (50 and 100 mmHg).
- (2) Pulse. Between 50 and 100 beats per minute and regular.
- (3) Temperature. Oral temperature not exceeding 37.5°C.

(4) Weight. Donors weighing less than 50 kg may be bled proportionately less than 450 ml per unit, provided all other donor requirements are met.

In some countries it is not required to take the body temperature, but the decision to do so or not should be made by the national control authority.

Donors shall be free from any infectious skin disease at the venepuncture site and from skin punctures or scars indicative of addiction to narcotics.

3.1.5 *Determination of haemoglobin or erythrocyte volume fraction*

The haemoglobin shall not be less than 125 g/l of blood for women and 135 g/l of blood for men. If erythrocyte volume fraction measurement is substituted for haemoglobin measurement the values shall be not less than 0.38 and 0.41 for men and women, respectively.

These limits are not universally accepted, and the national control authorities should raise or lower them when considered to be appropriate.

3.1.6 *Donors for plasmapheresis*

All phases of plasmapheresis, including explaining to donors what is involved in the process and obtaining their informed consent, shall be performed under the direct supervision of a licensed physician.

There are two groups of plasmapheresis donors: those who donate at a frequency comparable to that allowed for whole blood donations and those who donate more frequently. The former group shall be accepted on the basis of the above criteria for donors of whole blood.

In addition to these criteria, donors participating in the plasmapheresis programme shall be examined by a licensed physician on the day of the first donation, or no more than 1 week prior to the first donation. This examination shall include urine analysis and blood sampling for liver function tests, and determination of plasma proteins by electrophoresis or another suitable method.

On the day of each donation, in addition to meeting the requirements for whole blood donors, plasmapheresis donors shall be shown to have a total serum protein of no less than 60 g/l.

The medical evaluation of plasmapheresis donors shall be repeated at monthly intervals, or as specified by the national control authority.

Whenever a laboratory value other than a liver function test is found to be outside the normal limits or any important abnormalities are noted in a donor's history or on physical examination, the donor shall be removed from the programme. The donor shall not return to the programme until the abnormal finding has returned to normal and the responsible physician has given approval.

If a plasmapheresis donor donates a unit of whole blood or does not have the red blood cells returned from a unit taken during the procedure, further plasmapheresis of the donor shall be deferred for 8 weeks unless special circumstances warrant approval by the responsible physician of earlier plasmapheresis. Plasmapheresis of donors of HBsAg-positive plasma will be permitted by the responsible physician, even if the liver function test values are above normal limits in individual donors, provided that the values obtained are stable.

The upper limits of these values should be specified by the national control authority.

Appropriate guidelines to define donor changes significant to justify discontinuation of plasmapheresis should be established by the responsible physician (6, p. 43).

The maximum volume of blood or plasma that may be taken in 1 year from chronic hepatitis B carriers shall be determined by the national control authority.

No guidelines have been established for the maximum volume of plasma that can be taken in any year from a chronic hepatitis B carrier.

In some countries it is permitted to take 500 ml per week.

Where plasmapheresis is regularly performed on HBsAg-positive donors, it is advisable to have equipment set aside specifically for this purpose.

3.2 Tests on single-donation plasma

Each single-donation plasma, whether obtained from whole blood or by plasmapheresis, shall be tested for HBsAg content by a method approved by the national control authority.

Several tests are suitable for this purpose (*J*). Potency should be established with reference to an appropriate potency standard included in all assays.

An international standard is available for the calibration of working reference reagents.

In some countries, unless the subtype of a particular donor has been identified, each single donation is tested for HBsAg subtype by a method approved by the national control authority.

The national control authority may require the data on the subtype composition.

In some countries the tests are done on plasma pools, when this is approved by the national control authority.

3.3 Pooling of single-donation plasma

Only acceptable plasma shall be included in a plasma pool.

3.4 Tests on plasma pools

3.4.1 Sterility tests

A volume of at least 10 ml of each plasma pool shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 48).

In some countries it is required that each plasma pool shall be tested for the presence of mycobacteria by culture methods approved by the national control authority.

3.4.2 Tests for extraneous viruses

3.4.2.1 Tests in animals and fertile eggs

(a) Tests in adult mice

Each plasma pool shall be tested in adult mice for adventitious agents pathogenic to mice. Each of at least 10 adult mice, of 15–20 g weight, shall be inoculated intracerebrally with 0.03 ml and at least 10 mice intraperitoneally with at least 0.5 ml of the plasma pool. The mice shall be observed for at least 21 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection, both macroscopically by direct observation and by subinoculation of

appropriate tissue suspensions by the intracerebral and intraperitoneal route into at least 5 additional mice, which shall be observed for 21 days.

The plasma pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the plasma pool.

(b) *Tests in suckling mice*

Each plasma pool shall be tested in suckling mice for adventitious agents pathogenic to mice. Each of at least 20 mice less than 24 hours old shall be inoculated intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the plasma pool. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined to determine the cause of death or illness.

In some countries a subinoculation is suggested and in others an additional blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The plasma pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the plasma pool.

(c) *Tests in embryonated eggs*

A sample of at least 5 ml of each plasma pool shall be tested in a group of embryonated hens' eggs by the allantoic route of inoculation and a similar sample in a separate group of eggs by the yolk-sac route of inoculation, using at least 0.25 ml of the pool per egg for each route of inoculation. The incubation of the eggs and the observation time shall be approved by the national control authority. The embryo shall remain normal throughout the observation period.

The plasma pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the plasma pool.

3.4.2.2 *Tests in cell cultures*

A sample of at least 5 ml of each plasma pool shall be tested for adventitious agents by inoculation into simian cell cultures. Similar

volumes of the plasma pool shall likewise be tested in human diploid cell cultures. The inoculated cell cultures and uninoculated control cultures shall be observed for at least 14 days. At the end of this observation period a subculture in the same cell system shall be made and cultures observed for at least 14 days.

In some countries larger volumes of the plasma pool are required to be tested.

Suitable simian cell cultures are prepared from the kidneys of *Cercopithecus* monkeys or from Vero cells. For the human cell cultures, either WI-38 or MRC-5 may be used.

The plasma pool passes the test if none of the cell cultures shows evidence of the presence of any adventitious agent attributable to the plasma pool.

3.4.2.3 *Other tests*

It may be desirable to carry out tests on the plasma pools for HBV DNA by sensitive DNA hybridization assays to monitor the elimination of HBV DNA by subsequent purification steps.

3.5 Concentration, purification, and inactivation

Each plasma pool shall be subjected to procedures that concentrate and purify HBsAg consistently and result in the inactivation of residual HBV and any extraneous agent that may be present in human blood. The methods used shall remove the bulk of extraneous substances and inactivate infectious agents so that the resultant purified product is safe when administered to humans.

In some countries the required HBsAg content of vaccines is not less than 95% of total protein content of the finished vaccine.

The national control authority shall approve the methods used for concentration and purification of HBsAg and for inactivation of HBV and other potential contaminating agents. The national control authority shall approve the number of inactivation steps that shall be used.

The national control authority shall ensure that the production process, including purification and inactivation, is reproducible and will give rise to consecutive lots that do not differ with respect to safety.

When new processes for HBV vaccine manufactured from human plasma are introduced, the efficiency of hepatitis B DNA removal at each step during the purification process shall be validated.

Removal of HBV DNA can be monitored by DNA hybridization assays.

Chimpanzee studies may be carried out to validate the inactivation process. In such tests titrated HBV stocks containing approximately 10^6 chimpanzee infectious doses are added to the material prior to inactivation and residual infectivity in the inactivated material is detected by the inoculation of two chimpanzees. Titrated HBV stocks are available from The New York Blood Center.

Should proteins other than HBsAg remain in the vaccine, the national control authority shall take into consideration data which identify such proteins and which show that they do not compromise the safety of the product.

In assessing the efficiency of a procedure or procedures for inactivation the national control authority shall take into consideration data demonstrating the ability of each method to inactivate infectious agents that may be found in human blood.

Precipitation by ammonium sulfate and polyethylene glycol has been found suitable for the concentration of HBsAg.

Much experience has now been gained in the consistent production of safe batches of vaccine. Reliance is placed predominantly on different methods (chemical treatment, physical separation, and heat treatment).

A procedure that has been successfully used includes purification by zonal centrifugation, followed by three chemical treatment procedures:

- (i) pepsin, 1 $\mu\text{g}/\text{ml}$ at pH 2.0 held at 37°C for 18 hours;
- (ii) urea, 8 mol/litre held at 37°C for 4 hours; and
- (iii) formalin 1:4000 (1:10 000 formaldehyde) at 37°C for 3 days.

A second method involves the separation of the HBsAg, including isopyknic zonal centrifugations through cesium chloride, followed by treatment with 1:4000 formalin (1:10 000 formaldehyde) at 30°C for 48 hours.

A third approach includes 3 isopyknic zonal centrifugation steps with KBr and rate zonal centrifugation through sucrose followed by heat treatment of the HBsAg at 60°C for 10 hours and treatment with 1:2000 formalin (1:5000 formaldehyde) at 37°C for 4 days.

One manufacturer uses differential precipitation with polyethylene glycol and ultracentrifugation, followed by heat inactivation for 90 s at 103°C . After adsorption to aluminium phosphate, the product is heated for 10 hours at 65°C . Another

manufacturer uses differential precipitations with polyethylene glycol, selective adsorption on hydroxylapatite and isopycnic centrifugation in KBr followed by 2 heating steps under defined conditions: first at 102°C for 2 min 40 s, then at 65°C for 10 hours.

3.6 Tests on purified, inactivated HBsAg batches

3.6.1 Sterility tests

A volume of at least 10 ml of each batch shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 48).

3.6.2 Tests for HBsAg and total protein

After purification, the protein content shall be measured for both total protein and HBsAg-specific protein. The latter shall be compared with that of a suitable reference preparation.

It has been found suitable to measure the total concentration of protein by means of extinction coefficient $E_{280}^{1\%}$ or by the micro-Kjeldahl technique, the Lowry test or another appropriate method.

The content of HBsAg shall be determined by a serological test in comparison with a suitable reference preparation. It is important that the method of production gives a reproducible content of HBsAg. The lower limit of concentration permitted shall be determined by the national control authority.

Both radioimmunoassay and ELISA methods have been shown to be suitable for this purpose. Other tests such as single radial immunodiffusion may be used.

The concentration of HBsAg shall be related to the total protein.

3.6.3 Tests for extraneous substances

Tests shall be made for the presence of blood group substances and other blood proteins, including liver-specific membrane proteins, by methods approved by the national control authority.

Agglutinins and agglutinogens are tested for by haemagglutination. Immunoassays, such as immunoelectro-

phoresis, agar gel diffusion, radioimmunoassay and ELISA, and polyacrylamide gel electrophoresis have been used to test for other extraneous proteins.

The preparation shall be free from detectable blood group substances.

The permitted concentration of non-HBsAg proteins present in the vaccine shall be determined by the national control authority.

3.6.4 *Test for HBV DNA*

The preparation shall be free from HBV DNA, as determined by a sensitive hybridization assay approved by the national control authority.

DNA is extracted from a volume of aqueous bulk concentrate that corresponds to 10 adult doses and tested for HBV DNA sequences using a sensitive and specific hybridization assay. The assay should be capable of detecting at least 1 pg of HBV DNA per 10 adult doses.

3.6.5 *Test for antigen purity*

A test shall be made for purity of HBsAg by polyacrylamide gel electrophoresis (PAGE).

In reduced preparations there should be 2 bands shown by polyacrylamide gel electrophoresis, one at 22 000–23 000 and another at 28 000–30 000 relative molecular mass. Additional HBV-specified or non-HBV-specified bands may also be present.

The national control authority shall determine the electrophoretic pattern permitted as a demonstration of purity.

3.6.6 *Tests for reagents used during manufacture*

A test shall be made for the presence of any potentially hazardous reagent, including inactivating reagents, that may have been used during the manufacture of the HBsAg.

The method used and the permitted concentration shall be approved by the national control authority.

3.7 **Final aqueous bulk**

The final aqueous bulk consists of one or more purified, concentrated HBsAg batches that have been treated to inactivate

infectious agents that may be present in human blood. Only batches that have satisfied the requirements in Part A, sections 3.5 and 3.6, shall be included in the final bulk.

3.7.1 *Safety test*

In view of the concern about the possibility of infectious agents being present in the plasma used for the production of hepatitis B vaccine, the national control authority shall ensure that the procedures for the collection of plasma and the production process, including purification and inactivation, are reproducible and will give rise to consecutive lots that will not differ with respect to safety.

The national control authority shall determine whether initial lots of vaccine shall be tested for the presence of infectious hepatitis B viruses in chimpanzees. The test shall be approved by the national control authority.

If a test in chimpanzees is not or cannot be carried out, alternative approaches may be considered by the national control authority. For example, clinical studies in man have been carried out that demonstrated the production of a vaccine acceptable to the national control authority.

When a chimpanzee safety test is used, the first 5 consecutive lots prepared by the same production procedures shall be tested in chimpanzees. These 5 lots shall be tested individually without pooling. If these 5 lots pass this test, safety testing of subsequent lots in chimpanzees may be discontinued. If an established manufacturing process is altered or the same process transferred under controlled conditions from one manufacturer to another after initial safety validation in chimpanzees, the national control authority may elect to reduce the number of chimpanzee safety tests required for the new production lots to less than 5.

Aqueous bulks that fail the test shall not be used to prepare vaccine for use in man. In such a case it shall be considered that the consistency has not been established and a further 5 consecutive lots must be tested. The reasons for failing the test shall be investigated and reported to the national control authority.

The chimpanzees used for testing shall have been under observation for at least 6 months before inoculation and shown to satisfy the conditions listed below. The chimpanzees shall:

(a) be free from hepatitis B virus infection, past or present, as shown by sensitive techniques (negative tests for HBsAg, anti-HBs, and anti-HBc);

(b) have normal levels of aminotransferases in at least 8 specimens taken during the 8 weeks that immediately precede the start of the study;

(c) have had at least 2 normal liver biopsies taken during the 8 weeks that precede the start of the study;

(d) be housed in adequate isolation quarters and attended by persons free from hepatitis B infection;

(e) have never received blood or blood products of human origin.

A satisfactory test involves 2 chimpanzees. One animal shall receive 1 human dose and the other shall receive 10 human doses by intravenous injection.

During the observation period of 6 months after inoculation the tests shall include:

(a) weekly determination of alanine aminotransferase (ALT), which shall remain normal for each individual chimpanzee; any abnormal finding shall be demonstrated to be unrelated to viral hepatitis;

(b) weekly determinations of the markers of HBV infection, using sensitive serological methods;

(c) antibody assays for HIV before inoculation and 4 and 6 months after inoculation;

(d) weekly weight determinations and daily checks of general health;

(e) biopsies for light microscopic examination to search for evidence of hepatitis taken monthly and at any time that the chimpanzees show any abnormality.

If after 6 months' observation the chimpanzees have shown normal alanine aminotransferase values throughout with no histological evidence of hepatitis or serological evidence of hepatitis B virus infection, the vaccine passes the chimpanzee safety test. If an animal develops only anti-HBs the test of this animal is invalidated, and an additional animal shall be added to the test.

In such circumstances consideration should be given to decreasing the amount of antigen administered.

3.7.2 *Sterility tests*

A volume of at least 10 ml of the final aqueous bulk shall be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (7, p. 49).

3.7.3 *Test for HBsAg*

The quantity of HBsAg compared with the total protein in the final aqueous bulk shall be determined by a quantitative serological procedure in comparison with a suitable reference reagent. The lower limit of HBsAg and the limit of total protein per human dose shall be approved by the national control authority.

The tests referred to in Part A, section 3.6.2, have been found to be suitable. The samples for this test should be taken before the addition of preservative.

3.7.4 *Pyrogenicity test*

Each final bulk shall be tested for pyrogenicity by a suitable test. The test shall be approved by the national control authority.

3.8 **Final bulk**

3.8.1 *Addition of adjuvant*

Where the final bulk contains an adjuvant, the adjuvant and the concentration used shall be approved by the national control authority. Where aluminium salts are used, the concentration of aluminium shall not exceed 1.25 mg per single human dose.

At this stage more preservative may need to be added.

In some countries the alum used as an adjuvant is formed in the presence of the HBsAg, whereas in others preformed alum salts are added to the aqueous bulk. Where preformed aluminium adjuvants are used, it may not be possible to resolubilize the aluminium compound, and the testing for purity and concentration of the HBsAg in the final bulk may not be possible.

3.8.2 *Test for completeness of adsorption to adjuvant*

Tests shall be carried out to confirm that all HBsAg is adsorbed to the adjuvant. The tests shall be approved by the national control authority.

3.8.3 *Tests for sterility*

A volume of at least 10 ml of the final bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (7, p. 49).

3.8.4 *Tests for preservative*

The final bulk shall be tested for the presence of preservative. The method used and the permitted concentration shall be approved by the national control authority.

4. Filling and Containers

The requirements concerning filling and containers in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 16) shall apply.

Care should be taken to ensure that the material of which the container is made does not adversely affect the HBsAg under the recommended conditions of storage.

Adjuvanted HBsAg vaccine can only be stored at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$.

5. Control Tests on Final Product

Samples shall be taken from each final lot for the tests in the following sections.

5.1 Sterility tests

The final lot shall be tested for sterility according to the requirements in Part A, section 5, of the revised Requirements for

5.2 Innocuity tests

Each final lot shall be tested for innocuity by appropriate tests in mice and guinea-pigs, using parenteral injections. The tests shall be those approved by the national control authority.

5.3 Test for preservative

Each final lot shall be tested for the presence of preservative. The test used and the permitted concentration shall be approved by the national control authority.

5.4 Assay of adjuvant

Each final lot shall be assayed for the content of adjuvant. The method used and permitted concentration shall be approved by the national control authority. Where aluminium compounds are used, the concentration of aluminium shall not be greater than 1.25 mg per single human dose.

5.5 Pyrogenicity test

Each final lot shall be tested for pyrogenicity by a suitable test. The test shall be approved by the national control authority.

5.6 Potency and identity test

The vaccine shall be identified as HBsAg by appropriate methods. An appropriate quantitative potency assay shall be performed on each final lot irrespective of how many filling lots are made. The vaccine potency shall be compared with that of the international reference reagent.

A suitable quantitative extinction test in mice is as follows:

Each of a group of at least 20 suitable mice, 5 weeks of age, is vaccinated intraperitoneally with a graded dose of adjuvanted hepatitis B vaccine diluted in the adjuvant used in the vaccine. Similar groups of mice are inoculated with the adjuvanted reference preparation. The mice are bled 28 days later and the sera are kept separate. Antibody determinations are performed

by a sensitive quantitative test such as radioimmunoassay. The lower limit should be less than 25% response. The data are analysed according to seroconversion as well as according to the geometric mean titre of anti-HBs for each antigen dose. The strain of mice used for this test must give a steep dose-response curve to the reference antigen.

In some countries a quantitative extinction test in guinea-pigs has been shown to be suitable.

The potency shall be measured in terms of quantity of vaccine giving an antibody response in 50% of the animals. The national control authority shall determine the lower limit of potency.

6. Records

The requirements in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 17) shall apply.

7. Samples

The requirements in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

8. Labelling

The requirements in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:

- the method used in the inactivation of the HBV;
- the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
- the volume of one recommended human dose, immunization schedules, and the recommended routes of administration; these

- shall be given for newborn babies, children, adults, and immuno-suppressed individuals and shall be the same for a given vaccine for all regions of the world;
- the amount of protein contained in one recommended human dose;
 - the amount of HBsAg contained in one recommended human dose.

9. Distribution and Shipping

The requirements in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

In addition, the condition of shipping shall be such that the vaccine does not freeze.

Temperature indicators should be packaged with each vaccine shipment to show that freezing did not occur.

If freezing has occurred, the vaccine should not be used.

10. Storage and Expiry Date

The requirements given in Part A, section 10, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

In addition, the conditions of storage shall be such that the vaccine does not freeze.

10.1 Storage conditions and stability

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccines, all vaccines in bulk form or in final containers shall be kept at $5 \pm 3^\circ\text{C}$. After distribution or issue, the vaccine shall be stored at a temperature not exceeding 8°C . The vaccine shall have been shown to maintain potency for a period equal to that between the date of issue and the expiry date. During storage the vaccine shall not be frozen.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of this test being that on which the test system was inoculated.

PART B

NATIONAL CONTROL REQUIREMENTS

1. General

The general requirements for control laboratories in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

The national control authority shall:

- approve the medical evaluation of donors;
- approve the methods of sterility control;
- approve the tests for HBsAg concentration and define its minimum value;
- approve the methods for concentration, purification, and inactivation;
- approve the purity of the final product;
- approve the tests for extraneous substances and total protein;
- approve the tests for the agents used for concentration and purification, free formaldehyde, and other inactivating agents and preservatives;
- approve the test for the presence of infectious hepatitis B virus;
- approve the tests used for freedom from abnormal toxicity in the final product;
- approve the adjuvant assay and define the permitted concentration of adjuvant in the final product; and
- approve the animals used in the assay of potency.

The national control authority shall also provide national reference preparations for the expression of activity of HBsAg contained in a given quantity of protein.

Where chimpanzees are not used in the proof of safety of the production process, the national control authority must accept responsibility for the use of the vaccine in man.

2. Release and Certification

A hepatitis B vaccine shall be released only if it fulfils Part A of the present Requirements.

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 or not the final lot of vaccine in question meets all national requirements as well as Part A of the present Requirements. The certificate shall state the date of the last satisfactory HBsAg potency test, 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 hepatitis B vaccine between countries.

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REFERENCES

1. WHO Technical Report Series, No. 602, 1977.
2. WHO Technical Report Series, No. 658, 1981, Annex 4.
3. WHO Technical Report Series, No. 725, 1985, Annex 3.
4. WHO Technical Report Series, No. 323, 1966.
5. *Weekly epidemiological record*, No. 18, 1986, 138-140.
6. WHO Technical Report Series, No. 626, 1978.
7. WHO Technical Report Series, No. 530, 1973.