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BIOLOGICAL PRODUCTS

DALTEPARIN SODIUM

Dalteparinum natricum

Dalteparin sodium is a heparin sodium salt of low molecular mass obtained by depolymerization with nitrous acid of heparin from porcine intestinal mucosa. Most components have a structure of 2-O-sulfo-α-L-idopyranose uronic acid in the non-reducing end and a structure of 6-O-sulfo-2,5-anhydro-D-mannitol in the reducing end of their chain.

Dalteparin sodium must be in compliance with the monograph *Low molecular mass heparin* with the modifications and additional requirements described below.

The mean relative molecular mass varies from 5600 Da to 6400 Da, with a characteristic value of approximately 6000 Da. The degree of sulfation is of 2.0 to 2.5 sulfates per disaccharide unit. Dalteparin sodium potency must be minimum 110 IU and maximum 210 IU of the anti-factor Xa activity per milligram, in relation to the dried substance. The anti-factor IIa activity must be minimum 35 IU/mg and maximum 100 IU/mg, in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must be between 1.9 and 3.2.

Dalteparin sodium must be produced through validated procedures of manufacturing and purification under conditions intended for minimizing the presence of N-NO groups. The manufacturing process must have been validated to reduce any contamination by N-NO groups to limits permitted using a validated quantification method.

IDENTIFICATION

A. Proceed as described on test **A.** of *Identification* from the monograph *Low molecular mass heparin*, using the dalteparin sodium RS. Calculate the potency in relation to the anti-factor Xa activity per milligram and to the anti-factor IIa activity. Calculate the ratio between the anti-factor Xa activity and the anti-factor IIa activity.

B. Proceed as described in test **B.** of *Identification* from the monograph *Low molecular mass heparin*. The spectrum obtained must be similar to the one from dalteparin sodium RS.

C. Proceed as described on test **C.** of *Identification* from the monograph *Low molecular mass heparin*. The following requirements are applied: the mean relative molecular mass varies between 5600 Da and 6400 Da; the percentage in mass of chains lower than 3000 Da is not less than 13,0% (m/m); the percentage in mass of chains with molecular masses higher than 8000 Da varies between 15.0% (m/m) and 25.0% (m/m).

CHARACTERISTICS

Aspect of the preparation. Dissolve 1 g of the sample in 10 mL of purified water. The preparation is clear **(5.2.25)**.

PURITY TESTS

Nitrite. Maximum 5 ppm. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use chromatograph with adequate electrochemical device with the following characteristics and settings: an adequate working electrode, a detector of potential + 1.00 V versus a reference electrode Ag/AgCl, and a detector with sensitivity of 0.1 uA and a column with 125 mm of length and 4.3 mm of internal diameter, packed with strong (anion) ion exchange (10 µm), stored at 40°C, *Mobile phase* flow of 1 mL/minute.

Mobile phase: solution composed of 13.61 g of sodium acetate CRS dissolved in purified water. Adjust pH to 4.3 with phosphoric acid RS and dilute to 1000 mL using purified water.

Note: *wash all volumetric flasks at least three times with purified water before preparing the solutions and before preparing the Reference solutions (c), (d) and (e); wash all pipettes with the Reference solution (b).*

Sample solution: dissolve 80 mg of the sample in purified water and complete the volume to 10 mL with the same solvent. Allow to stand for at least 30 minutes.

Reference solution (a): dissolve 60 mg of sodium nitrite CRS in purified water and dilute to 1000 mL with the same solvent.

Reference solution (b): use a pipette washed in advance with *Reference solution (a)*. Dilute 1 mL of the *Reference solution (a)* to 50 mL with purified water.

Reference solution (c): dilute 1 mL of the *Reference solution (b)* to 100 mL with purified water (corresponding to 1 ppm of nitrite in the sample).

Reference solution (d): dilute 3 mL of the *Reference solution (b)* to 100 mL with purified water (corresponding to 3 ppm of nitrite in the sample).

Reference solution (e): dilute 5 mL of the *Reference solution (b)* to 100 mL with purified water (corresponding to 5 ppm of nitrite in the sample).

Procedure: inject 100 µL of the *Reference solution (d)*. When the chromatograms are recorded in the conditions prescribed, the retention time for nitrite is 3.3 to 4.0 minutes. The test is only valid if: the number of theoretical plates calculated for the nitrite peak is not less than 7000 per meter of column (dalteparin sodium will block the bonding sites from the stationary phase, which will result in lower retention times and lower separation efficiency for the analyte; the initial performance of the column may be partially restored using 58 g/L of sodium chloride solution CRS, at a flow of 1 mL/minute for one hour; after the regeneration, the column is washed with 200 mL to 400 mL of purified water); the symmetry factor for the nitrite peak is below 3; the relative standard deviation of the peak area for the nitrite obtained from six injections is below 3%.

Inject 100 µL of each of the *Reference solutions (c)* and *(e)*. The test is only valid if: the correlation factor to a linear solution between the concentration and the response to the *Reference solutions (c)*, *(d)* and *(e)* is not less than 0.995; the signal-noise ratio for the *Reference solution (c)* is not less than 5 (if the noise level is excessively high, the electrode recalibration is recommended); a blank injection of purified water does not originate spurious peaks.

Inject 100 µL of the *Sample solution*. Calculate the content of nitrite from the peak areas on the chromatogram obtained with the *Reference solutions (c)*, *(d)* and *(e)*.

Boron. Proceed as described in *Inductively coupled plasma optical emission spectrometry* **(5.2.13.2.2)**. Use a wavelength specific for boron. The emission line to be used is 249.733 nm. Use an appropriate device, whose settings can be optimized according to the manufacturer's instructions. Not more than 1 ppm. Prepare the solutions described below.

Sample solution: dissolve 0.25 g of the substance to be analyzed in about 2 mL of water, add 100 µL of nitric acid CRS, and dilute to 10 mL using the same solvent.

Reference solution (a): prepare a solution 1% (v/v) of nitric acid in water.

Reference solution (b): prepare a solution of 11.4 µg/mL of boric acid in *Reference solution (a)*.

Reference solution (c): dissolve 0.25 g of standard dalteparin sodium without detectable boron in about 2 mL of water, add 100 µL of nitric acid and dilute to 10 mL with the same solvent.

Reference solution (d): dissolve 0.25 g of standard dalteparin sodium without detectable boron in about 2 mL of a solution of 1% (v/v) of nitric acid in water, add 10 µL of a solution of 5.7 mg/mL of boric acid and dilute to 10 mL with the same solvent. This solution contains 1 mg/mL of boron.

Calculate the content of boron on the substance to be examined, using the correction factor obtained according to the formula:

$$f = \frac{(\text{Standard}_{1} - \text{Standard}_{0}) \times 2}{(\text{Standard}_{cal} - \text{Blank})}$$

where

Standard_1 = *Reference solution (d)*;
Standard_0 = *Reference solution (c)*;
Standard_{cal} = *Reference solution (b)*;
Blank = *Reference solution (a)*.

Loss on drying (5.2.9.1). Weigh approximately 1 g of the sample. Dry the sample at 60°C on phosphorus pentoxide at a maximum pressure of 670 Pa for three hours. Not more than 5%.

ENOXAPARIN SODIUM

Enoxaparinum natricum

Enoxaparin sodium is the sodium salt of a low molecular mass heparin obtained through alkaline depolymerization of benzyl ester of heparin from the porcine intestinal mucosa. Enoxaparin consists of a complex compound of oligosaccharides that have not been completely characterized yet. Based on the present knowledge, most components have a 4-enopyranose uronate structure on the non-reducing end of their chain. 15% to 25% of the components have a 1,6-anhydrous structure in the reducing end of their chain.

Enoxaparin sodium must be in compliance with the monograph *Low molecular mass heparin* with the modifications and additional requirements described below.

The mean relative molecular mass varies from 3800 Da to 5000 Da, with a characteristic value of approximately 4500 Da. The degree of sulfation is of approximately two sulfates per disaccharide unit. The potency must be not less than 90 IU and not more than 125 IU of anti-factor Xa activity per milligram, calculated in relation to the dried substance. The anti-factor IIa activity must be not less than 20.0 IU and not more than 35.0 IU per milligram, calculated in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must be between 3.3 and 5.3.

Enoxaparin is produced by alkaline depolymerization of benzyl ether from heparin of the porcine intestinal mucosa, in conditions that result in a product in compliance with the structural requirements previously described.

IDENTIFICATION

A. Proceed as described on test **A.** of *Identification* from the monograph *Low molecular mass heparin*, using the standard enoxaparin sodium.

B. Proceed as described in test **B.** of *Identification* from the monograph *Low molecular mass heparin*. The spectrum obtained must be similar to the one from standard enoxaparin.

C. Proceed as described on test **C.** of *Identification* from the monograph *Low molecular mass heparin*. The following requirements apply: the mean relative molecular mass has mass variability between 3800 Da and 5000 Da; the percentage of chains with molecular mass inferior to 2000 Da must vary between 12.0% and 20.0%; the percentage of chains with molecular mass between 2000 Da and 8000 Da must be between 68.0% and 82.0%.

CHARACTERISTICS

Aspect of the preparation. The preparation is clear **(5.2.25)**.

PURITY TESTS

pH (5.2.19). 6.2 to 7.7. Dissolve 1,0 g of the sample in 10 mL of water free from carbon dioxide.

Specific absorbance. 14.0 to 20.0 in relation to the dried substance, determined at 231 nm. Dissolve 50.0 mg of the sample in 100 mL of hydrochloric acid 0.01 M.

Content of benzyl alcohol. Not more than 0.1% (m/m). Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use chromatograph with ultraviolet detector at 256 nm; pre-column with 20 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octylsilane group (5 µm); column with 150 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octylsilane group (5 µm), stored at temperature of 40°C, *Mobile phase* flow of 1 mL/minute.

Mobile phase: mix of methyl alcohol, acetonitrile and water (5:15:80).

Sample solution: dissolve approximately 0.500 g of the sample in 5.0 mL of sodium hydroxide 1 *M*. Allow to stand at room temperature for one hour. Add 1.0 mL of glacial acetic acid and dilute to 10.0 mL using water.

Reference solution: prepare solution with 0.25 g/L of benzyl alcohol in water. Separate 0.50 mL of this solution and dilute to 10.0 mL using water.

Procedure: inject equal volumes of *Reference solution* and *Sample solution* (20 µL), record the chromatograms and measure the peaks response. Calculate the percentage content (m/m) of benzyl alcohol according to the formula:

$$\frac{S_a}{S_r} \times \frac{C_r}{C_a} \times 100$$

where

S_a = area under the benzyl alcohol peak from the *Sample solution;*
S_r = area under the benzyl alcohol peak from the *Reference solution;*
C_r = concentration of benzyl alcohol in the *Reference solution* (mg/mL);
C_a = concentration of benzyl alcohol in the *Sample solution* (mg/mL);

Sodium. 11.3% to 13.5% in relation to the dried substance.

HEPARIN CALCIUM
Heparinum calcicum

Heparin calcium is a preparation with the calcium salt from a mixture of sulfated glycosaminoglycans, with variable weight, present in tissues from mammals. It is usually obtained from bovine lung or from porcine intestinal mucosa. It is comprised of polymers with units of *D*glucosamine (*N*-sulfated or *N*-acetylated) and uronic acid (*L*-iduronic acid or *D*-glucuronic acid) that alternate bonded by glycosidic bonds. It has the property of extending the blood coagulation time, especially by the formation of complex of some components from the mixture with specific proteins from plasma, boosting the thrombin inactivation (factor IIa). Other proteases involved in the coagulation process, such as the activated factor X (factor Xa), are also inhibited. The ratio between the anti-factor Xa activity and the anti-factor IIa potency must be between 0.9 and 1.1. The heparin calcium potency must not be less than 180 IU/mg, in relation to the dried substance. The animals from which heparin is derived must meet the sanitary requirements for the species in question and the production process must ensure the removal or inactivation of infection agents.

IDENTIFICATION

A. It meets the requirements from *Assay*, according to the method of *Determination of potency* or the method of *Anti-factor IIa potency.*

B. Use the nuclear magnetic resonance spectroscopy technique. Prepare solutions as described below:

Sample solution: not less than 20 mg/mL of the sample in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic.

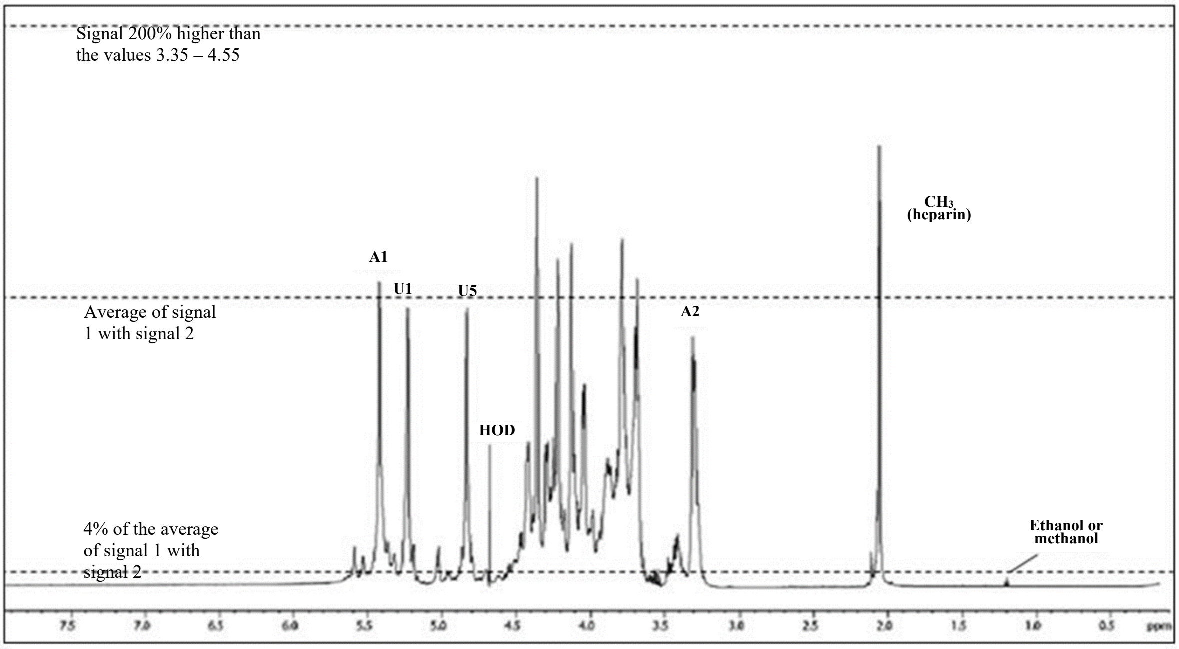
Standard solution: not less than 20 mg/mL of the heparin calcium CRS in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic.

Procedure: in the analysis of samples, it is necessary to use a nuclear magnetic resonance spectrometer with not less than 500 MHz operating the pulse (Fourier Transform) for acquisition of ^{1}H in free decay using 16 scans in 90° pulse. The test must be carried out under constant temperature of 25°C. The spectral window must be not less than 10 to -2 ppm. For all samples, methyl from the trimethylsilyl propionic compound must be referenced in 0.00 ppm. The chemical shifts of four typical regions of porcine heparin are: H1 of *N*acetylated glucosamine/*N*-sulfated glucosamine (signal 1) in 5.40 ppm, H1 of 2-sulfated iduronic acid (signal 2) in 5.21 ppm, H2 of *N*-sulfated glucosamine in 3.28 ppm, and methyl from *N*-acetylated glucosamine in 2.05 ppm. The ppm values observed for each signal must not vary ± 0.03 ppm.

The acceptance criteria are based on the average value of the height from signals 1 and 2. Any signal identified on the following ranges: 0.10 - 2.00, 2.10 - 3.20 and 5.70 - 8.00 ppm must not exceed 4% of the average value of the height from the two aforementioned signals. Likewise, signals higher than 200% of this value between 3.35 - 4.55 ppm must not be found.

Impurities: oversulfated chondroitin sulfate. The chemical shifts of the *N*-acetyl region from oversulfated chondroitin sulfate must be observed between 2.13 and 2.19 ppm.

Dermatan sulfate: The chemical shifts of the *N*-acetyl region from oversulfated chondroitin sulfate must be observed between 2.07 and 2.13 ppm.



C. Use the ion exchange liquid chromatography technique. Ion exchange chromatography is a test for determining the purity of heparin preparations, especially for detecting and separating dermatan sulfate, chondroitin sulfate, and oversulfated chondroitin sulfate. Use chromatograph with ultraviolet detector at 202 nm; pre-column with 50 mm of length and 2 mm of internal diameter, packed with anion-exchange resin (13 mm); column with 250 mm of length and 2 mm of internal diameter, packed with anion-exchange resin (9 mm), stored at temperature of 40°C, *Mobile phase* flow of 0,22 mL/minute.

Reference standards: Test solution (a) and *Reference solution (a)*, relative retention of reference heparin (retention time = approximately 26 min); dermatan and chondroitin sulfate = approximately 0.9; oversulfated chondroitin sulfate = 1.3 in relation to heparin.

Note: *reference solutions must be stabilized for 24 hours at room temperature.*

Suitability system: Reference solution; peak and valley ratio: minimum 1.3, where Hp = height above the peak baseline due to dermatan and chondroitin sulfate; Hv = height above the baseline from the lowest point of the curve that separates this top from the peak due to heparin.

The main peak on the chromatogram obtained with the *Test solution (a)* must be similar in form and retention time of the main peak in the chromatogram obtained with the *Reference solution (a).*

Prepare the solutions for the test as described below.

Solutions for test (a): dissolve approximately 50 mg of the substance to be examined accurately weighed in 5 mL of water for chromatography (deionized water with resistivity not less than 0.18 Mohms). Mix with a vortex until full dissolution.

Solutions for test (b): dissolve approximately 0.1 g of the substance to be examined accurately weighed in 1 mL of water for chromatography. Mix with a vortex until full dissolution.

Mix 0.5 mL of the solution and 0.25 mL of hydrochloric acid *M*, then add 50 µL of the sodium nitrite solution at 250 mg/mL. Mix gently and allow to stand at room temperature for 40 min before adding 0.2 mL of sodium hydroxide *M* to stop the reaction.

Reference solution (a): dissolve 250 mg of heparin CRS in water for chromatography and dilute to 2 mL with the same solvent. Mix using a vortex until full dissolution.

Reference solution (b): add 1.2 mL of *Reference solution (a)* and 0.3 mL of dermatan sulfate and oversulfated chondroitin sulfate. Mix with a vortex to homogenize.

Reference solution (c): 0.1 mL of *Reference solution (b)* and 0.9 mL of water for chromatography are added. Mix with a vortex to homogenize.

Reference solution (d): add 0.4 mL of *Reference solution (a)* to 0.1 mL of water for chromatography and mix with a vortex. Add 0.25 mL of hydrochloric acid *M*, then add 50 µL of the sodium nitrite solution at 250 mg/mL. Mix gently and allow to stand at room temperature for 40 minutes before adding 0.2 mL of sodium hydroxide *M* to stop the reaction.

Reference solution (e): to 0.5 mL of *Reference solution (b)*, add 250 µL of hydrochloric acid *M*, then add 50 µL of sodium nitrite solution at 250 mg/mL. Mix gently and allow to stand at room temperature for 40 minutes before adding 0.2 mL of sodium hydroxide *M* to stop the reaction.

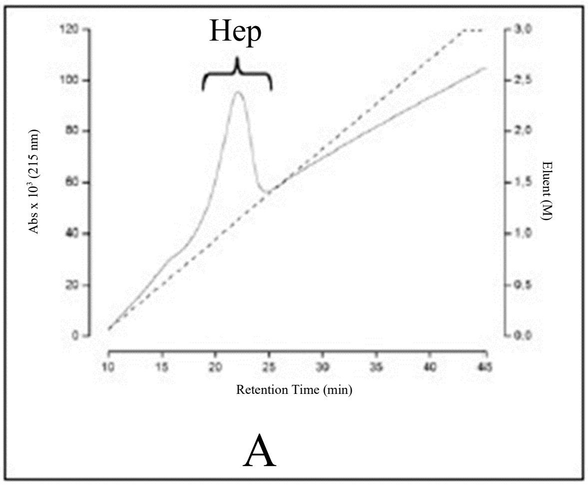
Mobile phase A: dissolve 0.4 g of sodium phosphate monobasic in 1000 mL of water for chromatography and adjust pH to 3.0 with diluted phosphoric acid solution;

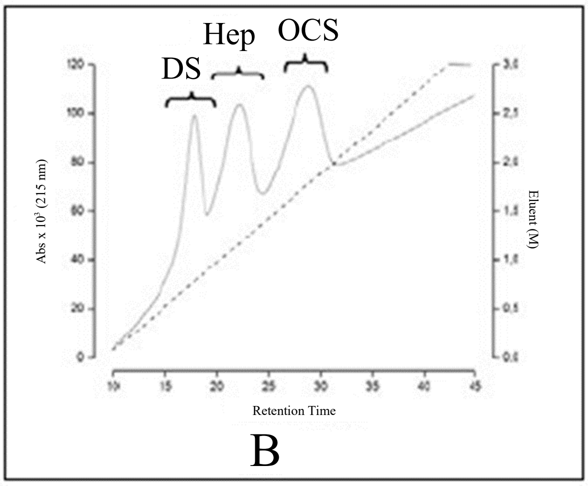
Mobile phase B: dissolve 0.4 g of sodium phosphate monobasic in 1000 mL of water for chromatography, add 140 g of sodium perchlorate and adjust to pH 3.0 with diluted phosphoric acid, filter and degas.

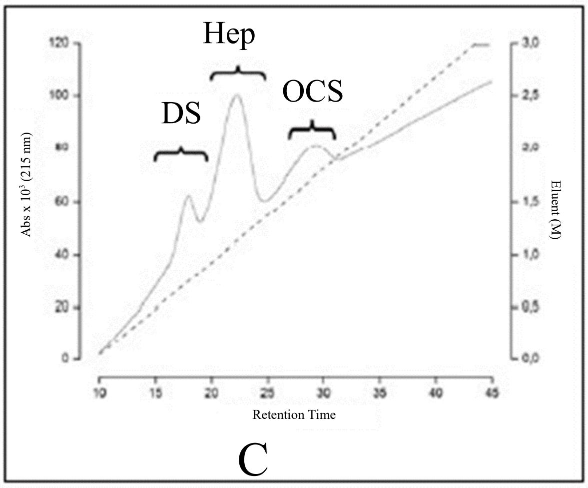
Mobile phase gradient: adopt the gradient system described on the following table:

Time (minutes)	***Mobile phase A% (v/v)***	***Mobile phase B% (v/v)***	***Elution***
0 - 10	75	25	isocratic
10 - 35	75 → 0	25 → 100	linear gradient
35 - 40	0	100	isocratic

Procedure: inject 20 µL of *Test solution (b)* and *Reference solutions (d)* and *(e)*. The relative retention in relation to heparin (retention time = approximately 26 minutes): dermatan sulfate and chondroitin sulfate = approximately 0.9; oversulfated chondroitin sulfate = 1.3. Equilibrium: at least 15 minutes. The resolution is of not less than 3.0 between the peaks related to dermatan sulfate plus chondroitin sulfate and oversulfated chondroitin sulfate in the chromatogram obtained as reference. The sum of the dermatan sulfate and the chondroitin sulfate areas is not higher than the area under the corresponding peak in the chromatogram obtained with the *Reference solution (e)* 2.0%. There must not be other peaks beyond the peak related to dermatan sulfate plus chondroitin sulfate and heparin, that is, there must not be impurities. *System suitability:* the chromatogram obtained with the *Reference solution (d)* does not present peaks in the heparin retention time. Example:







A – Chromatogram of the heparin solution CRS (Hep).

B – Chromatogram of the standard solution of the mixtures (DS - dermatan sulfate – 12%; Hep - heparin – 44% and OCS – oversulfated chondroitin sulfite – 54%).

C – Chromatogram of a sample rejected due to the presence of oversulfated chondroitin sulfate (OCS).

D. Responds to calcium ion reactions **(5.3.1.1)**.

CHARACTERISTICS

Physical characteristics. White or nearly white powder, moderately hygroscopic.

Solubility. Soluble in water.

PURITY TESTS

pH (5.2.19). 5.0 to 8.0. Determine in aqueous solution at 1% (w/v).

Proteins. Add five drops of trichloroacetic acid at 20% (w/v) to 1 mL of sample aqueous solution at 1% (w/v). There must be no formation of precipitate or turbidity.

Heavy metals (5.3.2.3). Use *Method I*. Not more than 0.003%.

Nitrogen (5.3.3.2). Use *Method I, macrodetermination*. Not less than 1.3% and not more than 2.5% of nitrogen, calculated in relation to the dried substance.

Calcium (5.3.2.7). Not less than 9.5% and not more than 11.5% of calcium, calculated in relation to the dried substance, determined at 0.2 g by *Complexometric titration* **(5.3.3.4)**.

Loss on drying (5.2.9.1). Determine in oven, under vacuum, at 60°C, for 3 hours. Not more than 5%.

Sulfated ashes (5.2.10). Not less than 28% and not more than 41%.

Nucleotidic impurities: Dissolve 40 mg in 10 mL of water. The absorbance measured at 260 nm and the result must not be higher than 0.2.

BIOLOGICAL SAFETY TESTS

Bacterial endotoxins (5.5.2.2). Not more than 0.03 EU/IU of heparin.

ASSAY

Potency determination.

Heparin anticoagulant activity is determined *in vitro*, comparing its ability in specific conditions to delay coagulation, from reference ovine plasma or reference human plasma, citrated and recalcified with the same capacity as a reference preparation of heparin, calibrated in international units.

An International Unit is the activity contained in an amount indicated in the international standard, consisting of an amount of lyophilized heparin calcium obtained from porcine intestinal mucosa. The equivalence in international units from the International Reference Standard is indicated by the World Health Organization.

The reference standard heparin calcium is calibrated in international units, against an International Standard, through the following test.

Carry out the test using a mechanical registration of the change of fluidity upon agitation, being careful to disturb the solution as little as possible during the initial coagulation phase (coagulometer).

Procedure: The volumes described in the text are presented as examples and can be adapted to the device used, provided that the ratio between different volumes is respected. Dilute the reference standard heparin calcium in a sodium chloride solution at 0.9% (w/v) so that it contains a precisely known number of International Units per milliliter and prepare a sample solution similar to the preparation to be examined, which shall have the same activity. Using a sodium chloride solution at 0.9% (w/v), prepare a series of dilutions in geometrical progression in such a way that the coagulation time obtained with the lowest concentration is not inferior to 1.5 times the blank recalcification time, and that, with the highest concentration obtained, a satisfactory dose-response logarithm curve is provided. Place 12 tubes in water bath with chilled water, labeling them in duplicates: A1, A2 and A3 for dilutions of the preparations to be examined, and P1, P2 and P3 for dilutions of reference preparation. For each tube, add 1 mL of thawed plasma substrate and 1 mL of an adequate dilution of the preparation to be examined or the reference preparation. After each addition, mix, but do not allow the formation of bubbles. Treat the tubes in the order P1, P2, P3, A1, A2, A3; transferring each tube to water bath at 37°C allows equilibrating at 37°C for approximately 15 minutes, and add to each tube 1 mL of a dilution of cephalin to each an adequate activator, such as kaolin, has been added, so that an adequate recalcification time obtained on blank is not longer than 60 seconds. When kaolin is used, it must be prepared immediately before use, in a mixture of equal volumes of cephalin and kaolin suspension at 0.4% (w/v) protected from light into a sodium chloride solution at 0.9% (w/v). After exactly 2 minutes, add 1 mL of a calcium chloride solution at 3.7 g/mL and record the coagulation time and the interval in seconds between this latest addition and the beginning of coagulation determined by the technique selected. Determine the blank recalcification time at the beginning and at the end of the process in a similar manner, using 1 mL of a sodium chloride solution at 0.9% (w/v) rather than one of the heparin dilutions. The two values obtained in blank must not differ significantly. Turn the coagulation time into logarithms, using the mean value for the tubes in duplicates. Repeat the procedure with fresh dilutions and carry out the incubation in the order A1, A2, A3, P1, P2, P3. Calculate the results through the habitual statistic methods. Carry out not less than three independent tests. For each test, prepare new reference solutions and the preparation to be examined and use another, recently thawed, portion of plasma. Carry out the heparin test. The estimated potency must not be less than 90% and not more than 111% of the stated potency. The confidence limits of the estimated potency must not be less than 80% and not more than 125% of the stated potency (P = 0.95).

Anti-factor IIa potency.

Buffer pH 8.4: dissolve 6.1 g of tromethamine, 10.2 g of sodium chloride, 2.8 g of edetate disodium and, if necessary, between 0 and 10 g of polyethylene glycol 6000 and/or 2 g of bovine albumin in 800 mL of water. Adjust with hydrochloric acid at pH of 8.4 and dilute with water to 1000 mL.

Note: *2 g of human albumin can be replaced by 2 g of bovine albumin.*

Antithrombin Solution: reconstitute a vial of antithrombin in water until obtaining an antithrombin solution of 5 IU/ mL. Dilute with *Buffer pH 8.4* to obtain a solution at an antithrombin concentration of 0.125 IU/mL.

Human thrombin solution: reconstitute the human thrombin (Factor IIa) in water to reach 20 IU/mL of thrombin, and dilute with *Buffer pH 8.4* to obtain a solution with 5 IU/mL thrombin concentration.

Note: *the thrombin must have a specific activity not lower than 750 IU/mg.*

Chromogenic substrate solution: prepare a solution from a thrombin substrate that is adequate for the amidolytic chromogenic assay in water to obtain a concentration of 1.25 *mM*.

Stop solution: prepare an acetic acid solution at 20% (v/v) in water.

Standard solutions: reconstitute the total content of an ampoule of heparin calcium CRS in water and dilute with *Buffer pH 8.4* to obtain at least four dilutions between the concentration range of 0.005 and 0.03 unit/mL of heparin.

Sample solutions: proceed as indicated in the solutions to obtain concentrations of heparin calcium similar to the ones obtained for the *Standard solutions.*

Each dilution of the *Standard solution* or of the *Sample solution* must be carried out in duplicates. Numeric labels must be placed depending on the number of repetitions to be tested. For example: if there are five blanks to be used - B1, B2, B3, B4 and B5; A1, A2, A3 and A4 for each duplicate of the samples in tests; and P1, P2, P3 and P4 for each duplicate of standard solutions in test. Distribute the blank spaces over the series in such a way that they accurately represent the reagents behavior during the experiments.

Note: *The tubes must be treated in the order B1, P1, P2, P3, P4, B2, A1, A2, A3, A4, B3, A1, A2, A3, A4, B4, P1, P2, P3, P4, B5.*

Notice that after each reagent addition, the incubated solution must be mixed without allowing the formation of bubbles. Add two times the volume (100 - 200 µL) of Antithrombin solution to each tube containing one volume (50-100 µL) of *Buffer pH 8.4* or an appropriate dilution of the sample solution or the standard solution. Shake, but do not allow the formation of bubbles, and incubate at 37°C for at least 1 minute. Add to each tube 25-50 µL of *Human thrombin solution* and incubate for at least 1 minute. Add 50 - 100 µL of *Chromogenic substrate solution*. Notice that all reagents, standard solutions and sample solutions must be pre-heated at 37°C shortly before their use.

Two different types of measurements can be recorded:

Endpoint measurement: Stop the reaction after at least 1 minute with 5-10 µL of *Stop solution*. Measure the absorbance of each solution at 405 nm through an adequate spectrometer. A relative standard deviation over the blank readings must be below 10%.

Kinetic Measurement: Following the change in absorbance for each solution over 1 minute at 405 nm through a spectrophotometer. Calculate the variation of absorbance per minute (ΔOD/minute). The blanks for kinetic measurement are also expressed as ΔOD/minute and must provide higher values in which they are carried out in the absence of heparin. The relative standard deviation over the blank readings must be below 10%.

The statistical models for analysis of ratio between straight line slopes or parallel lines may be used depending on the best model that describes the correlation between concentration and response.

Parallel line assay: For each series, calculate the regression of absorbance or change of absorbance per minute against the concentrations in logarithm of the sample solutions and the standard solutions and calculate the potency of reference heparin calcium in Units/mL using statistical methods for parallel line assays. Express the potency of heparin calcium as IU/mg of dried basis.

Ratio between Straight Line Slopes: For each series, calculate the regression of absorbance in logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of reference heparin calcium in Units/mL using statistical methods for assays on ratio between slopes. Express the potency of heparin calcium in IU/mg of dried basis.

Acceptance criteria: The potency of heparin calcium, calculated on dried basis, is not less than 180 units of heparin per mg.

Anti-factor Xa activity.

Buffer pH 8.4: dissolve 10.24 g of sodium chloride, 6.6 g of tromethamine and 2.8 g of edetate disodium in water. If necessary, adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Antithrombin solution: reconstitute the content of an ampoule of antithrombin with water (or as recommended by the manufacturer). Dilute with *Buffer pH 8.4* to obtain a solution at 1 IU of antithrombin per mL.

Bovine factor Xa solution: reconstitute the content of an ampoule of bovine factor Xa with water (or as recommended by the manufacturer). Dilute the solution obtained in *Buffer pH 8.4* to obtain a solution with absorbance values between 0.65 and 1.25, measured at 405 nm when tested as described below, but using 30 µL of *Buffer pH 8.4* rather than 30 µL of *Standard solution* or *Sample solution*.

The factor Xa solution has three nano-catalytic units per mL, but may vary depending on the manufacturer of factor Xa or on the substrate used.

Chromogenic substrate solution: prepare a chromogenic solution that is adequate for the amidolytic assay specific for factor Xa in water to obtain a concentration of about 1 *mM*.

Stop solution: prepare an acetic acid solution at 20% (v/v) in water.

Sample solution: dissolve the precise amount of the heparin calcium sample in *Buffer pH 8.4* and dilute with it to obtain solutions with activities that are approximately equal to the *Standard Solution*.

Standard solution: use heparin official standard. Another preparation whose potency has been calibrated in relation to the official standard can be used. Reconstitute the content of the official standard heparin ampoule in water and mix gently until its full dissolution. Prepare dilutions in *Buffer pH 8.4* to obtain five to seven solutions with known activities of 0.375; 0.3125; 0.25; 0.188; 0.125; 0.0625 and 0.0313 in heparin units per mL.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the ratio between volumes. Carry out the test with each *Standard solution* and *Sample solution* in duplicate. For each series of plastic tubes in water bath at 37°C, transfer 120 µL of *Buffer pH 8.4*. Separately, transfer 30µL of different dilutions of standard solutions or sample solutions to the tubes. Add 150 µL to each tube, mix and incubate for two minutes. Add 300 µL of *Chromogenic substrate solution*, pre-heated at 37°C for 15 minutes.
Add 150 µL of *Stop solution* to each tube and mix. Prepare the blank to zero the spectrophotometer adding the reagents in reverse order, starting with *Stop solution* and ending with the addition of 150 µL of *Buffer pH 8.4*, and excluding the standard solutions or the sample solutions. Record the absorbance measured at 405 nm against the blank.

Plot a chart with values from absorbances of *standard solutions* and *sample solutions* against concentrations of heparin in units. Plot separate straight lines of the best adjustment using analysis on linear regression of least squares for standard solutions and sample solutions and determine the slope of each regression straight line. Calculate the potency of heparin calcium by the formula.

$$P \times \left(\frac{SA}{SP}\right)$$

where

P = potency of the reference standard of heparin calcium;
SA and SP = are the slopes of straight lines from the *Sample solutions* and *Standard solutions*, respectively.

$$\left(\frac{\textbf{Anti-factor Xa activity}}{\textbf{Anti-factor IIa potency}}\right)$$

Express the potency of the anti-factor Xa from the sample solution with a percentage of the heparin concentration determined in the Assay. Calculate the ratio of anti-factor Xa against the potency of factor IIa by the formula. The ratio between the anti-factor Xa activity with anti-factor IIa potency must be not less than 0.9 and not more than 1.1.

PACKAGING AND STORAGE

According to legislation in force.

LABELLING

According to legislation in force.

THERAPEUTIC CLASS

Anticoagulant.

HEPARIN CALCIUM INJECTABLE SOLUTION

Injectable heparin calcium is a sterile solution of heparin calcium dissolved in water for injection. It displays minimum potency of 90.0% and maximum of 110.0% of the potency indicated on the label in terms of international units of heparin per mL.

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

pH (5.2.19). 5.0 to 8.0.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 0.03 EU/IU of heparin.

ASSAY

Proceed as described in *Assay* from the monograph *Heparin sodium, injectable* solution.

PACKAGING AND STORAGE

In containers for single or multiple doses, in type I glasses.

LABELLING

Comply with current legislation.

LOW MOLECULAR MASS HEPARIN

Heparinum massae molecularis minoris

Low molecular mass heparins are preparations with a mixture of polysaccharide chains with varied molecular mass. By definition, low molecular mass heparins must present mean molecular mass of less than 8000 Da and, accordingly, it is necessary that at least 60% of the total mass presents molecular mass less than 8000 Da. They are obtained through fractionation or depolymerization of porcine heparin. Low molecular mass heparins present chemical structural differences in the reducing or non-reducing ends from the polysaccharide chain resulting from the fractionation method used. The anticoagulant activity results from the inhibition of several factors from the coagulation cascade, extending the blood coagulation time. This occurs mainly through the enhancement of the factor Xa inactivation. The anticoagulant potency is not less than 80 and not more than 125 units of anti-factor Xa activity per milligram, in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must be not less than 1.5. The animals from which heparin is derived must meet the sanitary requirements for the species in question and the production process must ensure the removal or inactivation of infection agents.

IDENTIFICATION

A. Meets the requirements described in *Assay*, according to the methods of *Anti-factor Xa activity* and *Anti-factor IIa activity.*

B. Use the one-dimensional proton nuclear magnetic resonance spectroscopy method. Prepare solutions as described below.

Standard solution: prepare solution in a concentration of not less than 20 mg/mL of low molecular mass heparin used as internal standard in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid.

Sample solution: prepare solution in a concentration of not less than 20 mg/mL of the sample in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid.

Procedure: in the analysis of samples, a nuclear magnetic resonance spectrometer is required with at least 500 MHz operating the pulse (Fourier Transform) for acquisition of $_1$H in free decay using 16 scans in 90° pulse. The test must be carried out under constant temperature of 25°C and a water suppression program. The spectral window must be not less than 10 to -2 ppm. For all samples, the methyl group from the trimethylsilyl propionic compound must be referenced in 0.00 ppm. The spectra obtained must be similar to the one from the specific reference low molecular mass heparin.

The sample spectrum must be similar to the standard spectrum. Chemical shift between 2.13 and 2.16 ppm, corresponding to the *N*-acetyl regions, must not be observed.

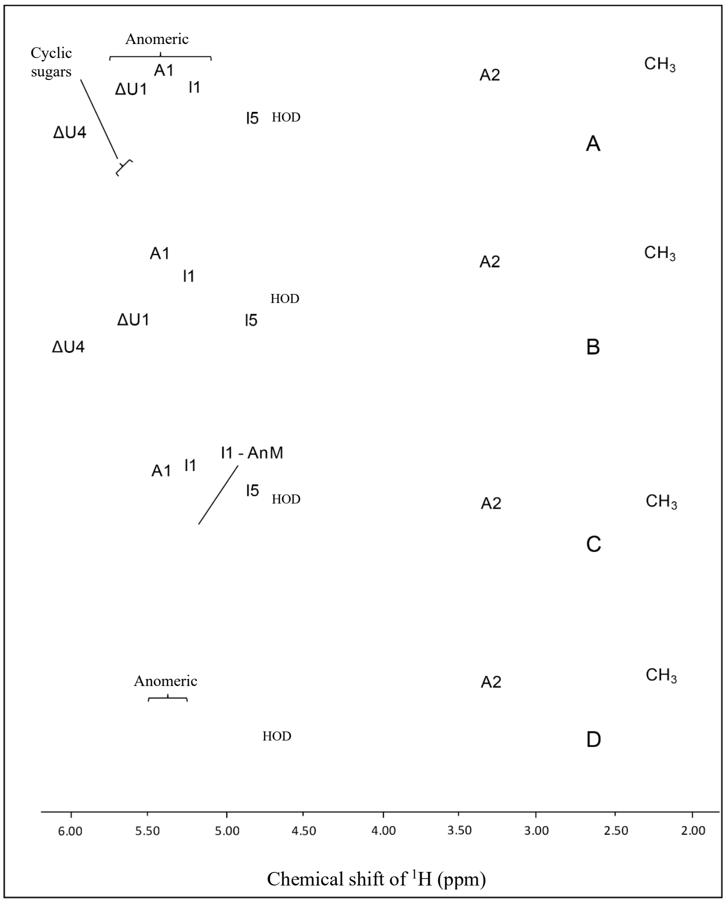


Figure 1 - 1D NMR spectra of ^{1}H of four different types of low molecular mass heparin.

Panel A – enoxaparin; Panel B – tinzaparin; Panel C – dalteparin; Panel D – nadroparin. The signals designated as A1 and A2 correspond to H1 and H2 from the α-glucosamine 6- and N-disulfated units; The signals I1 and I5 correspond to H1 and H5 from 2-sulfated α-iduronic acid; □U1 and □U4 correspond to H1 and H4 from uronic acid 4,5 unsaturated and 2- sulfated. The signals related to cyclic sugars (N-sulfated, 1,6 anhydrous β-glucosamine or β-mannosamine) are also indicated in the panels. HOD – deuterated water.

C. Use the gel filtration chromatography method to determine the distribution of molecular mass of oligosaccharides present in the low molecular mass heparin solution. Use high performance liquid chromatography equipped with refraction index detector and ultraviolet reader at 234 nm; a pre-column with 6.0 mm of diameter x 4.0 cm of height, packed with silica matrix of 7 µm of diameter and two analytical columns in series, with 7.5 mm of diameter x 30 cm of height, packed with silica matrix of 5 µm or 10 µm of diameter that fractionates proteins in the range of approximately 10 000 to 500 000 Da. Not less than 20 000 theoretical plates per meter. Mobile phase flow of 0.3 mL/minute.

Prepare solutions as described below.

Mobile phase: ammonium acetate solution 0.1 *M*, pH 6.0.

Standard solution for calibrating the molecular mass for low molecular mass heparins: dissolve the standard low molecular mass heparin sample for calibrating the molecular mass in the concentration of 10 mg/mL in the mobile phase when carrying out the assay.

Sample solution: dissolve the test sample at the concentration of 10 mg/mL in the mobile phase.

Procedure: inject 20 µL of the *Standard solution* to determine the elution profile of the calibration standard and, later, inject 20 µL of the test sample to compare with the elution profile of the *Standard solution.*

Calibration: for detection, use a refraction index detector connected in series to an ultraviolet reader at 234 nm. For both chromatograms to be correctly aligned, carefully measure the time elapsed between the detection by the two readers. The retention time used for calibration calculations must be the time from the refraction index (RI) detector.

A normalization factor must be calculated to determine the relative molecular mass from the RI/UV ratio as follows: calculate the total area under the UV_{234} curve ($\sum UV_{234}$) and under the RI curve ($\sum RI$) through numerical integration of the area of interest (excluding salt peaks and solvents at the end of the chromatogram). Calculate the ratio *r* using the following formula:

$$r = \frac{\sum RI}{\sum UV_{234}}$$

Calculate the factor *f* using the following formula:

$$f = \frac{M_{na}}{r}$$

where

M_{na} = mean relative molecular mass of the low molecular mass heparin standard for CRS calibration determined by the manufacturer.

Make sure that the responses from UV_{234} and RI are aligned and calculate the relative molecular mass (M) from each point using the following formula:

$$M = f\ \frac{RI}{UV_{234}}$$

The resulting table of retention times and relative molecular mass for each point must be used to derive a calibration for the chromatography system creating a mathematical ratio for the recorded values. It is recommended to plot a 3rd degree polynomial curve. Extrapolating the values from this calibration curve plotted for higher molecular mass values is not valid.

Inject 20 µL of the *Sample solution* and record the chromatogram for the necessary time period for complete elution of the sample and solvent peaks.

The mean relative molecular mass is defined by the formula:

$$\frac{\sum (\mathrm{RI}_i\, M_i)}{\sum \mathrm{RI}_i}$$

where

RI_i = mass of the substance that elutes in the fraction *i*;
M_i = relative molecular mass corresponding to the fraction *i*.

The values of the mean relative molecular mass must be not more than 8000, and not less than 60% of the total mass must have a relative molecular mass below 8000. Additionally, the molecular mass parameters (mean relative molecular mass and percentage of the chains comprised within specific values) must correspond to the reference preparation.

Compare the retention time from the test sample peaks with the peaks derived from the standard sample. The validation criterion is met if the test sample peaks correspond to the standard sample peaks.

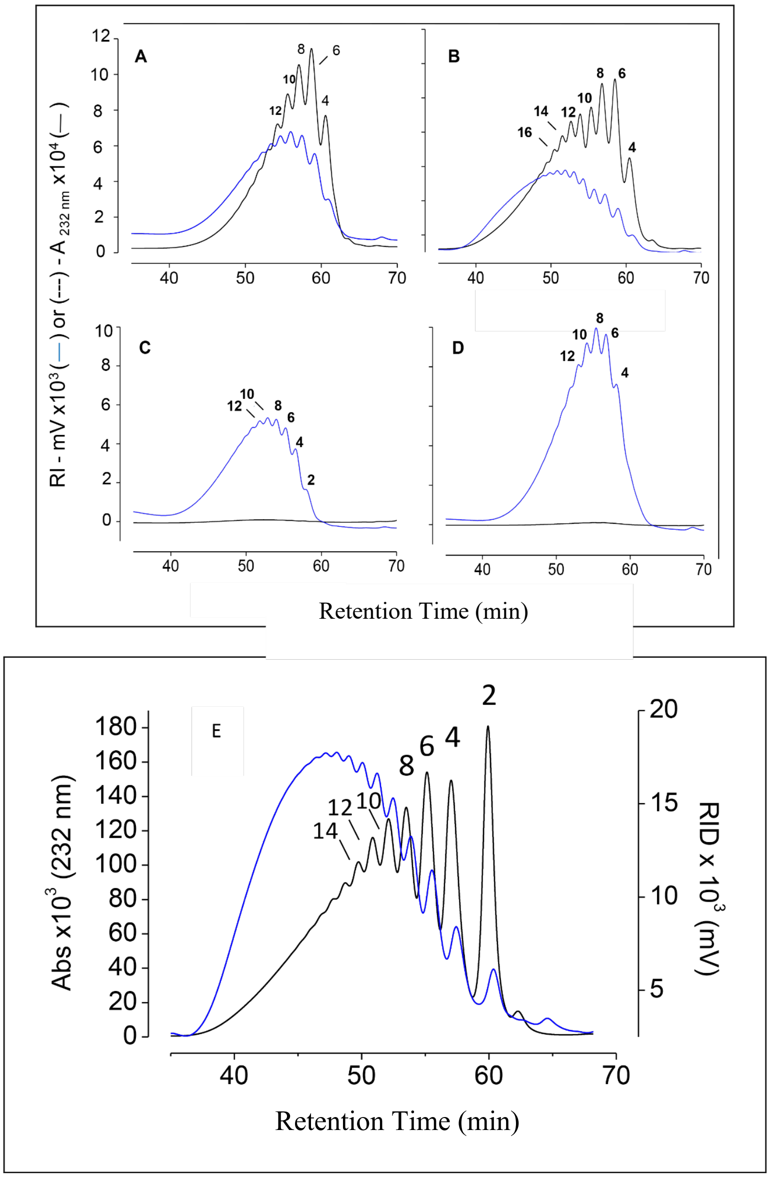


Figure 2 – Elution profile from different low molecular mass heparins by the gel filtration chromatography method.

Analysis of the distribution of molecular masses of oligosaccharides found in different preparations of LMWH. Preparations of enoxaparin (A), tinzaparin (B), dalteparin (C), nadroparin (D) and molecular mass calibration standard (E). The numbers represent the disaccharide units (2– disaccharide, 4– tetrasaccharide, 6- hexasaccharide, 8- octasaccharide, 10- decasaccharide, 12- dodecasaccharide).

CHARACTERISTICS

Physical characteristics. White or nearly white powder, hygroscopic.

Solubility. Soluble in water.

PURITY TESTS

pH (5.2.19). 5.5 to 8.0. Determine in aqueous solution at 1% (w/v).

Proteins. Add five drops of trichloroacetic acid at 20% (w/v) to 1 mL of sample aqueous solution at 1% (w/v). There must be no formation of precipitate or turbidity.

Nucleotidic impurities. Dissolve 40 mg of the material in 10 mL of water. The absorbance measured at 260 nm and 280 nm must be not greater than 0.20 and 0.15, respectively.

Nitrogen (5.3.3.2). Use *Method I, macrodetermination*. Not less than 1.5% and not more than 2.5% of nitrogen, calculated in relation to the dried substance.

Sodium. Between 10.5% and 13.5% of sodium determined as described in *Atomic absorption spectrophotometry* **(5.2.13.1)**.

Heavy metals (5.3.2.3). Use the *Identification test C*. Prepare the reference solution using 1.5 mL of lead standard solution (10 ppm of Pb). 0.5 g in compliance with the test from C. Not greater than 30 ppm.

Loss on drying (5.2.9.1). Not greater than 10%, determined from 1000 g in vacuum oven at 60°C, for three hours, in pressure not exceeding 0.67 kPa.

BIOLOGICAL SAFETY TESTS

Bacterial endotoxins (5.5.2.2). Not greater than 0.01 EU per IU of anti-Xa for low molecular mass heparin.

ASSAY

Anti-factor Xa activity

Buffer tris (hydroxymethyl) aminomethane pH 7.4: dissolve quantities of tris (hydroxymethyl) aminomethane to obtain concentration of 20 m*M*, sodium chloride to 150 m*M* and calcium chloride to 10 m*M* in distilled water containing polyethylene glycol 8000 at 0.1%. If necessary, adjust pH to 7.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of human antithrombin as recommended by the manufacturer. Homogenize. Dilute with *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 0.02 IU/mL (equivalent to 40 n*M*) of human antithrombin.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solution at 1.0 IU/mL (equivalent to 20 n*M*) of human factor Xa.

Chromogenic substract solution for factor Xa: dissolve an amount of *N*-α-benzyloxycarbonyl-Darginyl-L-glycyl-L-arginine-*p*-nitroaniline with dihydro-hydrochloride in sterile distilled water, obtaining a 2 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Stop solution: prepare a solution at 50% (v/v) of acetic acid in water.

Standard solution: use standard low molecular mass heparin solution. Another preparation, whose potency has been determined in relation to the standard, can be used. Reconstitute the content of the low molecular mass heparin ampoule as recommended by the manufacturer and mix gently until complete dissolution. From the reconstituted solution, prepare dilutions in *buffer tris (hydroxymethyl) aminomethane pH 7.4* solution, to obtain at least five solutions with concentration ranging from 0.2 to 0.005 IU/mL of anti-Xa activity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solutions with activities that are approximately equal to the ones from the *Standard solution*.

Procedure: two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining a constant ratio among volumes. Run the test in microplates and carry out the spectrometric reading at 405 nm in a microplate reader at 37°C. The test must be carried out with each *Standard solution* and *Sample solution* in duplicate. In each microplate well, add 40 µL of *Buffer tris (hydroxymethyl)aminomethane pH 7.4* or of *Sample solution*, 25 µL of the *Human antithrombin solution* and 10 µL of the *Human factor Xa solution*. After two minutes of incubation at 37°C, add 25 µL of *the Chromogenic substrate solution for factor Xa*. Record the absorbance measured at 405 nm against the blank for five minutes. The blank is the test carried out when no low molecular mass heparin is added, using 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4*.

Endpoint measurement: proceed as described in the *Kinetic measurement*. After adding the chromogenic substrate, wait four minutes and stop the reaction by adding 50 µL of *Stop solution*.

The statistical models for analyzing the ratio between straight line slopes or parallel lines may be used depending on the best model that describes the correlation between concentration and response.
Assay on parallelism: for each series, calculate the absorbance regression or absorbance change per minute against the concentrations in logarithm of the *Sample solutions* and the *Standard solutions* and calculate the potency of the sample using statistical methods for parallel line assays. Express the potency of low molecular mass heparin in IU/mg.

Relation between straight line slopes: for each series, calculate the absorbance regression in logarithm or the logarithm of changes in absorption per minute against the concentrations of the *Sample solutions* and the *Standard solutions* and calculate the potency of the sample using statistical methods for assays on relation between straight line slopes. Express the potency of low molecular mass heparin in IU/mg of dried basis.

Acceptance criteria: the potency of low molecular mass heparins must present not less than 80 and not greater than 125 of anti-factor Xa activity per mg.

Anti-factor IIa activity

Proceed as described in *Anti-factor Xa* activity, except for the *Human factor Xa solution*, which must be replaced by *Human factor IIa solution*, and the *Chromogenic substrate solution* N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroaniline dihydrochloride, which must be replaced by *Chromogenic substrate solution* H-D-phenylalanine-L-arginine-p-nitroaniline dihydrochloride. The buffer to be used in this test is *tris (hydroxymethyl) aminomethane pH 7.4,* but without adding calcium chloride. Prepare the *Human factor IIa solution* and the *Chromogenic substrate solution for factor IIa* as described below.

Human factor IIa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, without adding calcium chloride, to obtain solution at 2.0 IU/mL (equivalent to 20 n*M*) of human factor IIa.

Chromogenic substrate solution for factor IIa: dissolve an amount of H-D-phenylalanine-Larginine-*p*-nitroaniline dihydrochloride in sterile distilled water obtaining a 2 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Proceed with the statistical analyses and calculations indicated in *Anti-factor Xa activity*.

Acceptance criteria: the ratio between the anti-factor Xa/anti-factor IIa activity must be not less than 1.5.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

THERAPEUTIC CLASS

Anticoagulant.

INJECTABLE LOW MOLECULAR MASS HEPARIN SOLUTION

Injectable preparations of low molecular mass heparin are sterile solutions of low molecular mass heparin diluted in water for injection. The anticoagulant potency is not less than 90% and not greater than 110% of the anti-Xa potency declared on the label in units per milliliter. The ratio between the anti-factor Xa/anti-factor IIa activity must be observed according to the type of low molecular mass heparin solution analyzed. Enoxaparin presents a range of variation for the anti-factor Xa/anti-factor IIa activity between 3.3 and 5.3. For tinzaparin, the ratio varies between 1.5 and 2.5. Dalteparin presents a ratio between 1.9 and 3.2 and nadroparin, between 2.5 and 4.0.

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

pH (5.2.19). 5.5 to 7.5.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not greater than 0.01 EU/IU of low molecular mass heparin.

ASSAY

Anti-factor Xa activity

Buffer tris (hydroxymethyl) aminomethane pH 7.4: dissolve quantities of tris (hydroxymethyl) aminomethane to obtain concentration of 20 m*M*, sodium chloride to 150 m*M* and calcium chloride to 10 m*M* in distilled water containing polyethylene glycol 8000 at 0.1%. If necessary, adjust pH to 7.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of human antithrombin as recommended by the manufacturer. Homogenize. Dilute with *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 0.02 IU/mL (equivalent to 40 n*M*) of human antithrombin.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solution at 1.0 IU/mL (equivalent to 20 n*M*) of human factor Xa.

Chromogenic substract solution for factor Xa: dissolve an amount of *N*-α-Benzyloxycarbonyl-Darginyl-L-glycyl-Larginine-*p*-Nitroaniline dihydrochloride in sterile distilled water, obtaining a 2 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Stop solution: prepare a solution at 50% (v/v) of acetic acid in water.

Standard solution: use standard low molecular mass heparin solution. Another preparation, whose potency has been determined in relation to the standard, can be used. Reconstitute the content of the low molecular mass heparin ampoule as recommended by the manufacturer and mix gently until complete dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris(hydroxymethyl) aminomethane pH 7.4* solution, to obtain at least five solutions with concentration ranging from 0.2 to 0.005 IU/mL of anti-Xa activity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solutions with activities that are approximately equal to the ones from the *Standard solution*.

Procedure: two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining a constant ratio among volumes. Run the test in microplates and carry out the spectrometric reading at 405 nm in a microplate reader at 37°C. The test must be carried out with each *Standard solution* and *Sample solution* in duplicate. In each microplate well, add 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4* or of sample solution, 25 µL of the *Human antithrombin solution* and 10 µL of the *Human factor Xa solution*. After two minutes of incubation at 37°C, add 25 µL of *the Chromogenic substrate solution for factor Xa*. Record the absorbance measured at 405 nm against the blank for five minutes. The blank is the test carried out when no low molecular mass heparin is added, using 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4*.

Endpoint measurement: proceed as described in the *Kinetic measurement*. After adding the chromogenic substrate, wait four minutes and stop the reaction by adding 50 µL of *Stop solution*.

The statistical models for analysis of ratio between straight line slopes or parallel lines may be used depending on the best model that describes the correlation between concentration and response.

Assay on parallelism: for each series, calculate the absorbance regression or change of absorbance/minute against the concentrations in logarithm of the sample solutions and of the standard solutions and calculate the potency of reference heparin sodium in IU/mL using statistical methods for parallel line assays. Express the potency of low molecular mass heparin in IU/mL.

Ratio between straight line slopes: for each series, calculate the absorbance regression in logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and of the standard solutions and calculate the potency of standard low molecular mass heparin in IU/mL using statistical methods for assays on ratio between straight line slopes. Express the potency of low molecular mass heparin in IU/mL.

Acceptance criteria: the potency of injectable preparations of low molecular mass heparin must present not less than 90% and not greater than 110% of the activity stated on the label. The potency must be expressed in IU/mL.

Anti-factor IIa activity

Proceed as described in *Anti-factor Xa* activity, except for the *Human factor Xa solution*, which must be replaced *by Human factor IIa solution*, and the *Chromogenic substrate solution* N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroaniline dihydrochloride, which must be replaced by *Chromogenic substrate solution* H-D-phenylalanine-L-arginine-p-nitroaniline dihydrochloride. The buffer to be used in this test is *tris (hydroxymethyl) aminomethane pH 7.4,* but without adding calcium chloride. Prepare the *Factor IIa solution* and the *Chromogenic substrate solution* as described below.

Human factor IIa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* without adding calcium chloride, to obtain the solution at 2.0 IU/mL (equivalent to 20 n*M*) of human factor IIa.

Chromogenic substrate solution for factor IIa: dissolve an amount of H-D-phenylalanine-Larginine-p-nitroaniline dihydrochloride in sterile distilled water obtaining solution 2 m*M*. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Proceed with the statistical analyses and calculations indicated in *Anti-factor Xa activity*.

Acceptance criteria: the potency of injectable preparations of low molecular mass heparin must present not less than 90% and not greater than 110% of the activity stated on the label. The potency must be expressed in IU/mL. The ratio between the anti-factor Xa/anti-factor IIa activity must be observed according to the type of low molecular mass heparin solution analyzed. Enoxaparin presents a range of variation for the anti-factor Xa/anti-factor IIa activity between 3.3 and 5.3. For tinzaparin, the ratio varies between 1.5 and 2.5. Dalteparin varies between 1.9 and 3.2 and nadroparin, between 2.5 and 4.0.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

BOVINE HEPARIN SODIUM

The bovine heparin sodium is extracted from the bovine intestinal mucosa and contains heterogeneous polysaccharide chains with varied molecular mass. It is mostly comprised of units of α-D-glucosamine N,6 sulfate and 2-O-sulfate iduronic acid. The α-D-glucosamine units present a more heterogeneous standard of sulfation when compared to porcine heparin. We especially observe a higher proportion of non-sulfated α-glucosamine units on position 6. It has anticoagulant activity by the inhibition of several factors from the coagulation system, extending the blood coagulation time. This occurs especially through enhancing the inactivation of factor Xa and of thrombin by antithrombin. It has not less than 100 units of anti-factor IIa activity per mg of heparin, respectively, in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must range between 0.9 and 1.1. As acceptance criterion for each test carried out for the anti-factor IIa and Xa activity, the potency calculated based on the dry weight must range between 90% and 110% of the potency declared. The animals from which heparin is extracted must meet the sanitary requirements for the species in question and the production process must ensure the removal or inactivation of infection agents.

IDENTIFICATION

A. Meets the requirements described in *Assay*, according to the *methods I* or *II* of **Anti-factor Xa activity** and **Anti-factor IIa activity**.

B. Use the one-dimensional proton nuclear magnetic resonance spectroscopy method. Prepare solutions as described below.

Standard solution: prepare solution in a minimum concentration of 20 mg/mL of heparin (CRS) in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid. An internal reference standard of heparin from bovine intestinal mucosa can be used.
Solution for system suitability: prepare solution at 1% (w/w) of oversulfated chondroitin sulfate CRS in *Standard solution*.

Sample solution: prepare solution in a concentration of not less than 20 mg/mL of the sample in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid.

Procedure: a nuclear magnetic resonance spectrometer of at least 500 MHz, minimum acquisition time of 2 seconds, and minimum repetition time (waiting time plus acquisition time) of 4 seconds must be used in the analysis of samples. The test must be carried out under constant temperature of 35°C with a water suppression program. The spectral window must be not less than 10 to -2 ppm. For all samples, the methyl group from the trimethylsilyl propionic compound must be referenced in 0.00 ppm. The spectra obtained must be similar to the one from the *Standard solution*.

System suitability: the chemical shifts corresponding to the *N*-acetyl regions from heparin and oversulfated chondroitin sulfate in the *Solution for system suitability* must be observed between 2.02 and 2.08 ppm and between 2.13 and 2.19 ppm, respectively. The chemical shifts of signals corresponding to H1 and H2 from the α-glucosamine 6- and *N*-disulfated α-glucosamine units (A1 and A2), to H1 from 2-sulfated α-iduronic acid (I1), to H1 from the *N*-sulfated α-glucosamine units (C1) and to the methyl grouping from *N*-acetylated α-glucosamine (CH_3) of the standard solution are present at 5.40; 3.28; 5.22; 5.31; and 2.05 ppm, respectively. The ppm values observed for each signal must not vary more than ± 0.03 ppm.

Samples acceptance criterion: the chemical shifts of signals A1, C1, I1, A2 and CH_3 must be observed at 5.40; 5.31; 5.22; 3;28; and 2.05 ppm, respectively. The ppm values observed for each signal must not vary more than ± 0.03 ppm. The integers of signals A1, C1, I1 must be obtained according to guidance from the panels on **Figure 1**. The bovine heparin sodium spectra present two I1 signals, but they must be integrated together. Spectrometers with higher resolution show the separation of A1 into two signals (as exemplified on panel B of **Figure 1**), but they must also be integrated together. The integer of A1 is taken as a reference. Proceed to the calculation according to the formula:

$$\frac{C1 \times 100}{A1} = 42 — 58$$

The value obtained must necessarily be between 42 and 58. No unidentified signal in the spectrum, in the region of 0.10 – 2.00; 2.10 – 3.20; and 5.70 – 8.00 ppm, must exceed 4% of the height of signal A1 (5.40 ppm). The chemical shift between 2.13 and 2.19 ppm, corresponding to the *N*-acetyl regions of oversulfated chondroitin sulfate, must not be observed.

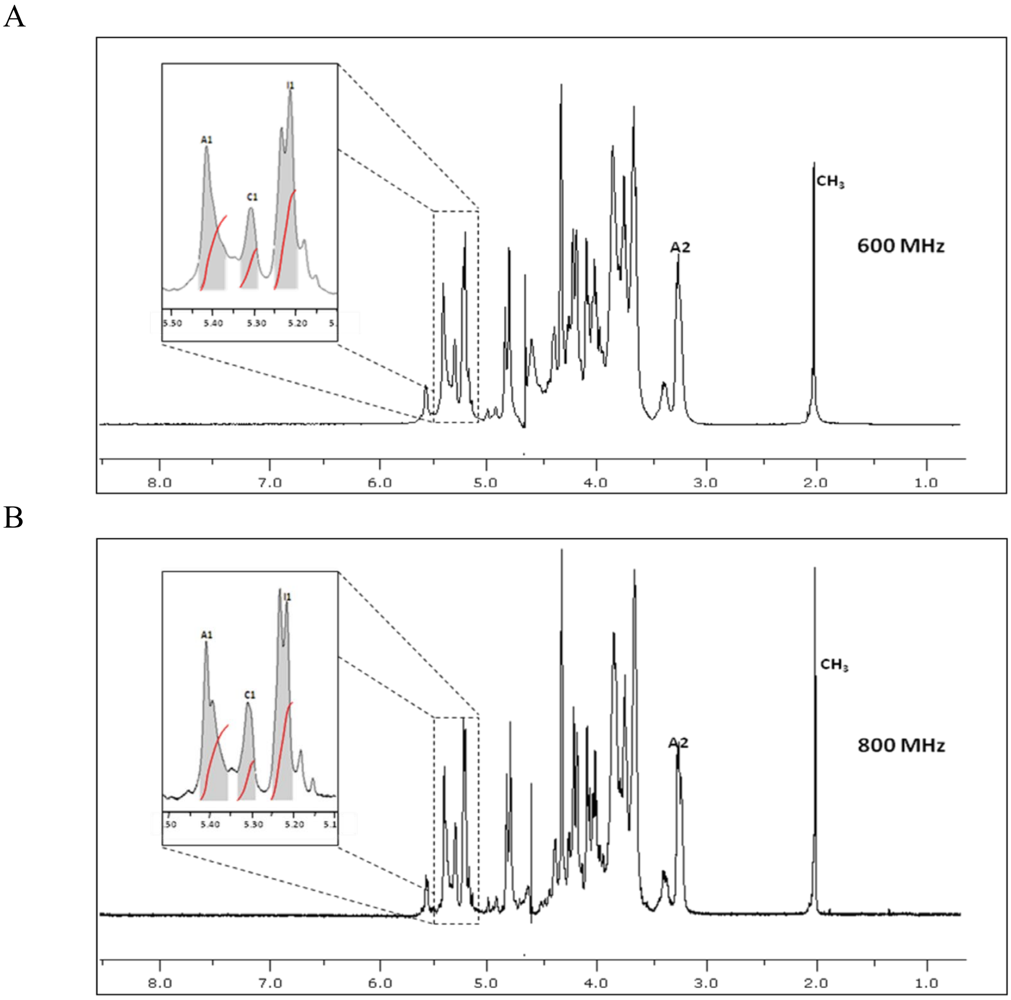


Figure 1 – **Bovine heparin sodium analyzed in spectrometer.**

Panel A – 600 MHz; Panel B – 800 MHz. The signals designated as A1 and A2 correspond to H1 and H2 from the α-glucosamine 6- and N-disulfated units at 5.40 and 3.28 ppm, respectively; signal I1 corresponds to H1 from 2-sulfated α-iduronic acid at 5.21 ppm; C1 corresponds to H1 from the *N*-sulfated α-glucosamine units at 5.31 ppm; and methyl from *N*-acetylated glucosamine at 2.05 ppm. The ppm values observed for each signal must not vary more than ± 0.03 ppm. By the inserts, the expansion of regions between 5.10 and 5.50 ppm of same spectra is observed, to guide the integration of signals A1, C1 and I1.

C. Use the ion exchange liquid chromatography method for detecting and separating possible contaminants of bovine heparin sodium, such as dermatan sulfate, chondroitin sulfate, and oversulfated chondroitin sulfate. Use a chromatograph with ultraviolet detector. The reading can be done at 202 nm or 215 nm, provided that it complies with the system suitability. Use a pre-column with 50.0 mm of length and 2.0 mm of internal diameter packed with Ionpac AG11 HC resin (9 µm); column with 250 mm of length and 2.0 mm of internal diameter, packed with Ionpac AS11 HC (9 µm), stored at 40°C; mobile phase flow of 0.5 mL/minute.

Prepare solutions as described below.

Eluent A: prepare TRIS solution at 20 m*M*. Adjust pH to 7.4 with diluted hydrochloric acid.

Eluent B: prepare TRIS solution at 20 m*M* and NaCl at 2.5 *M*. Adjust pH to 7.4 with diluted hydrochloric acid.

Mobile phase gradient: adopt the gradient system from 0 to 2.5 *M* of NaCl, as described on the following table:

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0 – 4	83.4	16.6	isocratic
4 – 22	83.4 → 0	16.6 → 100	linear gradient
22 – 40	0	100	isocratic

Reference bovine heparin sodium solution: dissolve heparin from bovine intestinal mucosa at the concentration of 4.0 mg/mL in mobile phase A at the moment of carrying out the assay **(Figure 2C)**.

System suitability solution: dissolve dermatan sulfate CRS and oversulfated chondroitin sulfate CRS at the concentration of 0.6 mg/mL and 0.4 mg/mL, respectively, in the *Bovine heparin sodium reference solution* in the concentration selected, at the moment of carrying out the assay **(Figures 2B and 2C)**.

Sample solution: dissolve the test sample at the concentration of 4.0 mg/mL in the mobile phase A.

Procedure: inject 50 µL of each one of the three solutions mentioned above to determine the elution profile. The peak obtained in the chromatogram with the *Sample solution* must be similar in form and retention time to the peak obtained with the *Reference bovine heparin solution* **(Figure 2A)**. The retention time of bovine heparin sodium is of approximately 18 minutes.

System suitability: the relative retention in relation to the bovine heparin sodium (retention time = approximately 18 minutes) is of 0.9 for dermatan sulfate and 1.2 for oversulfated chondroitin sulfate. In the *System suitability solution*, the ratio between the height of the peak of dermatan sulfate and the valley (baseline between the peaks of dermatan sulfate and bovine heparin) is of not less than 1.3. Bovine heparin, when compared to porcine heparin (see specific monograph), elutes in ion exchange liquid chromatography with a wider and less resolved peak in relation to dermatan sulfate. This demands special attention in the analysis to meet the system suitability criteria.

Acceptance criteria: the presence of oversulfated chondroitin sulfate must not be detected and the amount of dermatan sulfate must be less than 4% of total glycosaminoglycans. There cannot be other peaks apart from the peak related to heparin and to dermatan sulfate.

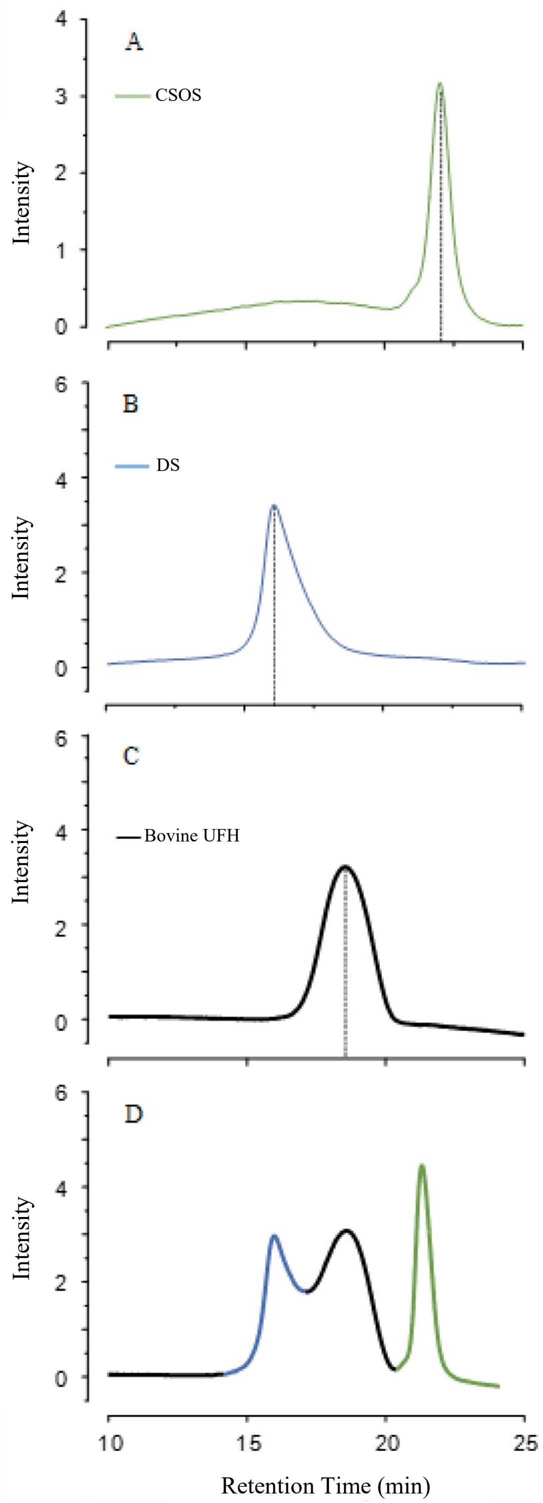


Figure 2 – **Bovine heparin sodium elution profile in the liquid chromatography in ion exchange column**

Elution profile of oversulfate chondroitin, dermatan sulfate and bovine heparin in ion exchange chromatography. 20 µg of CSOS (A), 30 µg of DS (B), 200 µg of bovine heparin (C) and a mixture with the same amounts of each polysaccharide (D) were applied. The retention times are equal for samples analyzed separately or mixed.

CHARACTERISTICS

Physical characteristics. White or nearly white powder, hygroscopic.

Solubility. Soluble in water.

PURITY TESTS

pH (5.2.19). 5.0 to 8.0. Determine in aqueous solution at 1% (w/v).

Proteins.

A. Add five drops of trichloroacetic acid at 20% (w/v) to 1 mL of sample aqueous solution at 1% (w/v). No precipitate or turbidity must be formed.

B. Proceed as described in *Ultraviolet, visible and infrared spectrophotometry* **(5.2.14)**.

Solution A: mix two volumes of sodium hydroxide 1% with two volumes of sodium carbonate 5% and dilute to five volumes with water.

Solution B: mix two volumes of copper sulfate ($CuSO_4.5H_2O$) 1,25% with two volumes of sodium tartrate ($C_4H_4Na_2O_6.2H_2O$) 2.98% and dilute to five volumes with water.

Solution C: mix one volume of *Solution B* with 50 volumes of *Solution A.*

Solution D: dilute adequately the phosphomolybdotungstic reagent in water so that the sample solution and the standard solution have pH value between 10.00 and 10.50 after adding the S*olutions C and D*.

Sample solution: prepare a sample solution with concentration of 5 mg/mL in water.

Reference solution: prepare bovine albumin R solution (approximately 96% of protein) with concentration of 100 mg/mL in water. Make dilutions with water to obtain at least five reference solutions with protein concentrations distributed evenly within the range of 5 µg/mL to 100 µg/mL.

Procedure: add 5 mL of *Solution C* to each 1 mL of the *Reference solutions, Sample solution* and blank (water), respectively. Allow to stand for 10 minutes. Add 0.5 mL of *Solution D*, mix and allow to stand at room temperature for 30 minutes. Measure the solutions absorbances at 750 nm, using the blank solution for zero adjustment.

Calibration curve: the relation between absorbance and protein concentration is not linear; however, if the concentration range used to plot the standard curve is sufficiently small, it will ultimately be close to linearity. Plot a standard curve, plotting the absorbances from the reference solutions against their concentrations, using linear regression, plot a linear straight line of best adjustment to the points plotted. Determine the protein concentration in the sample solution, through its absorbance and the standard curve. Not greater than 0.5% in relation to the dried substance.

Nucleotidic impurities. Dissolve 40 mg in 10 mL of water. The absorbance is measured at 260 nm and the result must be not greater than 0.15.

Nitrogen (5.3.3.2). Use *Method I, macrodetermination*. Not less than 1.5% and not more than 2.5% of nitrogen, calculated in relation to the dried substance.

Sodium. Between 10.5% and 13.5% of sodium determined as described in *Atomic absorption spectrophotometry* **(5.2.13.1)**.

Heavy metals (5.3.2.3). Use *Method I.* Not more than 0.003% (30 ppm).

Loss on drying (5.2.9.1). Determine in a vacuum oven at 60°C for three hours. Not more than 8%.

BIOLOGICAL SAFETY TESTS

Bacterial endotoxins (5.5.2.2). Not more than 0.03 EU/IU of bovine heparin sodium.

ASSAY

Use one of the methods described below.

A. *Method I*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 7.4: dissolve amounts of tris (hydroxymethyl) aminomethane to obtain concentration of 20 m*M*, sodium chloride to 150 m*M* in distilled water with polyethylene glycol 8000 at 0.1%. If necessary, adjust pH to 7.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of human antithrombin as recommended by the manufacturer. Homogenize. Dilute with *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 0.02 IU/mL (equivalent to 40 n*M*) of human antithrombin.

Human factor IIa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 2.0 IU/mL (equivalent to 20 n*M*) of human factor IIa.

Chromogenic substrate solution for human factor IIa: dissolve an amount of HD-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride (H-D-Phe-Pip-Arg-pNA.2HCl) in sterile distilled water obtaining a 2.0 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Stop solution: prepare a solution at 50% (v/v) of acetic acid in water.

Standard solution: for assays, use heparin (CRS), regardless of tissue origin. Reconstitute the content of the heparin CRS ampoule as recommended by the manufacturer and mix gently until complete dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* solution, to obtain at least ten solutions with concentration ranging from 0.1 to 0.0001 IU/mL of anti-IIa activity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solutions with activities that are approximately equal to the ones from the *Standard solution*.

Procedure: two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions between the standard preparations, sample being tested, and reagents. Run the test in microplates and carry out the spectrometric reading at 405 nm in a microplate reader at 37°C. The test must be carried out with each heparin CRS solution and *Sample solution* in duplicate. In each microplate well, add 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4* or of sample solution, 25 µL of the *Antithrombin solution* and 10 µL of the *Human factor IIa solution*. After two minutes of incubation at 37°C, add 25 µL of *the Chromogenic substrate solution for human factor IIa*. Record the absorbance measured at 405 nm against the blank for five minutes. The blank is the test carried out when no heparin is added, using 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4*.

Endpoint measurement: proceed as described in the *Kinetic measurement*. After adding the *Chromogenic substrate solution for human factor IIa*, wait four minutes and stop the reaction by adding 50 µL of *Stop solution*.

The statistical models for analyzing the ratio between straight line slopes or parallel lines may be used depending on the best model that describes the correlation between concentration and response.

Assay on parallelism: for each series, calculate the absorbance regression or change of absorbance/minute against the concentrations in logarithm of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for parallel line assays. Express the potency of bovine heparin sodium in IU/mg of dried basis.

Ratio between straight line slopes: for each series, calculate the absorbance regression in logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for assays on ratio between straight line slopes. Express the potency of bovine heparin sodium in IU/mg of dried basis.

Acceptance criteria: the potency of bovine heparin sodium must present not less than 100 IU of anti-factor IIa activity per mg.

Anti-factor Xa activity

Proceed as described in *Anti-factor IIa* activity, except for the *Human factor IIa solution*, which must be replaced by *Human factor Xa solution*, and the *Chromogenic substrate solution* H-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride (H-D-Phe-Pip-Arg-pNA.2HCl), which must be replaced by *Chromogenic substrate solution* N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroaniline dihydrochloride (N-α-Z-D-Arg-Gly-Arg-pNA.2HCl). The buffer to be used in this test is *tris (hydroxymethyl) aminomethane pH 7.4,* but adding calcium chloride 10 m*M*. Prepare the *Human factor Xa solution* and the *Chromogenic substrate solution for human factor Xa* as described below.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* with addition of calcium chloride, to obtain solution at 1.0 IU/mL (equivalent to 20 n*M*).

Chromogenic substrate solution for human factor Xa: dissolve an amount of Nα-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroaniline dihydrochloride (N-α-Z-D-Arg-Gly-ArgpNA.2HCl) in sterile distilled water obtaining a 2.0 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Proceed with the statistical analyses and calculations indicated in *Anti-factor IIa activity*.

Acceptance criteria: the potency of bovine heparin sodium must present not less than 100 IU of anti-factor IIa activity per mg. The ratio between the anti-factor Xa/anti-factor IIa activity must be not less than 0.9 and not more than 1.1.

B. *Method II*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 8.4: dissolve 6.10 g of tris (hydroxymethyl) aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium and, if necessary, 0 to 10.0 g of polyethylene glycol 6000 and/or 2.0 g of bovine or human serum albumin in 800 mL of distilled water. Adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide and complete with water to 1000 mL.

Antithrombin solution: reconstitute the content of an ampoule of antithrombin as recommended by the manufacturer. Homogenize and dilute with *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain an antithrombin solution at 5.0 IU/mL. Dilute with the same buffer to obtain a concentration of 0.125 IU/mL.

Human thrombin solution: reconstitute the content of the human thrombin (factor IIa) ampoule in distilled water to a concentration of 20 IU/mL and dilute in *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, to obtain a thrombin solution at 5.0 IU/mL.

Chromogenic substrate solution: dilute a chromogenic thrombin substrate for amidolytic assay in water to obtain 1.25 m*M*.

Stop solution: prepare a solution at 20% (v/v) of acetic acid in water.

Standard solution: reconstitute the content of the primary or secondary reference standard heparin sodium ampoule and mix gently until full dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* solution to obtain at least four solutions with concentration ranging from 0.005 to 0.03 IU/mL.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain solutions with activities approximately equal to the ones from the standard solution.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions among the standard preparations, sample being tested, and reagents. The test must be carried out with each heparin solution and sample solution in duplicate. The tubes must be identified according to the number of replicates to be tested. Distribute the blanks in the columns so that they represent the behavior of reagents during the assay. The blank is the test carried out when no heparin is added, using 50-100 µL of *Buffer tris (hydroxymethyl) aminomethane pH 8.4*.

All reagent, standard and sample solutions in test must be pre-heated at 37°C for 15 minutes before being added to the tubes. Transfer to each of the plastic tubes, separately, a fixed volume (for example, 50-100 µL) of each of the different dilutions of *Standard solution,* or *Sample solution* or *Buffer tris (hydroxymethyl) aminomethane pH 8.4*. Add to each of the tubes a doubled volume of the *Antithrombin solution* (100-200 µl). Homogenize all tubes, gently, without producing bubbles, and incubate at 37°C for at least one minute. Add to each tube 25-50 µL of the *Human thrombin solution* and incubate at 37°C for at least one minute. Add 50-100 µL of the *Chromogenic substrate solution* and homogenize.

Two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Endpoint measurement: stop the reaction after one minute with 50-100 µL of stop solution. Record the absorbance of each solution at 405 nm. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Kinetic measurement: record the change in absorbance for each solution during one minute, measured at 405 nm. Express as a change in absorption per minute (ΔOD/minute) of solutions and blanks, which must present higher values due to the absence of heparin. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Calculations: the statistical models for analysis of ratio between straight line slopes or parallel lines may be used depending on the model that best describes the correlation between concentration and response.

Assay on parallelism: for each series, determine the absorbance regression or change of absorbance/minute against the logarithm of concentrations of the sample solutions and the standard solutions and calculate the sample potency using statistical methods for parallel line assays. Express the potency of bovine heparin sodium in IU/mg of dried basis.

Ratio between straight line slopes: for each series, determine the regression of the absorbance logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for assays on ratio between straight line slopes. Express the potency of bovine heparin sodium in IU/mg of dried basis.

Acceptance criteria: the potency of bovine heparin sodium must present not less than 100 IU of anti-factor IIa activity per mg.

Anti-factor Xa activity

Buffer tris (hydroxymethyl) aminomethane pH 8.4: dissolve amounts of tris (hydroxymethyl) aminomethane, edetic acid or edetate sodium, and sodium chloride in distilled water with polyethylene glycol 6000 at 0.1% to obtain concentrations of 0.050 *M*; 0.075 *M* and 0.175 *M*, respectively. If necessary, adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Antithrombin solution: reconstitute the content of an ampoule of antithrombin as recommended by the manufacturer. Homogenize and dilute with *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain an antithrombin solution at 1.0 IU/mL.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, to obtain a solution that results in 0.65 to 1.25 of absorbance at 405 nm when tested as described below, replacing 30 µL of sample solution with 30 µL of buffer solution pH 8.4.

Chromogenic substrate solution for human factor Xa: dilute in water a chromogenic substrate for amidolytic assay, specific for factor Xa, to obtain a concentration of 1 m*M*.

Stop solution: prepare a solution at 20% (v/v) of acetic acid in water.

Standard solution: reconstitute the content of the primary or secondary reference standard heparin sodium ampoule and mix gently until full dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* solution, to obtain at least five solutions with concentration ranging from 0.03 to 0.375 IU/mL of anti-factor Xa activity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain solutions with activities approximately equal to the ones from the standard solution.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions among the standard preparations, sample being tested, and reagents. Transfer 120 µL of *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to plastic tubes stored at 37°C. Add separately to each tube 30 µL of the different dilutions of *Standard solution* or *Sample solution*. Add to each tube 150 µL of the *Antithrombin solution* pre-heated at 37ºC for 15 minutes, homogenize and incubate for two minutes. Add 300 µL of the *Human factor Xa solution* pre-heated at 37ºC for 15 minutes, homogenize and incubate for two minutes. Add 300 µL of the *Chromogenic substrate solution for human factor Xa* pre-heated at 37ºC for 15 minutes, homogenize and, after incubating for two minutes, add to each tube 150 µL of the *Stop solution* and homogenize. To zero the spectrophotometer, prepare a blank, adding the reagents in reverse order, from the *Stop solution* to the final addition of 150 µL of the *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, omitting the *Standard solution* and *Sample solution.* Register the absorbance measured at 405 nm against the blank.

Calculations: determine the values of the absorbance logarithm against the concentrations of sample solutions and standard solutions. Calculate the sample activity using the statistical methods for assay on ratio between straight line slopes. Calculate the anti-factor Xa activity according to the formula:

$$P = (S_A/S_P)$$

where

P = potency of bovine heparin sodium in IU/mg of dried basis;
S_A = straight line slope for the *Sample solution;*
S_P = straight line slope for the *Standard solution;*
Express the anti-factor Xa activity of bovine heparin sodium in IU/mg of dried basis.

Acceptance criteria: calculate the ratio of the anti-factor Xa activity against the potency of anti-factor IIa (anti-factor Xa / anti-factor IIa), which must be between 0.9 and 1.1.

PACKAGING AND STORAGE

In tightly closed containers.

LABELLING

Comply with current legislation.

THERAPEUTIC CLASS

Anticoagulant.

PORCINE HEPARIN SODIUM

The porcine heparin sodium is extracted from the porcine intestinal mucosa and contains a mix of polysaccharide chains with varied molecular mass. It is composed mainly of alternate units of *N*- and 6- disulfated *α*-D-glucosamine units and 2-sulfated α-iduronic acid. It has anticoagulant activity due to the inhibition of several factors from the coagulation system, extending blood coagulation time. This occurs especially through enhancing the inactivation of factor Xa and of thrombin by antithrombin. It has at least 180 units of anti-factor IIa activity per mg of heparin in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must range between 0.9 and 1.1. As acceptance criterion for each test carried out for the anti-factor IIa and Xa activity, the potency calculated based on the dry weight must be between 90% and 110% of the potency declared. The animals from which heparin is extracted must meet the sanitary requirements for the species in question and the production process must ensure the removal or inactivation of infection agents.

IDENTIFICATION

A. Meets the requirements described in *Assay*, according to *methods I* or *II* of *Anti-factor Xa activity* and *Anti-factor IIa activity.*

B. Use the one-dimensional proton nuclear magnetic resonance spectroscopy method. Prepare solutions as described below.

Standard solution: prepare solution in a minimum concentration of 20 mg/mL of porcine heparin CRS in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid.

Solution for system suitability: prepare solution at 1% (w/w) of oversulfated chondroitin sulfate CRS in *Standard solution*.

Sample solution: prepare solution in a concentration of not less than 20 mg/mL of the sample in deuterium oxide 99.9% with 0.02% (w/v) of sodium trimethylsilyl propionic acid.

Procedure: a nuclear magnetic resonance spectrometer of at least 500 MHz, minimum acquisition time of 2 seconds, and minimum repetition time (waiting time plus acquisition time) of 4 seconds must be used in the analysis of samples. The test must be carried out under constant temperature of 35°C with a water suppression program. The spectral window must be not less than 10 to -2 ppm. For all samples, the methyl group from the trimethylsilyl propionic compound must be referenced in 0.00 ppm. The spectra obtained must be similar to the one from the *Standard solution*.

System suitability: the chemical shifts corresponding to the *N*-acetyl regions from heparin and oversulfated chondroitin sulfate in the *Solution for system suitability* must be observed between 2.02 and 2.08 ppm and between 2.13 and 2.19 ppm, respectively. The chemical shifts of signals corresponding to H1 and H2 from the α-glucosamine 6- and *N*-disulfated units (A1 and A2), to H1 from 2-sulfated α-iduronic acid (I1), to H1 from the *N*-sulfated α-glucosamine units (C1) and to the methyl grouping from *N*-acetylated α-glucosamine (CH_3) of the standard solution are present at 5.40; 3.28; 5.22; 5.31; and 2.05 ppm, respectively. The ppm values observed for each signal must not vary more than ± 0.03 ppm.

Samples acceptance criteria: the chemical shifts of signals A1, C1, I1, A2 and CH_3 must be observed at 5.40; 5.31; 5.22; 3.28; and 2.05 ppm, respectively. The ppm values observed for each signal must not vary more than ± 0.03 ppm. The integers of signals A1, C1, I1 must be obtained according to guidance from the panels on **Figure 1**. Spectrometers with higher resolution suggest separations of these signals (as shown for I1 on the insert of panel B); however, they are integrated along with the main signal. The integer of A1 is taken as a reference. Proceed to the calculation according to the formula:

$$\frac{C1 \times 100}{A1} \leq 20$$

The value obtained must necessarily be lower than 20%. No unidentified signal in the spectrum, in the region of 0.10 - 2.00; 2.10 - 3.20; and 5.70 - 8.00 ppm, must exceed 4% of the height of signal A1 (5.40 ppm). The chemical shift between 2.13 and 2.19 ppm, corresponding to the *N*-acetyl regions of oversulfated chondroitin sulfate, must not be observed.

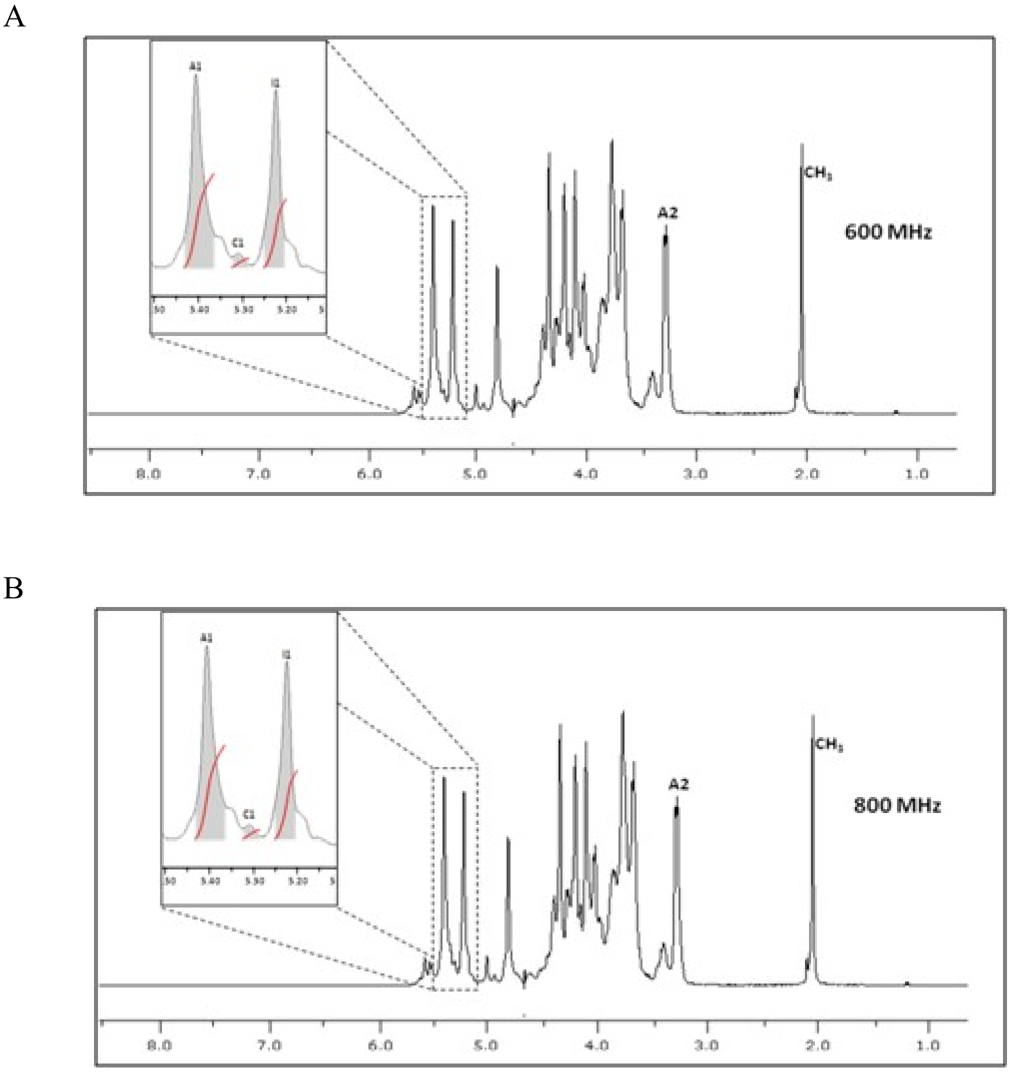


Figure 1 – **Porcine heparin sodium analyzed in spectrometer.**

Panel A – 600 MHz; Panel B – 800 MHz. The signals designated as A1 and A2 correspond to H1 and H2 from the α-glucosamine 6- and N-disulfated units at 5.40 and 3.28 ppm, respectively; signal I1 corresponds to H1 from 2-sulfated α-iduronic acid at 5.21 ppm; C1 corresponds to H1 from the *N*-sulfated α-glucosamine units at 5.31 ppm; and methyl from *N*-acetylated glucosamine at 2.05 ppm. The ppm values observed for each signal must not vary more than ± 0.03 ppm.

By the inserts, the expansion of regions between 5.10 and 5.50 ppm of same spectra is observed, to guide the integration of signals A1, C1 and I1.

C. Use the ion exchange liquid chromatography method for detecting and separating possible contaminants of porcine heparin sodium such as dermatan sulfate, chondroitin sulfate and oversulfated chondroitin sulfate. Use a chromatograph with ultraviolet detector. The reading can be done at 202 nm or 215 nm, provided that it complies with the system suitability. Use a pre-column with 50.0 mm of length and 2.0 mm of internal diameter packed with Ionpac AG11 HC resin (9 µm); column with 250 mm of length and 2.0 mm of internal diameter, packed with Ionpac AS11 HC (9 µm), stored at 40°C; and mobile phase flow of 0.5 mL/minute. Prepare solutions as described below.

Eluent A: prepare TRIS solution at 20 m*M*. Adjust pH to 7.4 with diluted hydrochloric acid.

Eluent B: prepare TRIS solution at 20 m*M* and NaCl at 2.5 *M*. Adjust pH to 7.4 with diluted hydrochloric acid.

Mobile phase gradient: adopt the gradient system from 0 to 2.5 *M* of NaCl, as described on the following table:

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0 – 4	83.4	16.6	isocratic
4 – 22	83.4 → 0	16.6 → 100	linear gradient
22 – 40	0	100	isocratic

Reference porcine heparin sodium solution: dissolve porcine heparin sodium CRS at the concentration of 4.0 mg/mL in mobile phase A when carrying out the assay **(Figure 2C)**.

System suitability solution: dissolve dermatan sulfate CRS and oversulfated chondroitin sulfate CRS at the concentration of 1.0 mg/mL and 0.4 mg/mL, respectively, in the *Porcine heparin sodium reference solution* in the concentration selected, when carrying out the assay (**Figures 2B** and **2A**).

Sample solution: dissolve the test sample at the concentration of 4.0 mg/mL in the mobile phase A.

Procedure: inject 50 µL of each one of the three solutions mentioned above to determine the elution profile. The peak obtained in the chromatogram with the *Sample solution* must be similar in form and retention time to the peak obtained with the *Reference porcine heparin solution* (**Figure 2C**). The retention time of porcine heparin sodium is of approximately 19 minutes.

System suitability: the relative retention in relation to the porcine heparin sodium (retention time = approximately 19 minutes) is of 0.8 for dermatan sulfate and 1.1 for oversulfated chondroitin sulfate. In the *System suitability solution*, the ratio between the height of the peak of dermatan sulfate CRS and the valley (baseline between the peaks of dermatan sulfate and heparin) is not less than 2.0. To meet such requirements, the adequate concentrations of the CRS must be selected.

Acceptance criteria: the presence of oversulfated chondroitin sulfate must not be detected. The amount of dermatan sulfate must be less than 4% of total glycosaminoglycans. There cannot be other peaks apart from the peak related to porcine heparin sodium and dermatan sulfate.

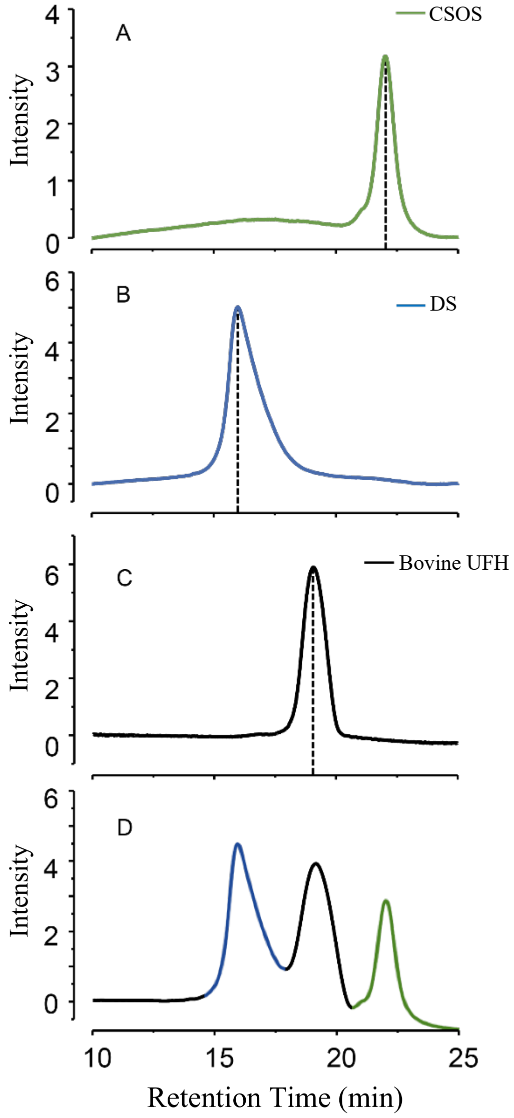


Figure 2 – **Porcine heparin sodium elution profile in the liquid chromatography in ion exchange column.**

Elution profile of oversulfate chondroitin, dermatan sulfate and porcine heparin sodium in ion exchange chromatography. 20 µg of CSOS (A), 50 µg of DS (B), 200 µg of porcine heparin sodium (C) and a mix with the same amounts of each polysaccharide (D) were applied. The retention times are equal for samples analyzed separately or mixed.

CHARACTERISTICS

Physical characteristics. White or nearly white powder, hygroscopic.

Solubility. Soluble in water.

PURITY TESTS

pH (5.2.19). 5.0 to 8.0. Determine in aqueous solution at 1% (w/v).

Proteins.

A. Add five drops of trichloroacetic acid at 20% (w/v) to 1 mL of sample aqueous solution at 1% (w/v). No precipitate or turbidity must be formed.

B. Proceed as described in *Ultraviolet, visible and infrared spectrophotometry* **(5.2.14)**.

Solution A: mix two volumes of sodium hydroxide 1% with two volumes of sodium carbonate 5% and dilute to five volumes with water.

Solution B: mix two volumes of copper sulfate ($CuSO_4.5H_2O$) 1,25% with two volumes of sodium tartrate ($C_4H_4Na_2O_6.2H_2O$) 2.98% and dilute to five volumes with water.

Solution C: mix one volume of *Solution B* with 50 volumes of *Solution A.*

Solution D: dilute adequately the phosphomolybdotungstic reagent in water so that the sample solution and the standard solution have pH value between 10.00 and 10.50 after adding S*olutions C* and *D*.

Sample solution: prepare a sample solution with concentration of 5 mg/mL in water.

Reference solution: prepare bovine albumin R solution (approximately 96% of protein) with concentration of 100 mg/mL in water. Make dilutions with water to obtain at least five reference solutions with protein concentrations distributed evenly within the range from 5 µg/mL to 100 µg/mL.

Procedure: add 5 mL of *Solution C* to each 1 mL of the *Reference solutions, Sample solution* and blank (water), respectively. Allow to stand for 10 minutes. Add 0.5 mL of *Solution D*, mix and allow to stand at room temperature for 30 minutes. Measure the solutions absorbances at 750 nm, using the blank solution for zero adjustment.

Calibration curve: the relation between absorbance and protein concentration is not linear; however, if the concentration range used to plot the standard curve is sufficiently small, it will ultimately be close to linearity. Plot a standard curve, plotting the absorbances from the reference solutions against their concentrations, using linear regression, plot a linear straight line of best adjustment to the points plotted. Determine the concentration of protein in the sample solution, through its absorbance and the standard curve. Not greater than 0.5% in relation to the dried substance.

Nucleotidic impurities. Dissolve 40 mg in 10 mL of water. The absorbance is measured at 260 nm and the result must be not greater than 0.15.

Nitrogen (5.3.3.2). Use *Method I, macrodetermination*. Not less than 1.5% and not more than 2.5% of nitrogen, calculated in relation to the dried substance.

Sodium. Between 10.5% and 13.5% of sodium determined as described in *Atomic absorption spectrophotometry* **(5.2.13.1)**.

Heavy metals (5.3.2.3). Use *Method I.* Not more than 0.003% (30 ppm).

Loss on drying (5.2.9.1). Determine in a vacuum oven at 60°C for three hours. Not more than 8%.

BIOLOGICAL SAFETY TESTS

Bacterial endotoxins (5.5.2.2). Not more than 0.03 EU/IU of porcine heparin sodium.

ASSAY

Use one of the methods described below.

A. *Method I*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 7.4: dissolve amounts of tris (hydroxymethyl) aminomethane to obtain concentration of 20 m*M*, sodium chloride to 150 m*M* in distilled water with polyethylene glycol 8000 at 0.1%. If necessary, adjust pH to 7.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of human antithrombin as recommended by the manufacturer. Homogenize. Dilute with *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 0.02 IU/mL (equivalent to 40 n*M*) of human antithrombin.

Human factor IIa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 2.0 IU/mL (equivalent to 20 n*M*) of human factor IIa.

Chromogenic substrate solution for human factor IIa: dissolve an amount of HD-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride (H-D-Phe-Pip-Arg-pNA.2HCl) in sterile distilled water obtaining a 2 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Stop solution: prepare a solution at 50% (v/v) of acetic acid in water.

Standard solution: use standard porcine heparin sodium CRS solution. Reconstitute the content of the porcine heparin sodium CRS ampoule as recommended by the manufacturer and mix gently until complete dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* solution, to obtain at least ten solutions with concentration ranging from 0.1 to 0.0001 IU/mL. The concentrations used must present not less than five points within a linear range. The concentration range used can be adapted to include this linearity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain solutions with activities approximately equal to the ones from the porcine heparin sodium CRS solution.

Procedure: two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions between the standard preparations, sample being tested and reagents. Run the test in microplates and carry out the spectrometric reading at 405 nm in a microplate reader at 37°C. The test must be carried out with each heparin CRS solution and sample solution in duplicate. In each microplate well, add 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4* or of *Sample solution*, 25 µL of *Human antithrombin solution* and 10 µL of *Human factor IIa solution.*

After two minutes of incubation at 37°C, add 25 µL of *the Chromogenic substrate solution for human factor IIa*. Record the absorbance measured at 405 nm against the blank for five minutes. The blank is the test carried out when no heparin is added, using 40 µL of *Buffer tris (hydroxymethyl) aminomethane pH 7.4*.

Endpoint measurement: proceed as described in the *Kinetic measurement*. After adding the *Chromogenic substrate solution for human factor IIa*, wait four minutes and stop the reaction by adding 50 µL of *Stop solution*.

The statistical models for analysis of ratio between straight line slopes or parallel lines must be used selecting the best model that describes the correlation between concentration and response.
Assay on parallelism: for each series, calculate the absorbance regression or change of absorbance/minute against the concentrations in logarithmic scale of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for parallel line assays. Express the potency of porcine heparin sodium in IU/mg of dried basis.

Ratio between straight line slopes: for each series, calculate the absorbance regression in logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for assays on ratio between straight line slopes. Express the potency of porcine heparin sodium in IU/mg of dried basis.

Acceptance criteria: the potency of porcine heparin sodium must present not less than 180 IU of anti-factor IIa activity per mg.

Anti-factor Xa activity

Proceed as described in *Anti-factor IIa* activity, except for the *Human factor IIa solution*, which must be replaced by *Human factor Xa solution*, and the *Chromogenic substrate solution* H-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride (H-D-Phe-Pip-Arg-pNA.2HCl), which must be replaced by *Chromogenic substrate solution* N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroaniline dihydrochloride (N-α-Z-D-Arg-Gly-Arg-pNA.2HCl). The buffer to be used in this test is *tris (hydroxymethyl) aminomethane pH 7.4,* but adding calcium chloride 10 m*M*. Prepare the *Human factor Xa solution* and the *Chromogenic substrate solution for Human factor Xa* as described below.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* with addition of calcium chloride, to obtain the solution at 1.0 IU/mL (equivalent to 20 n*M*).

Chromogenic substrate solution for human factor Xa: dissolve an amount of Nα-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroaniline dihydrochloride (N-α-Z-D-Arg-Gly-ArgpNA.2HCl) in sterile distilled water obtaining a 2 m*M* solution. Before using, dilute in *Buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Proceed with the statistical analyses and calculations indicated in *Anti-factor IIa activity*.

Acceptance criteria: the potency of porcine heparin sodium must present not less than 180 IU of anti-factor IIa activity per mg. The ratio between the anti-factor Xa activity/anti-factor IIa activity must be not less than 0.9 and not more than 1.1.

B. *Method II*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 8.4: dissolve 6.10 g of tris (hydroxymethyl) aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium and, if necessary, 0 to 10.0 g of polyethylene glycol 6000 and/or 2.0 g of bovine or human serum albumin in 800 mL of distilled water. Adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide and complete with water to 1 000 mL.

Human antithrombin solution: reconstitute the content of an ampoule of antithrombin as recommended by the manufacturer. Homogenize and dilute with *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain an antithrombin solution at 5.0 IU/mL. Dilute with the same buffer to obtain a concentration of 0.125 IU/mL.

Human thrombin solution: reconstitute the content of the human thrombin (factor IIa) ampoule in distilled water to a concentration of 20 IU/mL and dilute in *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, to obtain a thrombin solution at 5.0 IU/mL.

Chromogenic substrate solution: dilute a chromogenic thrombin substrate for amidolytic assay in water to obtain 1.25 m*M*.

Stop solution: prepare a solution at 20% (v/v) of acetic acid in water.

Standard solution: reconstitute the content of the primary or secondary reference standard porcine heparin sodium ampoule and mix gently until full dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* solution to obtain at least four solutions with concentration ranging from 0.005 to 0.03 IU/mL.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain solutions with activities approximately equal to the ones from the standard solution.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions among the standard preparations, sample being tested, and reagents. The test must be carried out with each heparin solution and sample solution in duplicate. The tubes must be identified according to the number of replicates to be tested. Distribute the blanks in the columns so that they represent the behavior of reagents during the assay. The blank is the test carried out when no heparin is added, using 50-100 µL of *Buffer tris (hydroxymethyl) aminomethane pH 8.4*.

All reagent, standard and sample solutions in test must be pre-heated at 37°C for 15 minutes before being added to the tubes. Transfer to each of the plastic tubes, separately, a fixed volume (for example, 50-100 µL) of each of the different dilutions of *Standard solution,* or *Sample solution* or *Buffer tris (hydroxymethyl) aminomethane pH 8.4*. Add to each of the tubes a doubled volume of the *Human antithrombin solution* (100-200 µl). Homogenize all tubes, gently, without producing bubbles, and incubate at 37°C for at least one minute. Add to each tube 25-50 µL of the *Human thrombin solution* and incubate at 37°C for at least one minute. Add 50-100 µL of the *Chromogenic substrate solution* and homogenize.

Two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: record the change in absorbance for each solution during one minute, measured at 405 nm. Express as a change in absorption per minute (ΔOD/min) of solutions and blanks, which must present higher values due to the absence of heparin. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Endpoint measurement: stop the reaction after one minute with 50-100 µL of stop solution. Record the absorbance of each solution at 405 nm. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Calculations: the statistical models for analysis of ratio between straight line slopes or parallel lines may be used depending on the model that best describes the correlation between concentration and response.

Assay on parallelism: for each series, determine the absorbance regression or change of absorbance/minute against the logarithm of concentrations of the sample solutions and the standard solutions and calculate the sample potency using statistical methods for parallel line assays. Express the potency of porcine heparin sodium in IU/mg of dried basis.

Ratio between straight line slopes: for each series, determine the regression of the absorbance logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for assays on ratio between straight line slopes. Express the potency of porcine heparin sodium in IU/mg of dried basis

Acceptance criteria: the potency of porcine heparin sodium must present not less than 180 IU of anti-factor IIa activity per mg.

Anti-factor Xa activity

Buffer tris (hydroxymethyl) aminomethane pH 8.4: dissolve amounts of tris (hydroxymethyl) aminomethane, edetic acid or edetate sodium, and sodium chloride in distilled water with polyethylene glycol 6000 at 0.1% to obtain concentrations of 0.050 *M*; 0.075 *M* and 0.175 *M*, respectively. If necessary, adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of antithrombin as recommended by the manufacturer. Homogenize and dilute with *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain an antithrombin solution at 1.0 IU/mL.

Human factor Xa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, to obtain a solution that results in 0.65 to 1.25 of absorbance at 405 nm when tested as described below, replacing 30 µL of sample solution with 30 µL of buffer solution pH 8.4.

Chromogenic substrate solution: dilute a chromogenic substrate for amidolytic assay in water, specific for factor Xa, to obtain a concentration of 1 m*M*.

Stop solution: prepare a solution at 20% (v/v) of acetic acid in water.

Standard solution: reconstitute the content of the primary or secondary reference standard heparin sodium ampoule and mix gently until full dissolution. From the reconstituted solution, prepare dilutions in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* solution, to obtain at least five solutions with concentration ranging from 0.03 to 0.375 IU/mL of anti-factor Xa activity.

Sample solution: dissolve an amount of sample in *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain solutions with activities approximately equal to the ones from the standard solution.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions among the standard preparations, sample being tested, and reagents. Transfer 120 µL of *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to plastic tubes stored at 37°C. Add separately to each tube 30 µL of the different dilutions of *Standard solution* or *Sample solution*. Add to each tube 150 µL of the *Antithrombin solution* pre-heated at 37ºC for 15 minutes, homogenize and incubate for two minutes. Add 300 µL of the *Factor Xa solution* pre-heated at 37ºC for 15 minutes, homogenize and incubate for two minutes. Add 300 µL of the *Chromogenic substrate solution for factor Xa* pre-heated at 37ºC for 15 minutes, homogenize and, after incubating for two minutes, add to each tube 150 µL of the *Stop solution* and homogenize. To zero the spectrophotometer, prepare a blank, adding the reagents in reverse order, from the *Stop solution* to the final addition of 150 µL of the *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, omitting the *Standard solution* and *Sample solution.* Register the absorbance measured at 405 nm against the blank.

Calculations: determine the values of the absorbance logarithm against the concentrations of sample solutions and standard solutions. Calculate the sample activity using the statistical methods for assay on ratio between straight line slopes. Calculate the anti-factor Xa activity according to the formula:

$$P = (S_A/S_P)$$

where

P = potency of porcine heparin sodium in IU/mg of dried basis;
S_A = straight line slope for the *Sample solution;*
S_P = straight line slope for the *Standard solution.*

Express the anti-factor Xa activity of porcine heparin sodium in IU/mg of dried basis.

Acceptance criteria: calculate the ratio of the anti-factor Xa activity against the potency of anti-factor IIa (anti-factor Xa / anti-factor IIa), which must range between 0.9 and 1.1.

PACKAGING AND STORAGE

In tightly closed containers.

LABELLING

Comply with current legislation.

THERAPEUTIC CLASS

Anticoagulant.

HEPARIN SODIUM INJECTABLE SOLUTION

The injectable preparation of heparin sodium is a sterile solution of heparin sodium diluted in water for injection. The anticoagulant potency is not less than 90.0% and not greater than 110.0% of the potency declared on the label in units per milliliter.

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

pH (5.2.19). 5.0 to 7.5.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 0.03 EU/IU of heparin.

ASSAY

Use one of the methods described below.

A. *Method I*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 7.4: dissolve amounts of tris (hydroxymethyl) aminomethane to obtain concentration of 20 m*M*, sodium chloride to 150 m*M* in distilled water with polyethylene glycol 8000 at 0.1%. If necessary, adjust pH to 7.4 with diluted solution of hydrochloric acid or sodium hydroxide.

Human antithrombin solution: reconstitute the content of an ampoule of human antithrombin as recommended by the manufacturer. Homogenize. Dilute with *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 0.02 IU/mL (equivalent to 40 nM) of human antithrombin.

Human factor IIa solution: reconstitute the content of the vial as recommended by the manufacturer. Dilute the solution obtained in *Buffer tris (hydroxymethyl) aminomethane pH 7.4*, to obtain solution at 2.0 IU/mL (equivalent to 20 nM) of human factor IIa.

Chromogenic substrate solution for human factor IIa: dissolve an amount of HD-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride (H-D-Phe-Pip-Arg-pNA.2HCl) in sterile distilled water obtaining a 2 m*M* solution. Before using, dilute in *buffer tris (hydroxymethyl) aminomethane pH 7.4* to obtain the 0.4 m*M* solution. Proceed protected from direct light.

Stop solution: prepare a solution at 50% (v/v) of acetic acid in water.

Standard solution: use standard heparin CRS solution. Reconstitute the content of the heparin CRS ampoule as recommended by the manufacturer and mix gently until complete dissolution. From the reconstituted solution, prepare dilutions in *buffer tris (hydroxymethyl) aminomethane pH 7.4* solution, to obtain at least ten solutions with concentration ranging from 0.1 to 0.0001 IU/mL. The concentrations used must present not less than five points within a linear range. The concentration range used can be adapted to include this linearity.

Sample solution: dilute an amount of sample in buffer *tris (hydroxymethyl) aminomethane pH 7.4* to obtain solutions with activities approximately equal to the ones from the heparin CRS solution.

Procedure: two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions between the standard preparations, sample being tested and reagents. Run the test in microplates and carry out the spectrometric reading at 405 nm in a microplate reader at 37°C. The test must be carried out with each heparin CRS solution and sample solution in duplicate. In each microplate well, add 40 µL of *buffer tris (hydroxymethyl) aminomethane pH 7.4* or of sample solution, 25 µL of *Human antithrombin solution* and 10 µL of *Human factor IIa solution***.** After two minutes of incubation at 37°C, add 25 µL of *the Chromogenic substrate solution for human factor IIa*. Record the absorbance measured at 405 nm against the blank for five minutes. The blank is the test carried out when no heparin is added, using 40 µL of *buffer tris (hydroxymethyl) aminomethane pH 7.4*.

Endpoint measurement: proceed as described in the *Kinetic measurement*. After adding the chromogenic substrate, wait four minutes and stop the reaction by adding 50 µL of *Stop solution*.

The statistical models for analysis of ratio between straight line slopes or parallel lines must be used selecting the best model that describes the correlation between concentration and response.
Assay on parallelism: for each series, calculate the absorbance regression or change of absorbance per minute against the concentrations in logarithmic scale of the sample solutions and standard solutions and calculate the potency of the sample using statistical methods for parallel line assays. Express the heparin sodium potency in IU/mL.

Ratio between straight line slopes: for each series, calculate the absorbance regression in logarithm or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and standard solutions and calculate the potency of the sample using statistical methods for assays on ratio between straight line slopes. Express the heparin sodium potency in IU/mL.

Acceptance criteria: the potency of injectable preparations of heparin sodium must present not less than 90% and not more than 110% of the activity stated on the label. The potency must be expressed in IU/mL.

B. *Method II*

Anti-factor IIa activity

Buffer tris (hydroxymethyl) aminomethane pH 8.4: dissolve 6.10 g of tris (hydroxymethyl) aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium and, if necessary, 0 to 10.0 g of polyethylene glycol 6000 and/or 2.0 g of bovine or human serum albumin in 800 mL of distilled water. Adjust pH to 8.4 with diluted solution of hydrochloric acid or sodium hydroxide and complete with water to 1 000 mL.

Antithrombin solution: reconstitute the content of an ampoule of antithrombin as recommended by the manufacturer. Homogenize and dilute with *Buffer tris (hydroxymethyl) aminomethane pH 8.4* to obtain an antithrombin solution at 5.0 IU/mL. Dilute with the same buffer to obtain a concentration of 0.125 IU/mL.

Human thrombin solution: reconstitute the content of the human thrombin (factor IIa) ampoule in distilled water to a concentration of 20 IU/mL and dilute in *Buffer tris (hydroxymethyl) aminomethane pH 8.4*, to obtain a thrombin solution at 5.0 IU/mL.

Chromogenic substrate solution: dilute a chromogenic thrombin substrate for amidolytic assay in water to obtain 1.25 m*M*.

Stop solution: prepare a solution at 20% (v/v) of acetic acid in water.

Standard solution: reconstitute the content of the primary or secondary reference standard heparin sodium ampoule and mix gently until full dissolution. From the reconstituted solution, prepare dilutions in *buffer tris (hydroxymethyl) aminomethane pH 8.4* solution, to obtain at least four solutions with concentration ranging from 0.005 to 0.03 IU/mL.

Sample solution: dissolve an amount of sample in buffer *tris (hydroxymethyl) aminomethane pH 8.4* to obtain solutions with activities approximately equal to the ones from the standard solution.

Procedure: the volumes described can be adapted to carry out the test in tubes or microplates, maintaining the proportions among the standard preparations, sample being tested, and reagents. The test must be carried out with each heparin solution and sample solution in duplicate. The tubes must be identified according to the number of replicates to be tested. Distribute the blanks in the columns so that they represent the behavior of reagents during the assay. The blank is the test carried out when no heparin is added, using 50 µL to 100 µL of *Buffer tris (hydroxymethyl) aminomethane pH 8.4*.

All reagent, standard and sample solutions in test must be pre-heated at 37ºC for 15 minutes before being transferred to the tubes. Transfer to each of the plastic tubes, separately, a fixed volume (for example, 50 µL to 100 µL) of each of the different dilutions of *Standard solution,* or *Sample solution* or *buffer tris (hydroxymethyl) aminomethane pH 8.4*.

Add to each of the tubes a doubled volume of the *Antithrombin solution* (100 µL to 200 µL). Homogenize all tubes, gently, without producing bubbles, and incubate at 37°C for at least one minute. Add to each tube 25 µL to 50 µL of the *Human thrombin solution* and incubate at 37ºC for at least one minute. Add 50 µL to 100 µL of the *Chromogenic substrate solution* and homogenize.

Two different types of measurements can be carried out: *Kinetic measurement* and *Endpoint measurement*.

Kinetic measurement: record the change in absorbance for each solution during one minute, measured at 405 nm. Express as a change in absorption per minute (ΔOD/min) of solutions and blanks, which must present higher values due to the absence of heparin. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Endpoint measurement: stop the reaction after one minute with 50 µL to 100 µL of *Stop solution*. Record the absorbance of each solution at 405 nm. The relative standard deviation between the readings obtained with the blank must be less than 10%.

Calculations: the statistical models for analysis of ratio between straight line slopes or parallel lines may be used depending on the model that best describes the correlation between concentration and response.

Assay on parallelism: for each series, determine the absorbance regression or change of absorbance per minute against the logarithm of concentrations of the sample solutions and standard solutions and calculate the potency of the sample using statistical methods for parallel line assays. Express the heparin sodium potency in IU/mL.

Relation between straight line slopes: for each series, determine the regression of the logarithm of absorbance or the logarithm of changes in absorption per minute against the concentrations of the sample solutions and the standard solutions and calculate the potency of the sample using statistical methods for assays on relations between straight line slopes. Express the heparin sodium potency in IU/mL.

Acceptance criteria: the potency of injectable heparin sodium preparations must present not less than 90% and not more than 110% of the activity stated on the label. The potency must be expressed in IU/mL.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

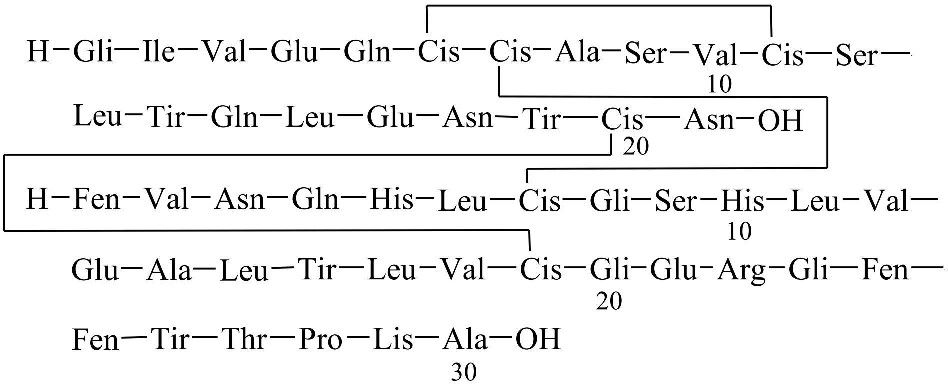
Comply with current legislation.

THERAPEUTIC CLASS

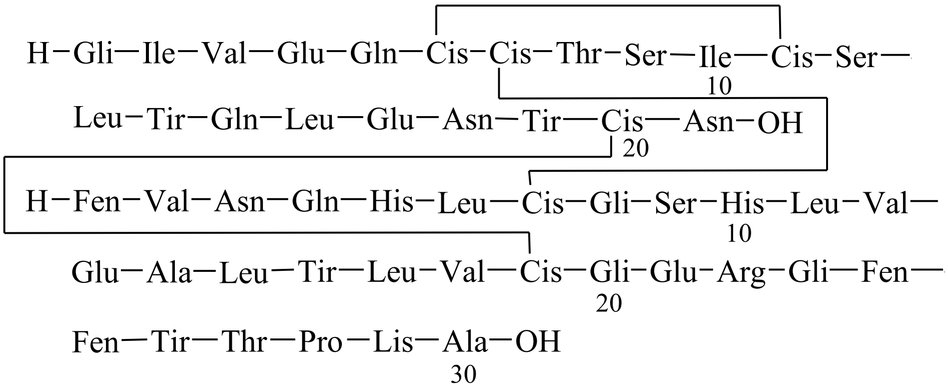
Anticoagulant.

INSULIN

Insulinum



BOVINE INSULIN



PORCINE INSULIN

$C_{254}H_{377}N_{65}O_{75}S_6$; 5733.50 (bovine)
$C_{256}H_{381}N_{65}O_{76}S_6$; 5777.55 (porcine)
insulin; 04908 Insulin (bovine)
[*11070-73-8*]
Insulin (porcine)
[*12584-58-6*]

Insulin is a protein that affects glucose metabolism. It is obtained from the pancreas of healthy bovines and swines, or both, used as food by humans. The amount is not less than 26.5 units of insulin in each milligram; insulin labeled as purified has not less than 27.0 units of insulin in each milligram, calculated on dried basis. The content of proinsulin is not more than 10 ppm.

***Note:** one unit of insulin is equivalent to 0.0342 mg of pure insulin derived from bovines or 0.0345 mg of pure insulin derived from swines.*

IDENTIFICATION

A. The retention time of the main peak from the *Assay preparation* chromatogram, obtained in *Assay*, corresponds to the one from the main peak of the *identification Preparation*.

***Note**: Applying the mixture from the Assay preparation with the identification Preparation may be required.*

B. Determination of peptide fragments. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 214 mm; column with 100 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 µm to 10 µm), stored at temperature of 40°C; *Mobile phase* flow of approximately 1.0 mL/minute.

Sulfate buffer: mix equal volumes of ammonium sulfate 2 *M* and sulfuric acid 0.5 *M* and filter.

Enzyme solution: prepare a *Staphylococcus aureus* V-8 protease solution in water, with an activity of 500 IU/mL.

HEPES buffer: dissolve 2.38 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) in approximately 90 mL of water in a 100 mL volumetric flask. Adjust pH to 7.5 using sodium hydroxide 5 *M*. Dilute with water until completing the flask volume and mix.

Eluent A: prepare a filtered and degassed mixture of 100 mL of acetonitrile, 700 mL of water and 200 mL of *Sulfate buffer*.

Eluent B: prepare a filtered and degassed mixture of 400 mL of acetonitrile, 400 mL of water and 200 mL of *Sulfate buffer*.

Standard digest solution: dissolve, according to the species indicated, 6 mg of insulin CRS in 3 mL of hydrochloric acid 0.01 *M* and transfer 500 µL of the resulting solution to a clean vial. Add 2 mL of *HEPES Buffer* and 400 µL of *Enzyme solution* and incubate at 25°C for six hours. Quench the digestion by adding 2.9 mL of *Sulfate buffer*.

Test digestion solution: for every 1 mg of insulin, add 500 µL of hydrochloric acid 0.01 *M* and mix to dissolve. Proceed as indicated for *Standard digest solution*, starting with "add 2 mL of *HEPES Buffer*".

Mobile phase gradient: adopt the gradient system described on the following table.

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0	90	10	equilibrium
0 – 60	90 → 30	10 → 70	linear gradient
60 – 65	30 → 0	70 → 100	linear gradient
65 – 70	0	100	isocratic
70 – 71	0 → 90	100 → 10	linear gradient
71 – 86	90	10	reequilibrium

Procedure: inject, separately, equal volumes of *Standard digest solution* and *Test digest solution*, record the chromatograms, and measure the area under the peaks. The chromatographic profile of the *Test digestion solution* corresponds to the one from the *Standard digestion solution*. Carry out the blank test and make the necessary corrections. The tailing factor is not more than 1.5. The resolution between the peaks of the digest fragment II and the digest fragment III is not less than 1.9.

***Note:** fragment I elutes at the same time in the porcine-derived insulin and in human insulin; fragment II elutes at the same time in all insulins; and fragment III elutes at the same time in insulin derived from bovines and swines.*

***Note:** the volume to be injected depends on the equipment resolution. Volume necessary for obtaining the separation and resolution of peaks must be injected.*

CHARACTERISTICS

Loss on drying (5.2.9.1). Determine in 0.2 g of sample. Dry in oven at 105°C for 16 hours. Not more than10%.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)**. Determine the zinc content of approximately 10 mg of the sample, accurately weighed. Not more than 1.0%, calculated on dried basis.

PURITY TESTS

Related substances. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4).** Use a chromatograph with ultraviolet detector at 214 mm; column with 250 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 µm to 10 µm), stored at temperature of 40°C; *Mobile phase* flow of approximately 1.0 mL/minute.

Diluent: dissolve 28.4 g of anhydrous sodium sulfate in 1000 mL of water. Add 2.7 mL of phosphoric acid. Adjust, if necessary, pH to 2.3 using ethanolamine and homogenize.

Eluent A: prepare a filtered and degassed mixture of the *Diluent* and acetonitrile (82:18).

Eluent B: prepare a filtered and degassed mixture of the *Diluent* and acetonitrile (50:50).

Mobile Phase gradient: adopt the gradient system described on the following table:

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0	81	19	equilibrium
0 – 60	81	19	isocratic
60 – 85	81 → 36	19 → 64	linear gradient
85 – 91	36	64	isocratic
91 – 92	36 → 81	64 → 19	linear gradient

Sample solution: transfer approximately 7.5 mg of insulin to a vial with adequate cap and add 2 mL of hydrochloric acid 0.01 *M*. Put the cap on the vial and gently shake for dissolution

***Note:** Sample solution can be stored at room temperature for up to two hours and in a refrigerator for up to 12 hours.*

Standard solution A: dissolve an amount, accurately weighed, of the appropriate species of insulin CRS in hydrochloric acid 0.01 *M* to obtain a solution with known concentration of about 3.75 mg/mL.

Standard solution B: pipet 1 mL of *Standard solution A*, transfer to a 10 mL volumetric flask, complete the volume with hydrochloric acid 0.01 *M* and homogenize.

Standard solution C: pipet 1 mL of *Standard solution B*, transfer to a 10 mL volumetric flask, complete the volume with hydrochloric acid 0.01 *M* and homogenize.

Note: *the three standard solutions can be stored at room temperature for up to 12 hours and in a refrigerator for up to 48 hours.*

System suitability solution: proceed as described for the *System suitability solution* at the *Physicochemical method* in *Assay*.

Adjust the *Mobile phase* composition and the duration of the isocratic elution to obtain a retention time of approximately 31 minutes for insulin, with the elution of A-21 desamido insulin shortly before the start of the elution phase by linear gradient. Inject the *Standard solution A*, *Standard solution B* and *Standard solution C*, record the chromatograms, and measure the peak responses as indicated in *Procedure*. Calculate factor X_1 (ten times the ratio between areas from *Standard solution B* by *Standard solution A*) according to the formula:

$$10 \times \left({}^{r_B}\!/_{r_A} \right)$$

where

r_B = area of response from peak obtained for *Standard solution B;*
r_A = area of response from peak obtained for *Standard solution A*.

X_1 value must be between 0.91 and 1.09.

Calculate factor X_2 (one hundred times the ratio between areas from *Standard solution C* by *Standard solution A*) according to the formula:

$$100 \times \left({}^{r_C}\!/_{r_A} \right)$$

where

r_C = area of responses from peak obtained for *Standard solution C*.
r_A = area of responses from peak obtained for *Standard solution A*.

X_2 value must be between 0.7 and 1.3.

Inject the *System suitability solution* and record the responses from peak as indicated in *Procedure*. Resolution, *R*, between insulin and A-21 desamido insulin is not less than 2.0. The tailing factor for the insulin peak is not more than 1.8.

Note: *the volume to be injected depends on the equipment resolution. Volume necessary for obtaining the separation and resolution of peaks must be injected.*

Procedure: inject a volume of approximately 20 µL of *Sample solution*, record the chromatogram, and measure the response areas for the peak of main insulin, the peak of A-21 desamido insulin and peaks of any other impurities. Calculate the percentage of insulin (%I) in the insulin portion used according to the formula:

$$\%I = 100 \times \left({}^{r_I}\!/_{r_S} \right)$$

where

r_I = the response, in area, under the insulin peak.
r_S = the sum of responses, in area, under all peaks.

Calculate the percentage of A-21 desamido insulin (%D) in the insulin portion used according to the formula:

$$\%D = 100 \times \left({}^{r_D}\!/_{r_S} \right)$$

where

r_D = the response, in area, under the A-21 desamido insulin peak.
r_S = the sum of responses, in area, of all peaks.

Calculate the percentage of other compounds related to insulin in the insulin portion used according to the formula:

$$100 \times (\%I + \%D)$$

Not more than 10.0% of A-21 desamido insulin is found and not more than 5.0% of other insulin-related compounds are found. For insulin derived from a single species, measure the responses from any peaks corresponding to porcine or bovine insulin, and calculate the respective concentrations as percentage of r_s. The amount of cross contamination is not more than 1.0%.

Limit for high molecular mass proteins. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 276 mm; column with 300 mm of length and 7.8 mm of internal diameter, packed with silica chemically bonded to dihydroxypropane group (5 µm to 10 µm), *Mobile phase* flow of approximately 0.5 mL/minute.

Arginine solution: prepare a solution of L-arginine in water with 1 mg/mL.

Mobile phase: prepare a filtered and degassed mix of *Arginine solution*, acetonitrile and glacial acetic acid (65:20:15). Make adjustments, if necessary.

Sample solution: transfer approximately 4 mg of insulin to a small vial, add 1 mL of hydrochloric acid 0.01 *M* and mix until it dissolves. Store this solution in a refrigerator and use it within seven days.

Resolution solution: dissolve 4 mg of insulin that has more than 0.4% of high molecular mass proteins in 1 mL of hydrochloric acid 0.01 *M*. Store this solution in a refrigerator and use it within seven days.

***Note:** insulin with the indicated percentage of high molecular mass proteins can be prepared by storing the insulin at room temperature for 5 days.*

Perform the chromatography of the *Resolution solution* by injecting 100 µL and record the peak responses as indicated in *Procedure*. The retention times range between 13 and 17 minutes for insulin polymeric complexes, approximately 17.5 minutes for the insulin covalent dimer, and between 18 and 22 minutes for the insulin monomer, with salts eluting after the insulin monomer. The ratio between the insulin covalent dimer peak and the height of the valley between the insulin covalent dimer peak and the insulin monomer peak is not less than 2.0.

Procedure: inject 100 µL of the *Sample solution*, record the chromatogram and measure the peak response areas, without considering any peaks that have retention times greater than the one from the insulin monomer. Calculate the percentage of high molecular mass proteins in the insulin portion used according to the formula:

$$\frac{100\sum r_H}{(\sum r_{H} + r_M)}$$

where

$\sum r_H$ = sum of responses for all peaks with retention times lower than that of the insulin monomer;
r_M = response from insulin monomer peak (not more than1.0% is found).

BIOLOGICAL SAFETY TESTS

Count of the total amount of mesophilic microorganisms (5.5.3.1.2). The total bacterial count is not more than 300 CFU/g, with the test being carried out in a portion of approximately 0.2 g of the sample, accurately weighed.

Bacterial endotoxins (5.5.2.2). Not more than 10 EU/mg.

ASSAY

Physicochemical method

Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 214 mm; column with 150 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 µm to 10 µm), stored at temperature of 40°C; *Mobile phase* flow of approximately 1.0 mL/minute.

Sodium sulfate buffer: dissolve 28.4 g of anhydrous sodium sulfate in 1000 mL of water, add 2.7 mL of phosphoric acid, and adjust pH to 2.3 using ethanolamine, if necessary.

Mobile phase: prepare a filtered and degassed mixture of *Sodium sulfate buffer* with acetonitrile (74:26). Acetonitrile is heated at a temperature of 20°C or higher to avoid precipitation. Make adjustments, if necessary.

Standard preparation: dissolve an amount, accurately weighed, of appropriate insulin CRS in hydrochloric acid 0.01 *M* to obtain a solution with known concentration of approximately 1.5 mg/mL.

Identification Preparation: prepare a solution of porcine insulin CRS and bovine insulin CRS in hydrochloric acid 0.01 *M*, with approximately 0.6 mg of each per mL.

Assay preparation: transfer approximately 15 mg of insulin, accurately weighed, to a 10 mL volumetric flask, dissolve and dilute with hydrochloric acid 0.01 *M* to obtain a solution with a concentration of approximately 1.5 mg/mL.

System suitability solution: dissolve approximately 1.5 mg of insulin in 1 mL of hydrochloric acid 0.01 *M*. Allow to stand in room temperature for not less than three days to obtain a solution with not less than 5% of A-21 desamido insulin.

Note: *The Identification Preparation, Standard preparation and Assay preparation can be stored at room temperature for up to 12 hours or in refrigerator for up to 48 hours.*

Perform the chromatography of the *Standard preparation* by injecting 20 µL and record the peak responses as indicated in *Procedure*. The relative standard deviation for injection replicates is not more than1.6%. Perform the chromatography by injecting 20 µL of the *System suitability solution* and record the peak responses as indicated in *Procedure*. Resolution, *R*, between insulin and A-21 desamido insulin is not less than 2.0. The tailing factor for the insulin peak is not more than 1.8.

Procedure: inject, separately, equal volumes (approximately 20 µL) of the *Assay preparation*, *Identification Preparation* and *Standard preparation*, record the chromatograms and measure the peak responses for insulin and A-21 desamido insulin, using the chromatogram from the *Identification Preparation* to identify the insulin peaks. For insulin derived from a single species, calculate the amount on undried basis, in units of insulin per mg, of insulin in the *Assay preparation* according to the formula:

$$\left(\frac{CS}{CU}\right)\cdot\left(\frac{\sum rU}{\sum rS}\right)$$

where

CS = concentration of insulin CRS in *Standard preparation* (units of insulin/mL);
CU = concentration of insulin in *Sample preparation* (mg/mL);
$\sum rU$ and $\sum rS$ = sum of areas of peaks from insulin and A-21 desamido insulin obtained, respectively, from chromatograms of the *Sample preparation* and *Standard preparation*.

From the value obtained on the *Loss by drying* test, calculate the amount on dried basis. For insulin derived from the mixture of bovine with porcine insulin, calculate the total amount with the sum of the amounts of insulins determined, separately.

Biological method

Insulin assays

The most prominent manifestation of the insulin activity, an abrupt decrease of blood glucose, was the basis for biological assays upon the first clinical use. The procedure, although relatively laborious, has the great merit of reflecting the effect on a diabetic patient. The advent of sophisticated and also practical physicochemical methods (for example, high-efficiency liquid chromatography) to measure quantitatively the insulin potency resulted in a summarized, more precise and accurate test for insulin and related products. However, the bioidentity of insulin and its products cannot be assessed by these methods. A quantitative test in rabbits is therefore included in this monograph with its use being requested in relevant monographs.

The *quantitative rabbit blood sugar method* is used to determine the potency of insulin reference standards, to validate the stability of new insulin preparations and to determine the specific activities of insulin analogs.

Quantitative rabbit blood sugar method

Reference standards: glucose CRS, insulin CRS, bovine insulin CRS, human insulin CRS, porcine insulin CRS.

Diluent: prepare an aqueous solution with 0.1% to 0.25% (w/v) of cresol or phenol, 1.4% to 1.8% (w/v) of glycerin and enough hydrochloric acid to produce a pH between 2.5 and 3.5, unless indicated otherwise in an individual monograph.

Standard stock solution: dissolve an adequate and accurately weighed quantity of insulin (*Reference standard*) or a vial of lyophilized insulin (*Reference standard*) from the appropriate species in *Diluent* to make the *Standard stock solution* with 40 units of insulin per mL and pH between 2.5 and 3.5, unless indicated otherwise in an individual monograph. Store in a cold place, protected from freezing and use within six months.

Standard solutions: dilute portions of *Standard stock solution* with *Diluent* to obtain two solutions, one with one unit of insulin per mL (*Standard solution 1*) and the other with two units of insulin per mL (*Standard solution 2*).

Assay stock solution: proceed as indicated for the *Standard stock solution*, except for using the adequate quantity of the preparation being analyzed in place of insulin CRS. The *Assay stock solution* has approximately forty units of insulin per mL.

Assay solutions: dilute portions of the *Assay stock solution* with *Diluent* to obtain two dilutions of the test preparation, one of which is expected to have one unit of insulin per mL (*Assay solution 1*), based on the supposed potency, and the other with two units of insulin per mL (*Assay solution 2*). In the case of a neutral insulin injection, adjust to a pH of 2.5 to 3.5, before making the dilutions.

Doses of solutions to be injected: select, based on previous tests or experience, the dose of dilutions to be injected, whose volume will usually be between 0.30 mL and 0.50 mL. For each animal, the volume of the *Standard solution* is the same as the one of the *Assay solution*.

Preparation of animal: select adequate and healthy rabbits, each one weighing not less than 1.8 kg. Keep the rabbits in the laboratory for not less than one week before using them in the assay, maintaining them on an adequate constant diet, with water available at all times.

Procedure: separate the rabbits into four equal groups, preferably with not less than six rabbits each. On the preceding day, about 20 hours before the assay, give to each rabbit an amount of food to be eaten within six hours. Follow the same feeding scheme before each test day. During the assay, withhold all food until after the final blood sample is collected. Handle the rabbits carefully to avoid excessive excitement and inject, subcutaneously, the doses indicated on **Table 1.** The second injection must be made on the day after the first injection or not more than one week later. The time between the first and the second injections is the same for all rabbits.

Table 1 – **Doses to be injected in rabbits subcutaneously according to the Quantitative rabbit blood sugar method.**

Group	*First injection*	*Second injection*
1	*Standard solution 2*	*Assay solution 1*
2	*Standard solution 1*	*Assay solution 2*
3	*Assay solution 2*	*Standard solution 1*
4	*Assay solution 1*	*Standard solution 2*

Blood samples: one hour ± five minutes and two and a half hours ± five minutes after the injection, collect from each rabbit an adequate sample of blood from a marginal ear vein. Blood can also be effectively collected from the central auricular artery.

Determination of glucose content: determine the glucose content of blood samples through an adequate procedure that is adapted to automated analysis. The following procedure can be used.

Anticoagulant solution: dissolve 1 g of edetate disodium and 200 mg of sodium fluoride in 1000 mL of water and mix.

Glucose standard preparations: transfer known concentrations of glucose CRS to adequate containers and dilute, quantitatively and stepwise, with anticoagulant solution (1:9) to obtain a series of *Glucose standard glucose preparations* that have between 20 and 100 mg per 100 mL, with concentrations similar to the concentrations from the rabbit blood samples.

Test preparations: pipet and transfer to separate and adequate containers 0.1 mL of each blood sample and 0.9 mL of the *Anticoagulant solution*.

Procedure: subject the *Test preparations* to dialysis through a semipermeable membrane for a sufficient time so that glucose crosses the membrane in a solution of sodium chloride at 0.9% (w/v) with glucose oxidase, horseradish peroxidase (HRP peroxidase enzyme), 3-methyl-2-benzothiazolinone hydrazone hydrochloride RS and *N*,*N*-dimethylaniline. The absorbances of the *Test preparations* are determined at 600 nm in a colorimeter. The absorbances of the *Standard glucose preparations* are equally determined at the start and the end of each run.

Calculate the response from each rabbit to each injection, from the sum of the two blood sugar values. Calculate the individual differences, *y*, subtracting the responses as indicated in **Table 2**, disregarding the chronological order.

When data for one or more rabbits are missing on an assay, do not use the confidence interval from formulas described in the monograph, but refer to suitable statistical methods. The data can also be analyzed with adequate variance analysis.

When the number of rabbits, *f*, used in the assay is the same in each group, determine the sum of *y* for each group and calculate $T_a = -T_1 + T_2 + T_3 - T_4$ and $T_b = T_1 + T_2 + T_3 + T_4$. The logarithm of the relative potency of test dilutions is $M' = 0.301\ T_a/T_b$. The potency of injection in units per mg is equivalent to antilog (log R + M'), where $R = V_S/V_U$, V_S is the number of units per mL of *Standard solution* and V_U is the number of mg of insulin per mL of the corresponding *Assay solution*.

Determine the confidence interval of 95% for the log-relative potency using Fieller's Theorem (refer to the appendix at the end of the monograph and to the *Statistical procedures applicable to bioassays* described in chapter **8.**). If the confidence interval is more than 0.082, which corresponds to P = 0.95, for confidence limits of approximately ± 10.0% of the computed potency, repeat the test until the combined data of two or more assays, redetermined as described in *Statistical procedures applicable to bioassays*, meet this acceptable limit.

Table 2 – Registration of calculations of the responses from rabbits in the *Quantitative rabbit blood sugar method.*

Group	*Differences*	*Individual response (y)*	*Total response (T)*	*Standard deviations of differences (S)*
1	*Standard solution 2 – Assay solution 1*	y_1	T_1	S_1
2	*Assay solution 2 – Standard solution 1*	y_2	T_2	S_2
3	*Assay solution 2 – Standard solution 1*	y_3	T_3	S_3
4	*Standard solution 2 – Assay solution 1*	y_4	T_4	S_4

Bioidentity

Meet the requirements from the bioidentity test under insulin assays. Proceed as indicated by the *Quantitative rabbit blood sugar method* with the following changes.

Procedure: separate the rabbits into four equal groups with two rabbits each.

Proceed with the calculations as indicated in the *Quantitative rabbit blood sugar method*, but without the need to determine the confidence interval of the log-relative potency, M'.

If the potency value obtained is not less than 15 units/mg, the bioidentity test requirement is met. If the potency value is less than 15 units/mg, repeat the test using another eight rabbits. If the average potency of the two sets of tests is not less than 15 units per mg, the test requirement was met.

Appendix - Fieller's Theorem to determine the confidence interval for a ratio

This version of Fieller's Theorem applies to the case where numerator and denominator are not correlated. By using this equation, numerator and denominator are assumed to be normally distributed and the groups of rabbits have equal size.

Therefore, the confidence interval of 95% for the ratio is:

$$(L,U) = \frac{M' \pm \frac{t}{T_b}\sqrt{(1-g)S_N^2 + (M')^2 S_D^2}}{1-g}$$

where *f* (degrees of freedom from standard error) = 4 (k - 1), *k* is the number of rabbits in a group, *t* is the superior percentile 97.5 of the distribution *t* with degrees *f* of freedom, and:

$$g = \frac{t^2 S_D^2}{T_b^2}$$

If $g \geq 1$, the denominator is not significantly different from 0 and the formula does not apply.

$$S_N = 0.301\sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

$$S_D = \sqrt{k}\sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

HUMAN INSULIN

$C_{257}H_{383}N_{65}O_{77}S_6$; 5807.58
human insulin; 04918
[*11061-68-0*]

Human insulin is a protein corresponding to an active principle secreted in the human pancreas that affects the metabolism of carbohydrates (particularly glucose), lipids and proteins. It derives from enzyme modification of the insulin from porcine pancreas to suitably change the sequence of amino acids or produced by microbial synthesis via recombinant DNA process. The quantity, calculated on the dried basis is not less than 27.5 units of human insulin in each milligram. The proinsulin content of porcine-derived human insulin is not more than 10 ppm. The content of proteins from a host cell of human insulin derived from a recombinant DNA process, determined by a suitable and validated method, is not more than 10 ppm. The content of DNA derived from the host cell or from the vector and the limit for human insulin derived from a recombinant DNA process that uses eukaryote cells are determined by a validated method.

Note: *one unit of human insulin is equivalent to 0.0347 mg of pure human insulin.*

IDENTIFICATION

A. The retention time of the main peak from the *Assay preparation* chromatogram, obtained in *Assay*, corresponds to the one from the main peak of the *Standard preparation*.

B. Determine the peptide fragments, using the following procedure for mapping peptides. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 214 mm; column with 100 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 to 10 µm), stored at temperature of 40°C; *Mobile phase* flow of approximately 1.0 mL/minute.

Sulfate buffer: mix equal volumes of ammonium sulfate 2 *M* and sulfuric acid 0.5 *M* and filter.

Enzyme solution: prepare a *Staphylococcus aureus* V-8 protease solution in water, with an activity of 500 units per milliliter.

HEPES buffer: dissolve 2.38 g of HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) in approximately 90 mL of water in a 100 mL volumetric flask. Adjust pH to 7.5 using sodium hydroxide 5 *M*. Dilute with water until completing the flask volume and mix.

Eluent A: prepare a filtered and degassed mix of 100 mL of acetonitrile, 700 mL of water and 200 mL of *Sulfate buffer*.

Eluent B: prepare a filtered and degassed mix of 400 mL of acetonitrile, 400 mL of water and 200 mL of *Sulfate buffer*.

Standard digest solution: dissolve approximately 6 mg of human insulin CRS in 3 mL of hydrochloric acid 0.01 *M* and transfer 500 µL of the resulting solution to a clean vial. Add 2 mL of *HEPES Buffer* and 400 µL of *Enzyme solution* and incubate at 25°C for six hours. Quench the digestion by adding 2.9 mL of *Sulfate buffer*.

Test digestion solution: for every 1 mg of human insulin, add 500 µL of hydrochloric acid 0.01 *M* and mix to dissolve. Proceed as indicated for *Standard digest solution*, starting with "add 2 mL of *HEPES Buffer*".

Mobile phase gradient: adopt the gradient system described on the following table.

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0	90	10	equilibrium
0 – 60	90 → 30	10 → 70	linear gradient
60 – 65	30 → 0	70 → 100	linear gradient
65 – 70	0	100	isocratic
70 – 71	0 → 90	100 → 10	linear gradient
71 – 86	90	10	reequilibrium

Procedure: inject, separately, equal volumes of *Standard digest solution* and *Test digest solution*, record the chromatograms, and measure the area under the peaks. The chromatographic profile of the *Test digestion solution* corresponds to the one from the *Standard digestion solution*. Carry out the blank test and make the necessary corrections. The tailing factor is not more than 1.5. The resolution between the peaks of the digestion fragment II and the digestion fragment III is not less than 3.4.

Note: *fragment I elutes at the same time in the porcine-derived insulin and in human insulin; fragment II elutes at the same time in all insulins; and fragment III elutes at the same time in insulin derived from bovines and swines.*

PHYSICAL CHARACTERISTICS

Loss on drying (5.2.9.1). Weigh, accurately, approximately 200 mg and dry at 105°C for 16 hours. Not more than 10.0%.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)**. Determine the zinc content in accurately weighed 10 mg of the sample. Not more than 1.0% for anhydrous human insulin.

PURITY TESTS

Related substances. Proceed as described in *Related substances* in the monograph *Insulin*, except for the use of the following *Mobile Phase Gradient:* the program requires initially isocratic elution, for approximately 36 minutes, with the *Mobile phase* being comprised of a mixture of 78% of *Eluent A* and 22% of *Eluent B,* followed by the linear gradient elution. Later, the system returns to the initial conditions of 78% of *Eluent A* and 22% of *Eluent B*. Adjust the composition of the *Mobile phase* so that the retention time of the main peak of human insulin is between 15 and 25 minutes. The content of A-21 desamido insulin and other insulin-related compounds is not more than 2% for each of the total amounts of insulin and related compounds.

Limit for high molecular mass proteins. Proceed as described in *Limit of high molecular mass proteins* in the monograph *Insulin*. Not more than 1.0%.

BIOLOGICAL SAFETY TESTS

Count of the total amount of mesophilic microorganisms (5.5.3.1.2). Not more than 300 CFU/g, with the test being conducted in approximately 0.2 g of the sample, accurately weighed.

Bacterial endotoxins (5.5.2.2). Not more than10 EU/mg of human insulin.

ASSAY

Physicochemical method

Proceed as described in *Assay* in the monograph *Insulin*. Use the same *Mobile phase*, *Standard preparation*, *Sample preparation*, *Resolution solution*, chromatographic system and the *Procedure* described in the monograph *Insulin*, except for using human insulin CRS and any other substitute of human insulin for insulin throughout the assay.

*Fragment I consists of amino acids A5 to A17 and B1 to B13; fragment II consists of amino acids A18 to A21 and B14 to B21; fragment III consists of amino acids B22 to B30; fragment IV consists of amino acids A1 to A4. A refers to the A-chain of human insulin and B refers to the B-chain of human insulin.

Bioidentity

Complies with the *Bioidentity* test from the monograph *Insulin*.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

INJECTABLE HUMAN INSULIN

Injectable human insulin is a sterile, isotonic human insulin solution. It contains not less than 95.0% and not more than 105.0% of the labeled amount, expressed in units of insulin in each mL.

IDENTIFICATION

The retention time of the main peak in the *Sample preparation* chromatogram, obtained in *Assay*, corresponds to the one from the main peak in the *Standard preparation* chromatogram.

***Note**: Applying the mixture from the Sample preparation with the identification Preparation may be required.*

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. Between 10 µg and 40 µg for each 100 IU of insulin for the appropriate species.

PURITY TESTS

Contamination by particles (5.1.7). Meets the requirements for small volume injections for both methods.

pH (5.2.19). 7.0 to 7.8. Determine potentiometrically.

Limit for high molecular mass proteins. Prepare the *Arginine solution*, *Mobile phase*, *System suitability solution* and the chromatographic system as described in *Limit of high molecular mass proteins* in the monograph *Insulin* and use the same *Procedure*. Prepare the *Test solution* as described below. Not more than 2%.

Test solution: quantitatively add 4 µL of hydrochloric acid 6 *M* per milliliter of one volume of injection accurately weighed and mix.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Contains not more than 80 EU/100 IU of insulin.

ASSAY

Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Prepare the *Mobile phase*, *Identification preparation*, *Standard preparation*, *System suitability solution* and chromatographic system as described in *Assay* in the monograph *Insulin*. Prepare the *Sample preparation* as described in *Assay* in the monograph *Injectable Insulin*.

Procedure: inject, separately, equal volumes (approximately 20 µL) of the *Sample preparation*, *Identification preparation* and *Standard preparation* in the chromatograph, record the chromatograms, and measure the areas under the insulin and A-21 desamido insulin peaks, using the chromatogram from the *Identification Preparation* to identify the insulin peaks. For insulin injection prepared from a single species, calculate the amount, in units of insulin per mL, of the injection taken, according to the formula:

$$C \times D \times \left(\frac{\sum rU}{\sum rS} \right)$$

where

C = concentration, in units of insulin per mL, of insulin CRS in the *Standard preparation*; D = dilution factor;

$\sum rU$ and $\sum rS$ = sum of areas under peaks of insulin and A-21 desamido insulin obtained, respectively, from chromatograms of the *Sample preparation* and *Standard preparation*.

PACKAGING AND STORAGE

In closed containers, protected from light and in a refrigerator. Avoid freezing.

LABELLING

Comply with current legislation.

HUMAN INSULIN ISOPHANE SUSPENSION

Human insulin isophane suspension is a sterile suspension of human insulin crystals and zinc combined with protamine sulfate in buffered water for injection, combined in such a way that the solid phase of the suspension is composed of human insulin crystals, protamine and zinc. Protamine sulfate is prepared from sperm or maturating testes from fishes belonging to the genus *Oncorhynchus* Suckley or Salmo Linné (Salmonidae family). It contains, calculated from the sum of insulin and desamido insulin components, not less than 95.0% and not more than 105.0% of the amount stated on the label, expressed in units of human insulin in each milliliter.

IDENTIFICATION

Centrifuge 10 mL of the suspension at 1500 g for 10 minutes. Determine the insulin content in the supernatant as described in *Assay* in the monograph *Insulin*. The insulin concentration is not more than 1.0 IU/mL.

CHARACTERISTICS

Proceed as described in *Characteristics* in the monograph *Insulin*.

PHYSICOCHEMICAL TESTS

Zinc content (5.3.3.4). Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. Between 21 µg and 40 µg for each 100 IU of human insulin.

Zinc in solution. Centrifuge a portion of suspension sufficient for the test and determine the zinc content in the clear supernatant. Between 20% and 65% of the total zinc content.

PURITY TESTS

pH (5.2.19). Between 7.0 and 7.5.

Limit for high molecular mass proteins. Proceed as described in *Limit for high molecular mass proteins* in the monograph *Injectable Insulin*. Not more than 3.0%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Meets the requirements, when tested as indicated for *Filtration by membranes* in *Sterility Test of the product to be examined*, and the suspension to be filtered immediately after being reduced to a clear solution by adding one fresh-prepared 1 in 100 ascorbic acid solution in *Fluid A*.

Bacterial endotoxins (5.5.2.2). Contains not more than 80 EU/100 IU of human insulin.

ASSAY

Proceed as described in *Assay* in the monograph *Insulin*.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

HUMAN INSULIN ISOPHANE SUSPENSION AND HUMAN INSULIN INJECTION

Human insulin isophane suspension and human insulin injection is a sterile buffered suspension of human insulin, complexed with protamine sulfate, in a human insulin solution.

IDENTIFICATION

The retention time of the main peak from the *Sample preparation* chromatogram, obtained in *Assay*, corresponds to the one from the main peak of the *Standard preparation*.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. Between 0.02 mg and 0.04 mg for each 100 IU of human insulin.

PURITY TESTS

pH (5.2.19). Between 7.0 and 7.8, determined potentiometrically.

Limit for high molecular mass proteins. Prepare the *Arginine solution*, *Mobile phase*, *System suitability solution* and chromatographic system as described in *Limit for high molecular mass proteins* in the monograph *Insulin* and use the same *Procedure*. Prepare the *Test solution* as described below. Not more than 3.0%.

Test solution: quantitatively add 4 µL of hydrochloric acid 6 *M* per milliliter of one volume of injection accurately measured and mix.

Soluble human insulin content.

Use one of the methods described below.

A. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Prepare the *Mobile phase*, *System suitability solution* and chromatographic system as described in *Assay* in the monograph *Insulin*. Prepare the solutions described below.

Soluble insulin test solution: maintain the temperature between 24°C and 26°C throughout the procedure. Transfer 5 mL of the human insulin injection to a centrifuge tube. Add 20 µL of sodium hydroxide *M* and adjust with hydrochloric acid 0.05 *M* or sodium hydroxide 0.05 *M* to a pH between 8.18 and 8.22 if the total zinc concentration is approximately 20 µg/mL, or adjust to a pH between 8.33 and 8.37 if the total zinc concentration is 30 µg/mL. Record the volume, in µL, of acid or base required to adjust the pH. Mix and allow to stand for one hour. Centrifuge, transfer the supernatant to another centrifuge tube, and repeat the centrifugation. Transfer 2 mL of the supernatant to another tube, add 5 µL of hydrochloric acid 9.6 *M* and mix.

Total insulin test solution: transfer 2 mL of human insulin injection to an appropriate vessel, add 5 µL of hydrochloric acid 9.6 *M* and allow the suspension to clarify. Dilute the resulting solution with hydrochloric acid 0.01 *M* to the same theoretical concentration of insulin from the *Soluble insulin test* *solution* (for example, if the injectable human insulin is labeled to contain 20% of soluble insulin, the dilution factor is 100/20 = 5).

Procedure: inject, separately, equal volumes (approximately 20 µL) of the *Soluble insulin test solution* and the *Total insulin test solution* in the chromatograph, record the chromatograms. Measure the peak responses for insulin and A-21 desamido insulin. Calculate the amount of soluble human insulin as a percentage of total insulin content from the human insulin injection according to the formula:

$$\left(\frac{100}{D}\right)\times\left[\frac{(5020+VA)}{5000}\right]\times\left(\frac{rS}{rT}\right)$$

where

D = dilution factor for the *Total insulin test solution*;
VA = number of µL added to adjust pH of the *Soluble insulin test solution*;
rS and rT = responses, respectively, from the *Soluble insulin test solution* and *Total insulin test solution*.

The percentage of soluble human insulin is in the range (L ± 5), where L is the percentage of soluble human insulin specified on the product label.

B. Prepare the *Mobile phase*, *System suitability solution* and chromatographic system as described in *Assay* from the monograph *Insulin*.

Prepare the solutions described below.

Tris 0.1 M Buffer: dissolve (3.54 ± 0.01) g of tromethamine hydrochloride and (3.34 ± 0.01) g of tromethamine in 500 mL of water. The pH of the *Tris 0.1 M Buffer* must be between 8.15 and 8.35.

Soluble insulin test solution: dilute an adequate volume of human insulin injection in *Tris 0.1 M Buffer* to obtain a solution with approximately 6 units of soluble human insulin per milliliters of insulin (for example, 2 mL of 70/30 human insulin isophane suspension and human insulin injection with 100 units of human insulin per milliliter would be diluted with 8 mL of *Tris 0.1 M Buffer* to obtain a filtrate that has 6 units of human insulin from soluble insulin per milliliter). Immerse the container in water bath at (25 ± 1)°C for (30 ± 2) minutes. Immediately, pass this solution through a filter with pore diameter of 0.2 µm using a disposable syringe. Transfer two parts of the filtrate to an adequate vessel, add one part of hydrochloric acid 0.2 *M* (for example, the dilution factor for the *Soluble insulin test solution* that has 30% of soluble insulin is 5 × 3/2 = 7.5).

Total insulin test solution: for each milliliter of human insulin injection, add 3 µL of hydrochloric acid 9.6 *M*, mix and allow the suspension to clarify. Dilute the resulting solution with hydrochloric acid 0.01 *M* to four units of human insulin per milliliter (for example, if the product is labeled to contain a total of 100 units of human insulin per milliliter, the dilution factor is 25). Calculate the amount of soluble human insulin as a percentage of total insulin content from the human insulin injection according to the formula:

$$\left(\frac{100DS}{DT}\right)\times\left(\frac{rS}{rT}\right)$$

where

DS and DT = dilution factors for *Soluble insulin test solution* and *Total insulin test solution*, respectively; rS and rT = peak responses obtained, respectively, from the *Soluble insulin test solution* and *Total insulin test solution*.

The percentage of soluble human insulin is in the range (L ± 5), where L is the percentage of soluble human insulin specified on the product label.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 80 EU/100 IU of human insulin.

ASSAY

The determination of quantity is based on the sum of the insulin itself and components of desamido insulin, as determined in *Assay* in the monograph *Human Insulin* injection. Not less than 95.0% and not more than 105.0% of the amount specified on the label, expressed in units of human insulin in each mL.

PACKAGING AND STORAGE

Preserve in the unopened multiple-dose container, provided by the manufacturer. Store in a refrigerator, protected from sunlight, and avoid freezing.

LABELLING

Comply with current legislation.

HUMAN INSULIN ZINC SUSPENSION

Human insulin zinc suspension is a sterile suspension of human insulin in buffered water for injection, modified by the addition of a suitable zinc salt so that the solid phase of the suspension is composed of a mixture of crystalline and amorphous insulin in a ratio of about seven parts of crystals and three parts of amorphous material. It contains, calculated from the sum of insulin and desamido insulin components not less than 95.0% and not more than105.0% of the amount stated on the label, expressed in unit of human insulin per milliliter.

IDENTIFICATION

A. The insulin in the supernatant complies with the *Identification* test from the monograph *Human Insulin isophane suspension*.

B. Centrifuge an amount of suspension representing 1000 units of insulin and discard the supernatant. Suspend the residue in 8.4 mL of water, quickly add 16.6 mL of buffered acetone RS, shake vigorously and centrifuge within three minutes after adding the buffered acetone. Discard the supernatant, repeat the treatment with water and buffered acetone RS, centrifuge and discard the supernatant. Dissolve the crystalline residue in 5 mL de of diluted hydrochloric acid (1 in 100). Insulin not extracted by buffered acetone RS, determined by an adequate method, is between 63% and 77% of the total content of insulin from an equal amount of suspension.

CHARACTERISTICS

pH (5.2.19). Between 7.0 and 7.8. Determine potentiometrically.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. The total amount of zinc is between 0.12 mg and 0.25 mg for each 100 IU of insulin.

Zinc in solution. Centrifuge a portion of suspension sufficient for the test and determine the zinc content in the clear supernatant. Between 20% and 65% of the total zinc content.

PURITY TEST

Limit for high molecular mass proteins. Proceed as described in *Limit for high molecular mass proteins* in the monograph *Insulin injection*. Not more than 1.5%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 80 EU/100 IU of human insulin.

ASSAY

Proceed as described in *Assay* in the monograph *Insulin*.

PACKAGING AND STORAGE

Preserve in the unopened multiple-dose container, provided by the manufacturer. Store in a refrigerator, protected from sunlight and avoid freezing.

LABELLING

Comply with current legislation.

EXTENDED HUMAN INSULIN ZINC SUSPENSION

Extended human insulin zinc suspension is a sterile suspension of human insulin in buffered water for injection, modified by the addition of a suitable zinc salt so that the solid phase of the suspension is predominantly crystalline. It contains, calculated from the sum of its insulin and desamido insulin components, not less than 95.0% and not more than 105.0% of the amount stated on the label, expressed in units of human insulin per milliliter.

IDENTIFICATION

A. The insulin in the supernatant complies with the *Identification* test from the monograph *Human Insulin isophane suspension*.

B. Proceed as described in test **B.** of *Identification* from the monograph *Human insulin zinc suspension*.

C. The concentration of insulin, determined by a method described in the monograph *Insulin*, is not less than 90% of the total insulin content.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. Between 0.12 mg and 0.25 mg for each 100 IU of insulin.

Zinc in solution. Centrifuge a portion of suspension sufficient for the test and determine the zinc content in the clear supernatant. Between 20% and 65% of the total zinc content.

PURITY TEST

pH (5.2.19). Between 7.0 and 7.8.

Limit for high molecular mass proteins. Proceed as described in *Limit for high molecular mass proteins* in the monograph *Insulin injection*. Not more than 1.5%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 80 EU/100 IU of human insulin.

ASSAY

Proceed as described in *Assay* in the monograph *Insulin injection*.

PACKAGING AND STORAGE

Comply with current legislation.

LABELLING

Comply with current legislation.

INSULIN INJECTION

Insulin injection is a sterile, isotonic insulin solution. It contains not less than 95.0% and not more than 105.0% of the amount stated on the label, expressed in units of insulin.

IDENTIFICATION

The retention time of the main peak from the *Sample preparation* chromatogram, obtained in *Assay*, corresponds to the one from the main peak of the *Identification preparation*

***Note**: Applying the mixture from the Sample preparation with the identification Preparation may be required.*

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)** or *Atomic absorption spectrometry* **(5.2.13.1)**. Between 10 µg and 40 µg for each 100 IU of insulin for the appropriate species.

PURITY TEST

Contamination by particles (5.1.7). Meets the requirements for small volume injections. Apply both methods.

pH (5.2.19). 7.0 to 7.8. Determine potentiometrically.

Limit for high molecular mass proteins. Prepare the *Arginine solution*, *Mobile phase*, *System suitability solution* and chromatographic system as described in *Limit for high molecular mass proteins* in the monograph *Insulin* and use the same *Procedure*. Prepare the *Test solution* as described below. Not more than 2%.

Test solution: quantitatively add 4 µL of hydrochloric acid 6 *M* per milliliter of one volume of injection accurately weighed and homogenize.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 80 EU/100 IU of insulin.

ASSAY

Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Prepare the *Mobile phase*, *Identification preparation*, *Standard preparation*, *System suitability solution* and chromatographic system as described in *Assay* from the monograph *Insulin*.

Note: *the Identification preparation, Standard preparation and Sample preparation can be stored at room temperature for up to 12 hours or in refrigerator for up to 48 hours.*

Sample preparation 1: (for injection labeled to contain 40 IU/mL) add 2.5 µL of hydrochloric acid 9.6 *M* per mL of one volume accurately weighed of the injection. Allow the suspension, if present, to clarify and mix.

Sample preparation 2: (for injection labeled to contain 100 IU/mL) add 2.5 µL of hydrochloric acid 9.6 *M* per mL of one volume accurately weighed of the injection. Allow the suspension, if present, to clarify and homogenize. Pipet 2 mL of this solution and transfer to a 5 mL volumetric flask, complete the volume with hydrochloric acid 0.01 *M* and homogenize.

Note: *pooling of different packages of units may be required to obtain a sufficient volume of the test material for the Sample preparation 2.*

Procedure: inject, separately, equal volumes (approximately 20 µL) of the *Sample preparation*, *Identification preparation* and *Standard preparation* in the chromatograph, record the chromatograms and measure areas under the peaks of insulin and A-21 desamido insulin, using the chromatogram from the *Identification preparation* to identify the insulin peaks. For injection of insulin prepared from a single species, calculate the amount in units of insulin per mg of the injection taken according to the formula:

$$C \times D \times \left(\frac{\sum rU}{\sum rS} \right)$$

where

C = concentration, in units of insulin per mL, of insulin CRS in the *Standard preparation*; D = dilution factor;

$\sum rU$ and $\sum rS$ = sum of areas under peaks of insulin and A-21 desamido insulin obtained, respectively, from chromatograms of the *Sample preparation* and *Standard preparation*.

Note: *for insulin derived from a mixture of bovine insulin and porcine insulin, calculate the total quantity as the sum of the quantities of insulin derived from bovines and swines, as indicated above.*

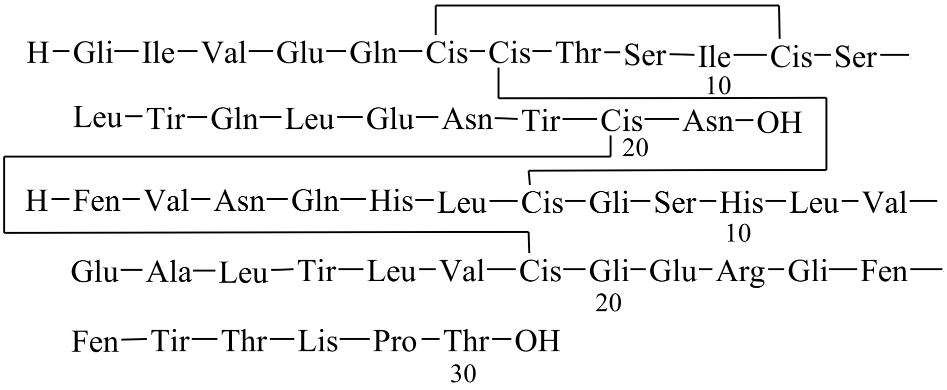
PACKAGING AND STORAGE

Preserve in the unopened multiple-dose container, provided by the manufacturer. Store in a refrigerator, protected from sunlight and avoid freezing.

LABELLING

Comply with current legislation.

INSULIN LISPRO



$C_{257}H_{383}N_{65}O_{77}S_6$; 5807.58
insulin lispro; 04920
28B-L-lysine-29B-L-proline
[*133107-64-9*]

Insulin lispro is identical in structure to human insulin, except for the fact it has lysine and proline at positions 28 and 29, respectively, of the B chain, whereas this sequence is inverted in human insulin. Insulin lispro is produced by microbial synthesis through a recombinant DNA process. The quantity, calculated on the dried basis, is of 27 units or more of insulin lispro per milligram. The proinsulin lispro content, determined by an adequate method, is not more than 10 ppm. The protein content derived from the host cell, determined by an adequate and validated method, is not more than 10 ppm.

Note: *one unit of insulin lispro is equivalent to 0.0347 mg of pure insulin lispro.*

IDENTIFICATION

A. The retention time of the main peak from the *Sample preparation* chromatogram, obtained in the *Assay* method, corresponds to the one from the main peak of the *Standard preparation.*

B. Determine the peptide fragments, using the following peptide mapping procedure. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Prepare the *Sulfate buffer*, *HEPES Buffer* and *Test digestion solution*, as described in test **B.** of *Identification* from the monograph *Human insulin*. Use the procedure described in the same monograph. Prepare the *Standard digestion solution* as described below.

Standard digestion solution: proceed as described in test **B.** of *Identification* from the monograph *Human insulin*, except for the use of insulin lispro CRS, in place of human insulin CRS.

Mobile Phase gradient: adopt the gradient system described in the following table. Prepare *Eluent A* and *Eluent B*, as described in test **B.** of *Identification* from the monograph *Human insulin*; *Mobile phase* flow of 0.8 mL/minute.

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0 – 3	95	5	isocratic

3 – 30	95 → 41	5 → 59	linear gradient
30 – 35	41 →20	59 → 80	linear gradient
35 – 40	20 → 95	80→ 5	return to initial
40 – 50	95	5	reequilibrium

CHARACTERISTICS

Loss on drying (5.2.9.1). Accurately weigh approximately 300 mg of the sample and dry at 105°C for 16 hours. Not more than 10.0%.

PHYSICOCHEMICAL TESTS

Zinc content. Proceed as described in *Complexometric titrations* **(5.3.3.4)**. Determine the zinc content in approximately 20 mg of the sample. Between 0.30% and 0.60%, in relation to the dried basis.

PURITY TESTS

Limit for high molecular mass proteins. Proceed as described in *Limit of high molecular mass proteins* in the monograph *Insulin*. Not more than 0.25%.

Related substances. Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 214 mm; column with 250 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 μm to 10 μm), stored at temperature of 40°C; *Mobile phase* flow of approximately 1.0 mL/minute.

Diluent: proceed as described in *Assay*.

Eluent A: prepare a filtered and degassed mix of *Diluent* and acetonitrile (82:18).

Eluent B: prepare a filtered and degassed mix of *Diluent* and acetonitrile (50:50).

Mobile Phase gradient: adopt the gradient system described in the following table.

Time (minutes)	***Eluent A (%)***	***Eluent B (%)***	***Elution***
0 - 3	95	5	isocratic
3 - 30	95 → 41	5 → 59	linear gradient
30 - 35	41 → 20	59 → 80	linear gradient
35 - 40	20 → 95	80 → 5	returns to initial state
40 - 50	95	5	reequilibrium

System suitability solution: dissolve an accurately weighed quantity of insulin lispro in hydrochloric acid 0.01 *M* to obtain a solution with approximately 3.5 mg per mL. Allow to stand at room temperature to obtain a solution with 0.8% to 11% of A-21 desamido insulin lispro.

Test solution: dissolve approximately 3.5 mg of insulin lispro in 1 mL of hydrochloric acid 0.01 *M*. Store the solution for not more than 56 hours in the refrigerator.

Procedure: proceed as described in *Procedure* from the *Related substances* test in the monograph *Insulin*. Not more than 1.0% of A-21 desamido insulin lispro; not more than 0.5% of any other substance related to insulin lispro; not more than 2.0% of total impurities, except A-21 desamido insulin lispro.

Adjust the *Mobile phase* composition and the duration of the isocratic elution to obtain a retention time of approximately 41 minutes for insulin lispro, with the A-21 desamido insulin lispro eluting just before the start of the linear gradient phase. Inject the *System suitability solution* and record the peak responses as indicated in *Procedure*. The resolution between insulin lispro and A-21 desamido insulin lispro is not less than 2.5; and the tailing factor for the insulin lispro peak is not more than 2.0.

BIOLOGICAL SAFETY TESTS

Count of the total amount of mesophilic microorganisms (5.5.3.1.2). Not more than 100 CFU/g, with the test being carried out in, accurately weighed, approximately 0.3 g of the sample.

Bacterial endotoxins (5.5.2.2). Not more than 10 EU/mg, use the chromogenic technique described in *Photometric techniques*.

ASSAY

Proceed as described in *High efficiency liquid chromatography* **(5.2.17.4)**. Use a chromatograph with ultraviolet detector at 214 mm; column with 100 mm of length and 4.6 mm of internal diameter, packed with silica chemically bonded to octadecylsilane group (1.5 µm to 10 µm), stored at temperature of 40°C; *Mobile phase* flow of approximately 0.8 mL/minute.

Diluent: dissolve 28.4 g of anhydrous sodium sulfate in 1000 mL of water, mix and adjust with phosphoric acid to a pH of 2.3.

Mobile phase: mix 745 mL of *Diluent* and 255 mL of acetonitrile. Make adjustments, if necessary, according to *System suitability* described in *High-efficiency liquid chromatography* **(5.2.17.4)**.

System suitability solution: dissolve a quantity, accurately weighed, of insulin lispro in hydrochloric acid 0.01 *M* to obtain a solution with a concentration of approximately 1.0 mg/mL. Allow to stand at room temperature to obtain a solution with 0.8% to 11% of A-21 desamido insulin lispro.

Standard preparation: dissolve a quantity, accurately weighed, of insulin lispro CRS in hydrochloric acid 0.01 *M* to obtain a solution with concentration of approximately 0.7 mg/mL.

Sample preparation: dissolve a portion, accurately weighed, of insulin lispro in hydrochloric acid 0.01 *M* to obtain a solution with concentration of approximately 0.8 mg/mL.

Procedure: inject, separately, equal volumes (approximately 20 µL) of the *Standard preparation* and *Sample preparation*, record the chromatograms, and measure the areas under the main peaks. Calculate the quantity, in the unit of insulin lispro per milligram, according to the formula:

$$\frac{\mathrm{CS}}{\mathrm{CU}} \times \left(\frac{\mathrm{rU}}{\mathrm{rS}} \right)$$

where

CS = concentration, in units of insulin lispro per mL, of insulin lispro CRS in the *Standard preparation*;
CU = concentration, in mg/mL, of insulin lispro in the *Sample preparation*;
rU and rS = areas under the peaks of insulin lispro obtained from the *Sample preparation* and the *Standard preparation*, respectively. From the value obtained on the *Loss on drying* test, calculate the amount in relation to the dried basis.

Adjust the composition of the *Mobile phase* to provide a retention time of approximately 24 minutes for the main peak of insulin lispro. Inject in triplicate the *System suitability solution* and record the peak responses as indicated in *Procedure*. The resolution between insulin lispro and A-21 desamido insulin lispro is not less than 3; the tailing factor for the insulin lispro peak is not more than 1.5; and the relative standard deviation to replicate the injections is not more than 1.1%.

Bioidentity

Proceed as described in the *Bioidentity* test in *Assay* from the monograph *Insulin*. Perform the blood collection as described below. Complies with the test.

Blood samples: (45 ± 5) minutes and two hours and (30 ± 5) minutes after the injection, collect from each rabbit an adequate sample of blood from a marginal vein of the ear. Blood can also be effectively collected from the central auricular artery.

PACKAGING AND STORAGE

In tightly closed containers, protected from light and in a refrigerator.

LABELLING

Comply with current legislation.

NADROPARIN CALCIUM
Nadroparinum calcicum

Nadroparin calcium is a preparation of low molecular mass heparin that contains calcium salt. This heparin, with low molecular mass, is obtained through depolymerization with nitrous acid from heparin of the porcine intestinal mucosa, followed by fractioning to selectively eliminate most of the chains with molecular mass below 2000 Da. Most chains have a 2-O-sulfo-α-L-idopyranose uronic acid in the non-reducing end and a structure of 6-O-sulfo-2,5-anhydro-D-mannitol in the reducing end of their chain.

Nadroparin calcium must be in compliance with the monograph *Low molecular mass heparin* with the modifications and additional requirements described below.

The relative mean molecular mass must vary from 3600 Da to 5000 Da, with a characteristic value of approximately 4300 Da. The sulfation degree must be of approximately two sulfates per disaccharide unit. The potency must be not less than 95 IU and not more than130 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity must be of 2.5 to 4.0.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Low molecular mass heparin*. Calculate the potency in relation to the anti-factor Xa activity per milligram and to the anti-factor IIa activity.

B. Proceed as described in test **B.** of *Identification* from the monograph *Low molecular mass heparin*. The spectrum obtained must be similar to the one from standard nadroparin.

C. Proceed as described in test **C.** of *Identification* from the monograph *Low molecular mass heparin*. The following requisites apply: the relative mean molecular mass must vary between 3600 Da and 5000 Da; the percentage of chains with molecular mass below 2000 Da must be not less than 15%; the percentage of chains with molecular mass between 2000 Da and 8000 Da varies between 75% and 95%; the percentage of chains with molecular mass between 2000 Da and 4000 Da varies between 35% and 55%.

PURITY TESTS

Ethyl alcohol. Not more than 1% (m/m). Proceed as described in *Gas chromatography in confined space (headspace)* **(5.2.17.5.1)**, using 2-propanol RS as internal standard. Use gas chromatograph with flame ionization detector, using nickel column with 1.5 m of length and 2 mm of internal diameter packed with copolymer ethylvinylbenzene and divinylbenzene with film thickness of 150 µm to 180 µm; column temperature of 150°C and injection door and detector temperature at 250°C; use helium or nitrogen gas as carrier gas; flow of 30 mL/minute.

Internal standard solution: dilute 1 mL of 2-propanol RS in 100 mL of purified water. Dilute 1 mL of this solution in 50 mL of purified water.

Reference solution: dilute 1 mL of ethyl alcohol RS in 100 mL of purified water. Dilute 0.5 mL of this solution in 20 mL of purified water.

Filling vials: put the solutions described below in four separate vials compatible with the injection system.

Blank vial: 1 mL of purified water.

Reference vial: 0.5 mL of *Reference solution* and 0.5 mL of *Internal standard solution*.

Test vial A: add to 10 mg of the sample 1 mL of purified water.

Test vial B: add to 10 mg of the sample 0.5 mL of purified water and 0.5 mL of the *Internal standard solution*.

Procedure: calibrate each vial in the headspace system at 90°C for 15 minutes. The pre-injection pressurization time is of one minute. The chromatogram obtained from the *Reference vial* presents two peaks that correspond to ethyl alcohol and to 2-propanol in the sense that the retention time increases (with retention times of approximately two and a half minutes and four minutes). Calculate the content of ethyl alcohol (m/m) taking into consideration its density at 20°C, which must be of 0.792 g/mL.

N-NO Groups. Not more than 0.25 ppm, determined by cleavage of the N-NO bond, with hydrobromic acid in ethyl acetate under a reflux condenser and posterior detection of NO (nitric oxide) released by chemiluminescence. Use the device illustrated on **Figure 1**.

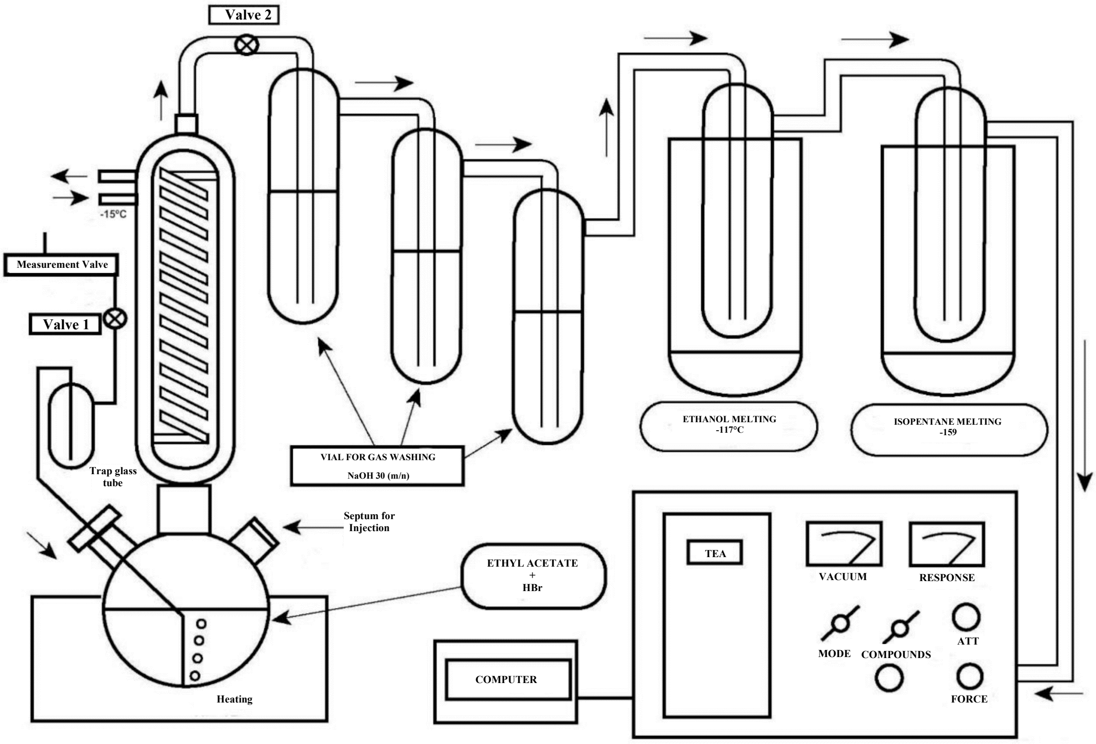


Figure 1 – Scheme of the device to be used in determining N-NO.

Device description (**Figure 1**). Use a 500 mL borosilicate glass vial, with round bottom and three joints, above which a condenser is connected, equipped with:

- on one side, a torion joint through which an argon stream can be introduced by a cannula;

- on the other side, a screw set with a piston equipped with a septum through which the *Reference solution* and *Sample solution* will be injected.

The round-bottomed flask is serially connected to three bubble traps that are connected to two cold traps, which in their turn are connected to a chemiluminescence detector. The adequate tubing ensures that the junctions are leak-proof.

Preparation of chemiluminescence detector: turn the chemiluminescence detector on 48 hours before using and turn the vacuum pump on. The vacuum must be below 0.5 mmHg. One hour before using, open the oxygen valve at a pressure of 0.2 MPa and a flow rate of 9.4 mL/minute.

Preparation of bubble trap: in each bubble trap, put 30 mL of a 300 g/L solution of sodium hydroxide RS in purified water.

- Trap at -120°C: slowly add liquid nitrogen in an isothermal flask with 250 mL of ethyl alcohol, using a wooden spatula to maintain agitation until a paste is obtained. Put the cold trap in the isothermal flask prepared as described.

- Trap at -160°C: slowly add liquid nitrogen to an isothermal flask with 250 mL of 2-methylbutane, using a wooden spatula to maintain agitation until a paste is obtained. Put the cold trap in the isothermal flask prepared as described.

Drying in 500 mL round bottom borosilicate glass flask and condenser: boil 50 mL of ethyl acetate under reflux for one hour under argon atmosphere without turning the chemiluminescence detector system on.

Sample solution: dry the substance to be examined for 12 hours in diphosphorus pentoxide at 60°C under vacuum. Dissolve 0.10 g of the treated substance so that it is examined in 1 mL of formamide. Shake the solution obtained for 30 minutes.

Reference solution: dilute 0.1 mL of nitrosodipropylamine solution in 6 mL of ethyl alcohol. Dilute 0.1 mL of the solution obtained in 1.0 mL of formamide (this solution is equivalent to 0.05 ppm of N-NO groups).

Introduce 50 mL of ethyl acetate in a dry 500 mL borosilicate glass flask with round bottom and equipped with a septum. Connect the round bottom flask to the condenser, previously chilled at -15°C for two hours.

Connect the argon cannula and adjust the flow rate to 0.1 liter/minute. Check if the system is leak-free. Only the connector for the chemiluminescence remains open to avoid excessive pressure. Heat ethyl acetate until ebullition.

Evacuate the system by slowly turning the chemiluminescence detector valve. At the same time, press the entrance of the chemiluminescence detector. When the system is calibrated, vacuum reaches 4 mmHg. The zero adjustment signal in the chemiluminescence detector is defined as 10% of the full recorder scale. Through the septum of the 500 mL round bottom borosilicate glass flask, inject sequentially 0.5 mL of purified water, 2 mL of diluted hydrobromic acid and, then, 2 mL of another sample of diluted hydrobromic acid, making sure that the recorder pen has returned to the base line between each injection. Inject 50 µL of the *Reference solution* and, then, 50 µL of the *Sample solution* after the recorder pen returns to the base line. Calculate the concentration of the N-NO groups from the substance to be examined.

Free sulfates. Not more than 0.5% (m/v). Proceed as described in *High-efficiency liquid chromatography* **(5.2.17.4)**, using a device equipped to a conductivity detector. Use a detector with sensitivity of 30 µS. Use an anion separation column with 50 mm of length and 4.6 mm of internal diameter; use as a chemical neutralization system a neutralization micro membrane according to the mobile phase for anion detection.

Sample solution: dissolve 30 mg of the sample in purified water and dilute to 10 mL using the same solvent.

Reference solution: dissolve 1.4787 g of anhydrous sodium sulfate in water and dilute to 1000 mL using the same solvent. Dilute 1 mL of this solution to 200 mL with distilled water (5 ppm of sulfate ions).

Procedure: elute with a solution of 1.91 g of disodium tetraborate in 1000 mL of purified water as mobile phase for 15 minutes, change to 100% of sodium hydroxide 0.1 *M* for half a minute; elute with this solution for 10 minutes; return to the initial conditions for a period of half a minute; the flow rate is 1 mL/minute.

Continuously pump the chemical neutralization system in counterflow with a solution of sulfuric acid at 2.45 g/L, at a flow rate of 4 mL/minute. Inject 50 µL of each solution. The chromatogram obtained with the reference solution presents a main peak that corresponds to the sulfate ion (retention time of approximately 7.5 minutes). Change the mobile phase composition, if necessary, to obtain the fixed retention time. Calculate the sulfate content in the substance to be examined.

ANTI-BOTHROPIC (PENTAVALENT) SERUM

Immunoserum bothropicum

Anti-bothropic (pentavalent) serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with antigen of the genus *Bothrops*, comprised of venoms from the serpents *Bothrops jararaca; Bothrops jararacussu; Bothrops moojeni; Bothrops alternatus* and *Bothrops neuwiedi*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains in each milliliter sufficient immunoglobulins to neutralize 5 mg of the reference venom from *B. jararaca*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from *B. jararaca*.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* in the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose **-** Effective Dose 50% (ED_{50}) to protect susceptible animals against the lethal effects from a fixed dose of the *Reference venom*.

Reference venom: homogeneous mixture of venoms that represent the geographic distribution of the species *B. jararaca*. It must be lyophilized and stored at -20°C. The venom is standardized by the determination of the Lethal Dose 50% (LD_{50}).

Determination of the venom's LD_{50}: reconstitute the lyophilized preparation of venom to a determined concentration of weight per volume, with saline solution 0.85% (w/v). Make dilutions in geometric progression with the same diluent, using constant dilution factor, not greater than 1.5 and equaling the final volumes. Inoculate, intraperitoneally, one volume of 0.5 mL per mouse of each dilution in groups of, at least, 10 Swiss albino mice of 18 g to 22 g. Observe the animals up to 48 hours after the inoculation and record the number of dead ones in each dilution. Calculate the LD_{50}

using an adequate statistical method. The response range (percentage of deaths) must be between the highest and the lowest dilution used, forming the regression curve that must present a linear relation. The confidence limits must not be wide, indicating a better accuracy of the assay the lower the limits are. Express the result in micrograms of venom per 0.5 mL.

Determination of the serum potency: make progressive dilutions of the sample in saline solution 0.85% (w/v), using constant dilution factor, not greater than 1.5, so that the final volume after the mixture with the venom challenge dose is identical in all test tubes. Reconstitute and dilute the *Reference venom* with saline solution 0.85% (w/v) and add to each tube a constant volume, so that each dose to be inoculated per animal contains 5 LD_{50}. homogenize and incubate the homogenizeture at 37ºC for 60 minutes. Inoculate, intraperitoneally, one volume of 0.5 mL per mouse of each mixture in groups of, at least, eight Swiss albino mice of 18 g to 22 g. Observe the animals up to 48 hours after the inoculation and record the number of live ones in each mixture of the sample being tested. Calculate the Effective Dose 50% (ED_{50}) in microliters, using an adequate statistical method. The response range produced (percentage of survival) must be between the highest and the lowest dilution used, forming the regression curve that must present a linear relation. The confidence limits must not be wide, indicating a better accuracy of the assay the lower the limits are. Calculate the potency in milligrams per milliliter of sample, according to the formula:

$$Potency\left(\frac{mg}{mL}\right) = \frac{\mathrm{Tv} - 1}{ED_{50}} \times \; LD_{50} \text{ of venom}$$

where

T_v = number of LD_{50} used per mouse in the venom test dose.

Not less than 5 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-BOTHROPIC (PENTAVALENT) AND ANTI-LAQUETIC SERUM

Immunoserum bothropicum-laqueticum

Anti-bothropic (pentavalent) and laquetic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venoms from *Bothrops jararaca; Bothrops jararacussu; Bothrops moojeni; Bothrops alternatus; Bothrops neuwiedi* and *Lachesis muta*. It meets the specifications and tests prescribed on the monograph *Hyperimmune sera for human use*. It contains in each milliliter sufficient immunoglobulins to neutralize 5 mg and 3 mg of the reference venoms from *B. jararaca* and *L. muta*, respectively.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigens venoms from *B. jararaca* and *L. muta*.

B. Complies with the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* in the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

Bothropic fraction

Determine the potency as described in the monograph *Anti-bothropic (pentavalent) serum*. Not less than 5 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

Laquetic fraction

The sample potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect susceptible animals against the lethal effects from a fixed dose of the *Reference venom*.

Reference venom: homogeneous mixture of venoms that represent the geographic distribution of the species *L. muta*. It must be lyophilized and stored at -20°C. The venom is standardized considering the determination of the Lethal Dose 50% (LD_{50}).

Determination of the venom's LD_{50}: proceed as described in *Determination of the venom's LD_{50}* in the monograph *Anti-bothropic (pentavalent) serum*.

Determination of the serum potency: proceed as described in *Determination of the serum potency* in the monograph *Anti-bothropic (pentavalent) serum*.

Not less than 3 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-BOTHROPIC (PENTAVALENT) AND ANTI-CROTALIC SERUM

Immunoserum bothropicum-crotalicum

anti-bothropic (pentavalent) and anti-crotalic serum; 09974

Anti-bothropic (pentavalent) and anti-crotalic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venoms from *Bothrops jararaca; Bothrops jararacussu; Bothrops moojeni; Bothrops alternatus; Bothrops neuwiedi* and *Crotalus durissus*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter, sufficient immunoglobulins to neutralize 5 mg and 1.5 mg of the reference venoms from *B. jararaca* and *C. durissus terrificus*, respectively.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigens venoms from *B. jararaca* and *C. durissus terrificus*.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* in the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

Bothropic fraction

Determine the potency, as described in *Assay* in the monograph *Anti-bothropic (pentavalent) serum*.

Crotalic fraction

Determine the potency, as described in *Assay* in the monograph *Anti-crotalic serum*.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-BOTHROPIC (PENTAVALENT), ANTI-CROTALIC AND ANTI-LAQUETIC SERUM

Immunoserum bothropicum-laqueticumcrotalicum

anti-bothropic (pentavalent), anti-crotalic and anti-laquetic serum; 09976

Anti-bothropic (pentavalent), anti-crotalic and anti-laquetic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venoms from *Bothrops jararaca; Bothrops jararacussu; Bothrops moojeni; Bothrops alternatus; Bothrops neuwiedi, Lachesis muta* and *Crotalus durissus*. It meets the specifications and controls prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter, sufficient immunoglobulins to neutralize not less than 5 mg, 3 mg and 1.5 mg of the reference venoms from *B. jararaca, L. muta* and *C. durissus terrificus*, respectively.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* in the monograph *Hyperimmune sera for human use*, using as antigens venoms from *B. jararaca, L. muta* and *C. durissus terrificus*.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Meets the *Characteristics* described on the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

It meets the *Physicochemical tests* described in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

It meets the *Biological safety tests* described in the monograph *Hyperimmune sera for human use*.

ASSAY

Bothropic fraction

Proceed as described in *Assay* in the monograph *Anti-bothropic (pentavalent) serum*.

Crotalic fraction

Proceed as described in *Assay* in the monograph *Anti-crotalic serum*.

Laquetic fraction

Proceed as described in *Assay* in the monograph *Anti-bothropic (pentavalent) and anti-laquetic serum*.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-BOTULINUM (TRIVALENT) SERUM

Immunoserum botulinicum

Anti-botulinum (trivalent) serum is a solution that contains purified immunoglobulins, obtained from plasma of animals hyper-immunized against type A, type B and type E toxins produced by *Clostridium botulinum*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter, a minimum of 375 IU, 275 IU and 425 IU of antitoxin for the types A, B and E, respectively.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigens the toxins type A, B and E produced by *C. botulinum*.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose to protect susceptible animals against the lethal effects from a test dose of each of the types of reference toxins. The serum dose being tested is compared with the dose of *Reference botulinum antitoxin* necessary to provide the same protection.

Reference botulinum antitoxins: international reference standards for antitoxins of types A, B or E are distributed to production and control laboratories in ampoules containing hyper-immune lyophilized equine serum, which specifically neutralizes the botulinum toxin of the type they refer to. The international standard equivalence in international units is established periodically by the World Health Organization.

Determination of the toxin test dose (L+/10): proceed as described in *Determination of the toxin test dose (L+/10)* from the monograph *Anti-tetanus serum* or extrapolating the values to *L+* or *L+/100*. The mixtures (toxin + antitoxin) are incubated at room temperature or between 20°C and 24ºC for 60 minutes.

Determination of the serum potency: dilute the reference toxin to a dose of L+/10, with phosphated gelatin solution (0.2% of gelatin dissolved in dibasic sodium phosphate buffer 0.05 *M*, pH 6.5 and autoclaved at 120ºC for 15 minutes). Distribute a constant volume of diluted botulinum toxin to a series of test tubes. Add variable volumes of the sample. Equalize the volumes to 5 mL with the same diluent. homogenize and incubate at room temperature or between 20°C and 24ºC for 60 minutes. Inoculate in each Swiss albino mouse or NIH of 18 g to 22 g, intraperitoneally, a volume of 0.5 mL in groups of at least eight mice per mixture. Observe the animals up to 96 hours after the inoculation and record the number of dead ones. In the same conditions described and in parallel, carry out the test with the *Reference botulinum antitoxin* to check the test validity and establish a correlation in the titer calculation. Determine the effective doses 50% (ED_{50}) of the sample and the *Reference botulinum antitoxin*, using an adequate statistical method. For the determination to be considered valid, it is necessary that: (a) the response range produced ($ED_{50)}$ is between the highest and the lowest dilution used in the test sample and standard, forming the regression curve that must present a linear relation; (b) the confidence limits (P = 0.95) must be between 50% and 200% of the potency calculated. Calculate the potency of the serum being tested, according to the formula:

$$Potency\ \left(\frac{IU}{mL}\right) = \frac{ED_{50}\ of\ the\ sample\ tested}{ED_{50}\ of\ the\ reference\ antitoxin} \times \frac{IU}{mL}\quad of\ the\ reference\ antitoxin$$

Not less than 375 IU/mL, 275 IU/mL and 425 IU/mL of antitoxin for the types A, B and E, respectively. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-CROTALIC SERUM

Immunoserum crotalicum

Anti-crotalic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venom from *Crotalus durissus*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter, sufficient immunoglobulins to neutralize 1.5 mg of the reference venom from *C. durissus terrificus*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from *C. durissus terrificus*.

B. Complies with the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect susceptible animals against the lethal effects from a fixed dose of the *Reference venom*.

Reference venom: homogeneous mixture of venoms that represent the geographic distribution of the species *C. durissus terrificus*. It must be lyophilized and stored at -20°C. The venom is standardized by the determination of the Lethal Dose 50% (LD_{50}).

Determination of the venom's LD_{50}: proceed as described in *Determination of the venom's* LD_{50} in the monograph *Anti-bothropic (pentavalent) serum*.

Determination of the serum potency: proceed as described in *Determination of the serum potency* in the monograph *Anti-bothropic (pentavalent) serum*.

Not less than 1.5 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-DIPHTHERIA SERUM

Immunoserum diphthericum

Anti-diphtheria serum is a solution that contains purified immunoglobulins, obtained from plasma of animals hyper-immunized against the toxin produced by *Corynebacterium diphtheriae*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter,not less than 1000 IU of antitoxin.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the toxin from *C. diphtheriae*.

B. Meets the requisites described in *Assay*.

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose to protect susceptible animals against the lethal effects from a test dose of *Reference diphtheria toxin*. The serum dose being tested is compared with the dose of *Reference diphtheria antitoxin* necessary to provide the same protection.

Reference diphtheria antitoxin: the international reference standard for diphtheria antitoxin is distributed to production and control laboratories in ampoules containing hyper-immune lyophilized equine serum, which specifically neutralizes the diphtheria toxin. The nominal titer of the secondary reference standard must be verified, periodically, in international units against the international reference standard.

Reference diphtheria toxin: it is prepared from sterile filtrates of supernatants from liquid cultures of *C. diphtheriae*. The filtrate must be concentrated, purified by physical or chemical methods and lyophilized. After reconstituting the toxin, add glycerol saline solution and store at -20ºC.

Determination of the toxin test dose (L+): dilute the *Reference diphtheria antitoxin* to 5 IU/mL, with saline solution at 0.85% (w/v). Dilute the *Reference diphtheria toxin* to a known concentration, with saline solution at 0.85% (w/v). In a series of test tubes, add variable volumes of toxin and constant volume of the diluted *Reference diphtheria antitoxin*. Equalize the volumes with saline solution at 0.85% (w/v). homogenize and incubate at 37ºC for 60 minutes. Inoculate in each guinea pig with 250 g to 350 g, subcutaneously, with a volume containing 1 IU of *Reference diphtheria antitoxin* in groups of, at least, four guinea pigs per mixture. Observe the animals up to 96 hours and record the number of dead ones in each dilution. The L+ (death limit) or toxin test dose is the lowest quantity of toxin that, when combined with 1 IU of *Reference diphtheria antitoxin*, causes the death of animals in the observation period stipulated.

Determination of the serum potency: dilute the *Reference diphtheria antitoxin* with physiological solution at 0.85% (w/v) for a dose of 10 L+. In a series of test tubes, distribute variable volumes of the sample. Add 1 mL of diluted *Reference diphtheria toxin*. Equalize the volumes to 10 mL with the same diluent. homogenize and incubate the homogenizetures at 37ºC for 60 minutes. Inoculate one dose of 2 mL in each of the guinea pigs with 250 g to 350 g, subcutaneously, in groups of at least four guinea pigs per mixture. Observe the animals up to 96 hours after the inoculation and record the number of dead ones. In the same conditions described and in parallel, carry out the test with the *Reference diphtheria antitoxin* to check the test validity and establish a correlation in the titer calculation. Determine the effective doses 50% (ED_{50}) of the sample and the *Reference diphtheria antitoxin*, using an adequate statistical method. For the determination to be considered valid, it is necessary that: (a) the response range produced (ED_{50}) is between the highest and the lowest dilution used in the test sample and standard, forming the regression curve that must present a linear relation; (b) the confidence limits (P = 0.95) must be between 50% and 200% of the potency calculated. Calculate the potency of the serum being tested, according to the formula:

$$Potency\ \left(\frac{IU}{mL}\right) = \frac{ED_{50}\ of\ the\ sample\ tested}{ED_{50}\ of\ the\ reference\ antitoxin} \times \frac{IU}{mL}\quad of\ the\ reference\ antitoxin$$

Not less than 1000 IU/mL. The manufacturer has the option of using the result obtained in the product before packaging.
Alternatively, an in vitro method, such as ELISA or VERO cell test, can be used, provided that they are validated against the serum neutralization test described.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-ELAPID (BIVALENT) SERUM

Immunoserum elapidicum

Anti-elapid (bivalent) serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venom from *Micrurus frontalis* and *Micrurus corallinus*. It meets the specifications and tests described in the monograph *Hyperimmune sera for human use*. It contains in each milliliter sufficient immunoglobulins to neutralize 1.5 mg of the reference venom from *M. frontalis*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from *M. frontalis*.

B. Complies with the requisites described in *Assay*.

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect susceptible animals against the lethal effects from a fixed dose of the *Reference venom*.

Reference venom: homogeneous mixture of venoms that represent the geographic distribution of the species *Micrurus frontalis*. It must be lyophilized and stored at -20°C. The venom is standardized by the determination of the Lethal Dose 50% (LD_{50}).

Determination of the venom's LD_{50}: proceed as described in *Determination of the venom's* LD_{50} in the monograph *Anti-bothropic (pentavalent) serum*.

Determination of the serum potency: proceed as described in *Determination of the serum potency* in the monograph *Anti-bothropic (pentavalent) serum*.

Not less than 1.5 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-SCORPION SERUM

Immunoserum escorpionicum

Anti-scorpion serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with venom from *Tityus serrulatus*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains in each milliliter sufficient immunoglobulins to neutralize 1.0 mg of the reference venom from *Tityus serrulatus*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from *T. serrulatus*.

B. Complies with the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect susceptible animals against the lethal effects from a fixed dose of the *Reference venom*.

Reference venom: homogeneous mixture of venoms that represent the geographic distribution of the species *Tityus serrulatus*. It must be lyophilized and stored at -20°C. The venom is standardized by the determination of the Lethal Dose 50% (LD_{50}).

Determination of the venom's LD_{50}: proceed as described in *Determination of the venom's* LD_{50} in the monograph *Anti-bothropic (pentavalent) serum*.

Determination of the serum potency: proceed as described in *Determination of the serum potency* in the monograph *Anti-bothropic (pentavalent) serum*.

Not less than 1.0 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTILONOMIC SERUM
Immunoserum lonomicum

Antilonomic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with extract from *Lonomia obliqua*. It meets the specifications and controls prescribed in the monograph *Hyperimmune sera for human use*. It contains in each milliliter sufficient immunoglobulins to neutralize 0.35 mg of the reference venom from *L. obliqua*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from the extract of *Lonomia obliqua* bristles.

B. Complies with the requisites described in *Assay*.

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect susceptible animals against the blood non-coagulability caused by a fixed dose of venom from *L. obliqua*.

Reference venom: venom extracted from *L. obliqua* by bristles maceration with buffered saline solution. After centrifugation of the extract, the supernatant with the venom is distributed to vials and must be kept at -20ºC. The venom is standardized by the determination of the Non-Coagulability Dose 50% (ND_{50}).

Determination of the venom's ND_{50}: make dilutions of the *Reference venom* with saline solution at 0.85% (w/v), using constant dilution factor of 1:1 to 1:5, and equalizing the final volumes with the same diluent. Inoculate, intraperitoneally, 0.5 mL per mouse of each dilution in groups of, at least, six male BALB/c mice of 18 g to 22 g. Observe the animals for two hours after the inoculation and collect, with the aid of a Pasteur pipette, approximately 300 µL of blood per puncture of the retro-orbital plexus. Transfer to a test tube and determine the coagulation time by visual observation. The maximum coagulation time is of two minutes. The blood samples that do not form clots within the time range established are considered non-coagulable. Record the number of animals with absence of blood coagulation and the total number of animals bled. Calculate the ND_{50} using an adequate statistical method. The response range (percentage of non-coagulable) must be between the highest and the lowest dilution used in the test sample, forming the regression curve where there must be a linear relation. The confidence limits must not be wide, indicating a better accuracy of the assay the lower the limits are. Express the result in micrograms of venom per 0.5 mL.

Determination of the serum potency: make progressive dilutions of the sample in saline solution at 0.85% (w/v), using constant dilution factor of 1:1 to 1:5, so that the final volume after the mixture with the challenge dose of 3 ND_{50} of the *Reference venom* is identical in all test tubes. homogenize and incubate the homogenizeture at 37ºC for 60 minutes. Inoculate, intraperitoneally, 0.5 mL per mouse of each mixture in groups of at least six male BALB/c mice of 18 g to 22 g. Observe the animals for up to two hours after the inoculation and, with the aid of a Pasteur pipette, collect approximately 300 µL of blood per puncture of the retro-orbital plexus. Transfer to a test tube and determine the coagulation time by visual observation. The blood samples that form clots within the range of up to two minutes are considered coagulable. Record the number of animals in which there is blood coagulation and the total number of animals bled. Calculate the Effective Dose 50% (ED_{50}) in microliters, using an adequate statistical method. The response range (percentage of coagulable) must be between the highest and the lowest dilution used in the test sample, forming the regression curve that must present a linear relation. The confidence limits must not be wide, indicating a better accuracy of the assay the lower the limits are. Calculate the potency in milligrams per milliliter, according to the formula:

$$Potency\left(\frac{mg}{mL}\right) = \frac{Tv - 1}{ED_{50}} \times \quad ND_{50}\ of\ venom$$

where

T_v = number of ND_{50} used per mice in the venom test dose.

The potency titer is expressed in milligrams of venom neutralized by mL of sample. Not less than 0.35 mg/mL. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-LOXOSCELIC (TRIVALENT) SERUM

Immunoserum loxoscelicum

anti-loxoscelic (trivalent) serum; 09983

Anti-loxoscelic serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized with antigen of the genus *Loxosceles,* comprised of venoms from *Loxosceles gaucho*, *Loxosceles intermedia* and *Loxosceles laeta* spiders. It meets the specifications and controls prescribed in the monograph *Hyperimmune sera for human use*. Each milliliter contains sufficient immunoglobulins to neutralize 15 minimum necrotizing doses (MND) of the reference venom from *L. intermedia*.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the venom from *L. intermedia*.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Meets the *Characteristics* described in the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

It meets the *Physicochemical tests* described in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

It meets the *Biological safety tests* described in the monograph *Hyperimmune sera for human use*.

ASSAY

The potency assay aims at determining the necessary neutralizing dose to protect susceptible animals against the dermonecrotic effects from a Minimum Necrotizing Dose (MND) of the *Reference venom*.

Reference venom: venom extracted from *L. intermedia*, which must be lyophilized or crystallized and kept at -20°C. The venom is standardized by determination of MND, which is the lowest quantity of venom capable of causing, in up to 72 hours, necrosis with approximately one centimeter of diameter, by intradermal injection in the inner part of a rabbit's ear.

Determination of the venom's MND: reconstitute the lyophilized or crystallized preparation of venom to a determined concentration (w/v) with saline solution at 0.85% (w/v). Make dilutions in geometric progression with the same diluent, starting with a dose of 3 mg of venom and using constant dilution factor, not greater than 1.5.

Inoculate, in two albino rabbits with 1.8 kg to 2.3 kg, intradermally, one volume of 0.1 mL from each dilution in the inner part of both ears from each rabbit. Observe the animals up to 72 hours after the inoculation, record the onset of skin necrosis and measure the lesions. The MND is calculated according to the formula:

$$MND = \frac{A + B}{2}$$

where

MND = minimum necrotizing dose (cm);
A = average between the maximum diameters in the four inoculated points; *B* = average between the minimum diameters in the four inoculated points.

The result is expressed by the lowest quantity, in mg, of venom capable of causing a dermonecrotic lesion of approximately 1 cm of diameter.

Determination of serum potency: make dilutions of the sample in saline solution at 0.85% (w/v), to determine the highest dilution that neutralizes 1 MND of the *Reference venom*, using a constant dilution factor, not greater than 1.5. Reconstitute and dilute the *Reference venom* with saline solution at 0.85% (w/v), so that each dose of 0.1 mL to be inoculated per animal contains 1 MND. Inject, intradermally, the dose of 0.1 mL of this dilution of *Reference venom* in the inner side of one of the ears from each of three rabbits. Then, administer 1 mL of diluted serum in the marginal vein of the ear opposed to the one where the venom was inoculated. In parallel, perform a venom control by inoculating 1 MND per year in, at least, one more rabbit. Observe the animals up to 72 hours after the inoculation as to the onset of skin necrosis. Record the highest serum dilution that does not cause necrosis. The potency titer is expressed in MND of venom neutralized per milliliter of serum.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-RABIES SERUM

anti-rabies serum; 09984

Anti-rabies serum is a solution that contains specific purified immunoglobulins, obtained from plasma of animals hyper-immunized against the rabies virus. Strains of fixed virus, inactivated or not, replicated in cultivation of cells different from the ones used in the preparation of the anti-rabies (inactivated) vaccine for human use, are used in the immunization of animals. It meets the specifications and controls prescribed in the monograph *Hyperimmune sera for human use*. It contains, in each milliliter, not less than 200 IU.

IDENTIFICATION

The *Assay* methods can be used.

CHARACTERISTICS

Proceed as described in *Characteristics* in the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

Use one of the methods described below.

A. Method of serum neutralization in mice.

The sample potency assay aims at determining the necessary neutralizing dose (Effective Dose 50%) to protect mice against the lethal effects from a challenge dose of rabies virus. For comparative assessment of the sample potency, reference lyophilized equine serum is used, checked in international units, by the international standard serum distributed by the World Health Organization.

Challenge virus: CVS (challenge virus standard) strain, of known Lethal Dose 50% (LD_{50}).

Determination of LD_{50}: make serial decimal dilutions of virus with a distilled water solution containing 2% (v/v) of normal serum of animal origin or 0.75% (w/v) of bovine albumin. homogenize and inoculate, intracerebrally, one volume of 30 mL of each dilution in groups of, at least, 10 Swiss albino mice of 10 g to 15 g. Observe the animals for 14 days. Calculate the LD_{50} by a statistically proven method, using the number in each group that dies or develops symptoms of rabies, between the 5th and the 14th days. The response range (percentage of deaths) produced must be between the highest and the lowest dilution used, forming the regression curve, which must present a linear relation.

Determination of the serum potency: make serial dilutions of the sample, with the same diluent described in the *Determination of* LD_{50}, using a constant dilution factor, not greater than 2, until the dilution where, supposedly, there is no neutralization. Transfer to test tubes a constant volume of each one of the four latest dilutions. Prepare dilution of the *Challenge virus* so that it contains initial 100 LD_{50} to 500 LD_{50}, using the same diluent. Add to each of the four tubes, already with the serum, the same volume of challenge dilution, so that doubled dilutions of virus are obtained, containing 50 LD_{50} to 250 LD_{50} of the sample being tested. homogenize the homogenizetures. Proceed identically for the reference serum. In parallel, to determine the real number of LD_{50} used as challenge, prepare four successive decimal dilutions with the same diluent, from the dilution used as challenge. Distribute a constant volume of diluent in each of the four test tubes and transfer to them, starting with the challenge dilution, the same volume of each of the serial virus dilutions. homogenize, obtaining doubled dilutions of the challenge virus. Incubate the mixtures of sera plus virus and virus plus diluent in water bath at (37 ± 0.5)°C, for 90 minutes. Inoculate, intracerebrally, one volume of 30 mL of each mix, in groups of, at least, eight Swiss albino mice of 10 g to 15 g. Observe the animals from each group for 14 days and record the number of animals who died or presented symptoms of rabies, in the period of 5 to 14 days after the challenge.

Calculate the Effective Doses 50% (ED_{50}) of the sample and reference serum, as well as the LD_{50} of the challenge virus, by a statistically validated method. The response range produced (percentage of survival) must be between the highest and the lowest dilution used in the test and standard sample, forming the regression curve, which must present a linear relation. The potency is determined according to the formula:

$$Potency\left|\frac{UI}{mL}\right| = \frac{ED_{50}\ of\ the\ sample\ being\ tested \times conc.\ in\ \frac{UI}{mL} of\ the\ reference\ serum}{ED_{50}\ of\ the\ reference\ serum}$$

The estimated potency must be not less than 200 IU/mL and the confidence limits must not be below 25% or above 400% of the activity determined.

B. Method of serum neutralization of rabies virus in BHK_{21} cells.

Pre-dilute the reference sera and the test sample to the approximate concentration of 1 IU/mL and make serial dilutions in the ratio 2, using Eagle-MEM medium with 2.5% of fetal bovine serum. Put 50 µL of each of these dilutions in a 96-well polystyrene microplate and add the same volume of one dilution of fixed virus CVS-11 in BHK_{21} cells, to obtain 30 to 300 fluorescent focus forming doses 50% (FFD_{50}) after the mixture with the sera. On the same plate, titer the CVS-11 virus with four serial dilutions on base 10, with the first being equal to the dilution added to test and reference sera. Incubate the microplate with the serum and virus mixtures in oven with 5% of CO_2 at 37°C for 90 minutes. Then, add to each well 100 µL of a suspension containing 3.7 x 104 BHK_{21} cells in Eagle MEM medium with 2.5% of fetal bovine serum. In two wells, put only the culture medium and the cells for their control. Incubate the microplate again at 37°C in oven with 5% of CO_2 for 22 hours. Wash the cells with saline solution buffered with phosphates pH 8.0 and fix them with 80% acetone chilled at -20°C for 15 minutes. Add an anti-rabies nucleocapsid immunoglobulin conjugated with fluorescein isothiocyanate and keep at 37°C for 30 minutes. Wash the plate twice in saline solution buffered with phosphate pH 8.0. Observe eight fields in each microplate well in a inverted fluorescence microscope with 200 times magnification. Consider the field with one or more fluorescent foci as positive.

Calculate the Effective Doses 50% (ED_{50}) of the sample and reference serum, as well as the LD_{50} of the challenge virus, by a statistically validated method. The response range produced (percentage of fluorescent foci) must be between the highest and the lowest dilution used in the test sample and standard, forming the regression curve, which must present a linear relation, and the statistical analysis must demonstrate a significant slope of the dose/response lines with no significant deviations from linearity and parallelism. The potency is determined according to the formula:

$$Potency\left|\frac{UI}{mL}\right| = \frac{ED_{50}\ of\ the\ sample\ being\ tested \times conc.\ in\ \frac{UI}{mL} of\ the\ reference\ serum}{ED_{50}\ of\ the\ reference\ serum}$$

The estimated potency must be not less than 200 IU/mL and the confidence limits must not be below 80% or above 125% of the activity determined.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-TETANUS SERUM

Immuno serum tetanicum ad usum humanum

Anti-tetanus serum is a solution that contains purified immunoglobulins, obtained from plasma of animals hyper-immunized against the toxin produced by *Clostridium tetani*. It meets the specifications and tests prescribed in the monograph *Hyperimmune sera for human use*. It contains in each milliliter not less than 1000 IU of antitoxin.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Hyperimmune sera for human use*, using as antigen the toxin from *C. tetani*.

B. Complies with the requisites described in *Assay.*

CHARACTERISTICS

Proceed as described in *Characteristics* from the monograph *Hyperimmune sera for human use*.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monograph *Hyperimmune sera for human use*.

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monograph *Hyperimmune sera for human use*.

ASSAY

The sample potency assay aims at determining the necessary neutralizing dose to protect susceptible animals against the lethal effects from a test dose of *Reference tetanus toxin*. The serum dose being tested is compared with the dose of *Reference tetanus antitoxin* necessary to provide the same protection.

Reference tetanus antitoxin: the reference standard of tetanus antitoxin is a preparation of lyophilized hyper-immune equine serum, which specifically neutralizes the tetanus toxin. The nominal titer of the secondary reference standard must be verified, periodically, in international units against the international reference standard.

Reference tetanus toxin: it is prepared from sterile filtrates of supernatants from liquid cultures of *C. tetani* incubated for five to seven days. The filtrate must be concentrated, purified by physical or chemical methods and lyophilized. After reconstitution, add glycerol saline solution and store at -20ºC.

Determination of the test toxin dose (Lp/10): dilute the *Reference tetanus antitoxin* to 1 IU/mL, with saline solution at 0.85% (w/v). Dilute the toxin to a determined concentration, in saline solution with peptone at 1% (w/v). In a series of test tubes, add variable volumes of toxin and constant volume of the diluted reference antitoxin. Equalize the volumes with the same toxin diluent. homogenize and incubate at 37ºC for 60 minutes. Inoculate in each Swiss albino mouse with 17 g to 22 g, subcutaneously, with a volume containing 0.1 IU of *Reference tetanus antitoxin* in groups of, at least, 10 mice per mixture. Observe the animals up to 96 hours after the inoculation and record the number of paralyzed animals per mixture. The *Pl/10* (paralyzing limit per 10) or test dose of toxin is the lowest amount of toxin that, when combined with 0.1 IU of *Reference tetanus antitoxin*, causes paralysis in animals in the observation period stipulated.

Determination of the serum potency: dilute the *Reference tetanus antitoxin* with buffered saline solution with peptone at 1% (w/v) for a dose of 10 *Pl/10*. To a series of test tubes, distribute variable volumes of the sample. Add 1 mL of the diluted *Reference tetanus toxin* and equalize the volumes to 2 mL with the same diluent. homogenize and incubate the homogenizetures at 37ºC for 60 minutes. Inoculate in each Swiss albino mouse of 17 g to 22 g, subcutaneously, a volume of 0.2 mL in groups of at least 10 mice per mixture. Observe the animals up to 96 hours after the inoculation and record the number of paralyzed animals. In the same conditions described and in parallel, carry out the test with the *Reference tetanus antitoxin* to check the validity of the test and establish a correlation in the titer calculation. Determine the average effective doses (ED_{50}) of the sample and the *Reference tetanus antitoxin*, using an adequate statistical method. For the determination to be considered valid, it is necessary that: (a) the response range produced (ED_{50}) is between the highest and the lowest dilution used in the test sample and standard, forming the regression curve that must present a linear relation; (b) the confidence limits (P = 0.95) must be between 50% and 200% of the potency calculated. Calculate the potency of the serum being tested, according to the formula:

$$\text{Potency } \left(\frac{IU}{mL}\right) = \frac{ED_{50} \text{ of the sample tested}}{ED_{50} \text{ of the reference antitoxin}} \times \frac{IU}{mL} \quad \text{of the reference antitoxin}$$

Not less than 1000 IU/mL. The manufacturer has the option of using the result obtained in the product before packaging.

Alternatively, an in vitro method, such as ELISA or ToBI (toxin-binding inhibition test), can be used, provided that they are validated against the serum neutralization test described.

PACKAGING AND STORAGE

Comply with the monograph *Hyperimmune sera for human use*.

LABELLING

Comply with current legislation.

ANTI-A BLOOD TYPING REAGENT SERUM FOR HUMAN USE

Immunoserum adversus A

DEFINITION

These are liquid, sterile preparations, with artificial blue color, clear, without the presence of particles in suspension, containing specific agglutinins for the blood type "A" or "A,B" (including the subtypes A1, A2, A1B and A2B), obtained from human plasma or serum or from cell culture (hybridoma). They must contain an antimicrobial preservative. When presented in lyophilized form, after reconstitution, they must have the same physical characteristics described for liquid preparations. They must not present contamination by other antibodies reactive against other human erythrocyte antigens, such as M^g, Wr^a , among others whose frequency is greater than 1% of the population. When obtained from human plasma fractionation, it must present high titers for this antibody. They must be tested with erythrocytes from types "O" and "B" proving their non-specificity for these blood types. Carry out a potency test comparing the reagent with a known anti-A reference serum, presenting an equivalent or superior result when tested in a same sample of type A red blood cells suspension.

CHARACTERISTICS

Aspect. Inspect visually during storage and immediately before use. If the color or physical appearance is abnormal or if there is any indication or suspicion of microbial contamination, the unit is unfit for use.

ASSAY

Laboratory tests. Duly validated laboratory tests are carried out to ensure the quality parameters detailed on **Table 1**.

Table 1 – Quality parameters for reagent serum of anti-A blood typing for human use.

Red blood cell panel	Agglutination intensity (minimum)	Avidity test	Titer
A_1	3+	Up to 15 seconds	256
A_2	2+	Up to 30 seconds	128
A_1B	3+	Up to 30 seconds	128
A_2B	2+	Up to 45 seconds	64

PACKAGING

Vial containing 10 mL of the anti-A blood typing reagent serum for human use, with dropper. Each drop is equivalent to approximately 50 µL.

LABELLING

Comply with current legislation. The package label must have white background and blue letters. The package insert must indicate that the serum was tested for hepatitis B, hepatitis C and for HIV viruses, however, no known methodology offers security for human blood derivative products and the biosafety precautions must be respected. When obtained from human plasma/serum, it must meet the requirements of the monograph *Human plasma for fractionation*. Additionally, the label or package insert must include, as a minimum, the following recommendations:

- blood typing must be conducted in a tube or a U-shaped plate;

- the validity period depends on the serum presentation form: liquid form - up to one year if kept between 2ºC and 6ºC and up to two years if kept at ≤ -20ºC; lyophilized form - up to five years and, after reconstituted, up to one year if kept between 2ºC and 6ºC.

ANTI-A,B BLOOD TYPING REAGENT SERUM FOR HUMAN USE

Immunoserum adversus A,B

DEFINITION

These are liquid, sterile, colorless, clear preparations, without the presence of particles in suspension, capable of agglutinating human erythrocyte from the blood type "A" or "A,B" (including the subtypes "A_1B" and "A_2B"), obtained from human plasma or serum or from cell culture (hybridoma). They must contain an antimicrobial preservative. When presented in lyophilized form, after their reconstitution, they must have the same physical characteristics described for liquid preparations. They must not present contamination by other antibodies reactive against other human erythrocyte antigens, such as M^g, Wr^a , among others whose frequency is greater than 1% of the population. When obtained from human plasma fractionation, it must present high titers for this antibody. They must be tested with erythrocytes from type "O" proving its non-specificity for this blood type. Carry out a potency test comparing the reagent with a known anti-A,B reference serum, presenting an equivalent or superior result when tested in a same sample of red cells suspension of type A,B blood group.

CHARACTERISTICS

Aspect. Inspect visually during storage and immediately before use. If the color or physical appearance is abnormal or if there is any indication or suspicion of microbial contamination, the unit is unfit for use.

ASSAY

Laboratory tests. Duly validated laboratory tests are carried out to ensure the quality parameters detailed on **Table 1**.

Table 1 – **Quality parameters for reagent serum of anti-A,B blood typing for human use**

Red blood cell panel	Agglutination intensity (minimum)	Avidity test	Titer
A_1	3+	Up to 15 seconds	256
A_1B	3+	Up to 15 seconds	256
B	3+	Up to 15 seconds	256
A_2	3+	Up to 30 seconds	128

PACKAGING

Vial containing 10 mL of the anti-A,B blood typing reagent serum for human use, with dropper. Each drop is equivalent to approximately 50 µL.

LABELLING

Comply with current legislation. The package label must have white background and orange letters. The package insert must indicate that the serum was tested for hepatitis B, hepatitis C and for HIV viruses, however, no known method offers security for human blood derivative products and the biosafety precautions must be respected. When obtained from human plasma/serum, it must meet the requirements of the monograph *Human plasma for fractionation*. Additionally, the label or package insert must include, as a minimum, the following recommendations:

- blood typing must be conducted in a tube or a U-shaped plate;
- the validity period depends on the serum presentation form: liquid form - up to one year if kept between 2ºC and 6ºC and up to two years if kept at ≤ -20ºC; lyophilized form - up to five years and, after reconstituted, up to one year if kept between 2ºC and 6ºC.

ANTI-B BLOOD TYPING REAGENT SERUM FOR HUMAN USE

Immunoserum adversus B

DEFINITION

These are liquid, sterile preparations, of artificial yellow color, clear, without the presence of particles in suspension, capable of agglutinating human erythrocyte from the blood type “B” or “A,B” (including the subtypes “A1B” and “A2B”), obtained from human plasma or serum or from cell culture (hybridoma). They must contain an antimicrobial preservative. When presented in lyophilized form, after reconstitution, they must have the same physical characteristics described for liquid preparations. They must not present contamination by other antibodies reactive against other human erythrocyte antigens, such as M^g, Wr^a , among others whose frequency is greater than 1% of the population. When obtained from human plasma fractionation, it must present high titers for this antibody. They must be tested with erythrocytes from types “A” and “O” proving their non-specificity for these blood types. Carry out a potency test comparing the reagent with a known anti-B reference serum, presenting an equivalent or superior result when tested in a same sample of type B red blood cells suspension.

CHARACTERISTICS

Aspect. Inspect visually during storage and immediately before use. If the color or physical appearance is abnormal or if there is any indication or suspicion of microbial contamination, the unit is unfit for use.

ASSAY

Laboratory tests. Duly validated laboratory tests are carried out to ensure the quality parameters detailed on **Table 1**.

Table 1 – **Quality parameters for reagent serum of anti-B blood typing for human use**

Red blood cell panel	Agglutination intensity (minimum)	Avidity test	Titer
B	3+	Up to 15 seconds	256
A_1B	3+	Up to 15 seconds	256

PACKAGING

Vial containing 10 mL of the anti-B blood typing reagent serum for human use, with dropper. Each drop is equivalent to approximately 50 µL.

LABELLING

Comply with current legislation. The package label must have white background and yellow letters. The package insert must indicate that the serum was tested for hepatitis B, hepatitis C and for HIV viruses, however, no known method offers security for human blood derivative products and the biosafety precautions must be respected. When obtained from human plasma/serum, it must meet the requirements of the monograph *Human plasma for fractionation*. Additionally, the label or package insert must include, as a minimum, the following recommendations:

- blood typing must be conducted in a tube or a U-shaped plate;

- the validity period depends on the serum presentation form: liquid form - up to one year if kept between 2ºC and 6ºC and up to two years if kept at ≤ -20ºC; lyophilized form - up to five years and, after reconstituted, up to one year if kept between 2ºC and 6ºC.

ANTI-RH (ANTI-D, ANTI-C, ANTI-E, ANTI-c, ANTI-e AND ANTI-C^W) BLOOD TYPING REAGENT SERUM FOR HUMAN USE

Immunoserum adversus Rh

DEFINITION

These are liquid, sterile, colorless, clear preparations, with no presence of particles in suspension, agglutinins for ABO system antigens and alloantibodies, obtained from human plasma or serum or from cell culture (hybridoma). They must contain an antimicrobial preservative. When presented in lyophilized form, after their reconstitution, they must have the same physical characteristics observed in the liquid preparations. No artificial coloring substances should be added. They can be presented as IgM immunoglobulin in saline solution with low protein concentration, as IgG in solution with high protein concentration, as mixtures of immunoglobulins IgG and IgM, or also chemically modified IgG.

CHARACTERISTICS

Aspect. Inspect visually during storage and immediately before use. If the color or physical appearance is abnormal or if there is any indication or suspicion of microbial contamination, the unit is unfit for use.

ASSAY

Laboratory tests. Duly validated laboratory tests are carried out to ensure the quality parameters listed on **Table 1** and on **Table 2**.

The main sera for Rh system typing are listed on **Table 1**, each one reacting with the antigen(s) designated by the corresponding letter (Fisher-Race terminology) and the

Table 1 – **Terminology according to Fisher-Race and Weiner.**

Antiserum	Antigen	
	Fisher-Race	Weiner
Anti-D	D	Rh_o
Anti-C	C	rh´
Anti-E	E	rh´´
Anti-CD	D and C	Rh_o and rh´
Anti-DE	D and E	Rh_o and rh´´
Anti-CDE	D, C and E	Rh_o, rh´ and rh´´
Anti-c	c	hr´
Anti-e	e	hr´´
Anti-C^w	C^w	rh’w

Each serum must meet the requirements from the potency test. In the case of saline sera, for test on test tubes, they are conducted in parallel with the anti-D, anti-C or anti-E reference standard serum, and the results cannot be below standard. There is no reference standard for anti-c and anti-e in saline medium, and the reactivity test must use red blood cell suspension of 3% to 5% with phenotypes according to **Table 2**.

In the case of sera, for use in plate or tube for quick testing, they are conducted in parallel with anti-D, anti-C, anti-E, anti-c or anti-e reference standard serum, with results not allowed to be below standard, and the reactivity test must use red blood cell suspension of 3% to 5% with phenotypes according to **Table 2**.

The avidity test for reagent sera, for use in plate and tube, meet the same requirements from the potency test described in the monographs *Anti-A blood typing reagent serum for human use, Anti-B blood typing reagent serum for human use* and *Anti-A,B blood typing reagent serum for human use*.

Type A, B, AB or O red blood cells can be used.

Table 2 - Relation between antibodies and cell phenotypes.

Serum	Cell phenotype
Anti-D	cDe
Anti-C	Ccde
Anti-E	cdEe
Anti-CD	cDe, Ccde
Anti-DE	cDe, cdEe
Anti-CDE	cdEe, cDe, Ccde
Anti-c	CcDEe
Anti-e	cdEe
Anti-C^w	rh_{w1}

The anti-D (anti-Rh(D) or anti-Rho) serum must present reactivity of not less than 3+, with red blood cells "O" R0r, "O" R1r and "O" R2r; avidity of up to 30 seconds and a titer of not more than 32. The anti-D serum in saline medium must present reactivity of not less than 1+, with red blood cells "O" R0r, "O" R1r and "O" R2r; and a titer of not more than 8.

The anti-D serum cannot react with Rh negative red blood cells (rr), at room temperature and at 37°C or in the human antiglobulin indirect test (indirect Coombs test), without booster. The serum must detect a weak D. The control serum must have in the same concentrations the substances used, including proteins and preservatives, and must not present reaction from agglutination with the "O" R0r, R1r, R2r red blood cells.

Each serum must meet the requirements from specificity assays for the most sensitive method, recommended by the manufacturer, where they must be tested with not less than four positive phenotypes and four negative phenotypes according to **Table 3**. The absence of contamination of antibodies reactive for M^g and Wr^a antigens, as well as other antigens that have incidence of 1% or more in the general population, must be demonstrated.

Table 3 – List of phenotypes to be tested.

Serum	Cells
Anti-D	CcDe, cDe, Ccde, cdEe, A_1cde, B cde, O cde, the test must be conducted by the indirect antiglobulin technique, using cells from three different donors
Anti-C	cDe, Ccde, cdEe, C + rh_i neg. cells, A_1cde, B cde, O cde
Anti-E	cDe, Ccde, cdEe, A_1 cde, B cde, O cde
Anti-CD	cDe, Ccde, cdEe, A_1cde, B cde, O cde, and where recommended for detection of G, $r^G r$ antigen
Anti-DE	cDe, Ccde, cdEe, A_1 cde, B cde, O cde
Anti-CDE	cDe, Ccde, cdEe, A_1 cde, B cde, O cde, and where recommended for detection of G, $r^G r$ antigen
Anti-c	Ccde, A_1 CDe, B CDe, O CDe, and CDEe or CDE or CdE

Anti-e	cdEe, A_1 cDE, B cDE, O cDE, and CcDE or CDE or CdE
Anti-C^w	rh^{w1}, A_1 rh^{w1}, B rh^{w1} and O CDe, and CDEe

All red blood cells in suspension used for these procedures must be validated and their records stored.

The antigens with highest incidence among the Brazilian population are: A,B ,H, Le^a, Le^b, I, K, k, Kp^a, Kp^B, Ic^C, P1, D, C ,E , c, e, C^w, M, N, S, s, U, Lu^a, Lu^b, Jk^A, Jk^b, Fy^a, Fy^b, Di^a and Di^B.

Ga, Do^a, Do^b, Yt^a, Yt^B, Lan, Co^a, Co^b, M^g, Wr^a and Sd^a are also known.

PACKAGING

Vial containing 10 mL of the specific blood typing reagent serum for human use, with dropper. Each drop is equivalent to approximately 50 µL.

LABELLING

Comply with current legislation. The label or package insert must include, as a minimum, the following recommendations:

- blood typing must be conducted in a tube or a U-shaped plate;
- the validity period depends on the form of presentation of the serum: liquid form - up to one year if kept between 2ºC and 6ºC and up to two years if kept at ≤ -20ºC; lyophilized form - up to five years and, after reconstituted, up to one year if kept between 2ºC and 6ºC.

HYPERIMMUNE SERA FOR HUMAN USE
Immunosera adusum humanum

Hyperimmune sera are preparations containing purified immunoglobulins of animal origin that specifically neutralize bacterial toxins, bacteria, viruses or toxic components of the venom of one or more species of venomous animals. An adequate preservative can be added and the final product is presented in liquid or lyophilized form. The liquid product is clear, colorless or slightly yellowish, not presenting lumps or particles. The lyophilized serum consists of a white or slightly yellowish powder that, once reconstituted, presents the same characteristics of liquid preparations.

Purified immunoglobulin are obtained by enzyme treatment and fractioned precipitation, or by other chemical or physical procedures, from plasma of healthy animals immunized with the specific antigens. During the immunization process, the animals must not be treated with penicillin or streptomycin.

To ensure the product quality in the different processing steps, sterility, pH, protein, activity or potency tests must be conducted, through *in vitro* or *in vivo* methods.

Before packaging, product samples are submitted to the following determinations.

Sodium chloride. 0.70% (w/v) to 0.90% (w/v).

Phenol. Not more than 0.35% (w/v).

Nitrogen and proteins. Not more than 0.30% (w/v) of non-protein nitrogen. Not more than 15% (w/v) of proteins.

Potency. It is determined according to procedures indicated in the respective monographs.

Total solids. Not more than 20%.

Ammonium sulfate. Not more than 0.20% (w/v). The preparation is distributed aseptically in ampoules or ampoule-vials. The product lyophilization, when required, must ensure water concentration not greater than 3% of the final product.

IDENTIFICATION

A. Based on the *in vitro* reaction of antigen-antibody by *Double radial immunodiffusion* (*Ouchterlony*). Prepare agar gel at 1% (w/v) and distribute on microscope slide, so that it results in a thin layer. Put in oven at 37°C, without drying. Add 4 mL of agar to the slide and put at temperature of 2°C to 8°C in a humid chamber for one hour. Punch the gel, keeping the same distance between the central and the peripheral holes. Fill the central hole with the specific antigen solution and the peripheral holes with the sample to be tested, in variable dilutions. Fill one of the holes with regular equine serum, for negative control. Incubate at 37°C for 24 hours in humid chamber and carry out reading in contrast light. Observe the presence of precipitation line, identity reaction among the components analyzed.

B. Meets the requisites described in *Assay.*

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

pH (5.2.19). 6.0 to 7.0.

PHYSICOCHEMICAL TESTS

Sodium chloride. In a 50 mL Erlenmeyer flask, add 10 mL of the sample diluted at 10% (v/v) in double-distilled water. Add, with agitation, three drops of diphenylcarbazone-bromophenol blue RS and, later, a few drops of nitric acid 0.20 *M* VS, until the solution turns greenish yellow. Conduct a blank test. Titrate with mercury (II) nitrate 0.01 *M* VS, until changeover, where a violet coloration indicates the endpoint. Each mL of mercury (II) nitrate 0.01 *M* VS is equivalent to 0.585 mg of sodium chloride. The manufacturer has the option of using the result obtained in the product before packaging. Between 0.70% (w/v) and 0.90% (w/v).

Phenol. Not more than 0.35% (w/v).

Use one of the methods described below.

A. Dilute the sample, so that the concentration of phenol is between 5 ppm and 30 ppm. Add 5 mL of borate buffer pH 9.0, 5 mL of 4-aminoantipyrine at 0.10% (w/v) and 5 mL of potassium ferricyanide at 5% (w/v). In parallel, prepare the blank and a phenol calibration curve with concentrations ranging from 5 ppm to 30 ppm. Read the absorbances **(5.2.14)** from the sample, the standards and the blank at a wavelength of 546 nm, 10 minutes after the reaction ends. Use the standard reading to plot the analytical curve and determine the phenol concentration in the sample by graphic interpolation or linear regression. The manufacturer has the option of using the result obtained in the product before packaging.

B. Add 1 mL of the sample to a volumetric flask and complete the volume to 200 mL with distilled water. Take 5 mL from this solution and transfer to a 25 mL volumetric flask. Add 3 mL of borate buffer pH 9.0, 2.5 mL of 4-aminoantipyrine at 0.15% (w/v) and 0.5 mL of potassium ferricyanide at 5% (w/v). Shake and complete the volume with distilled water. In parallel, prepare the blank and a phenol calibration curve with concentrations ranging from 0.6 µg to 3.9 µg of phenol per milliliter. Read the absorbances **(5.2.14)** from the sample, the standards and the blank, at a wavelength of 495 nm, 20 to 40 minutes after the reaction ends. Use the standard reading to plot the analytical curve and determine the phenol concentration in the sample, by graphic interpolation or linear regression.

Protein nitrogen and proteins. Proceed as described in *Determination of nitrogen by the Kjeldahl method* **(5.3.3.2)**. Not more than 0.3% (w/v) of non-protein nitrogen and 15% (w/v) of proteins. To determine the protein concentration, multiply the result of protein nitrogen by 6.25. The manufacturer has the option of using the result obtained in the product before packaging.

Total solids. On a previously tared weighing bottle, weigh accurately 1 g of the sample, in duplicate, and put in the fume hood over the heating plate, until the liquid evaporates. Transfer the weighing bottle with the sample to an oven at 105°C and allow to stand for one hour. Transfer the dried sample to the drier, allow to stand for 30 minutes and weigh. Repeat the drying procedure until constant weight. Calculate the percentage of total solids according to the formula:

$$\%\ \textit{of total solids} = B \times 100\ \mathrm{C}$$

where

B = difference between dried weighing bottle and empty weighing bottle;
C = sample weight.

The manufacturer has the option of using the result obtained in the product before packaging. Not more than 20%.

Ammonium sulfate. Dilute the sample at 1% (v/v) with purified water and transfer 10 mL of the solution to Nessler tube. Transfer 1 mL of the ammonium sulfate stock solution at 0.6% (w/v) to a 100 mL volumetric flask and complete the volume with purified water. Dilute the solution in proportions 1:2, 1:3, 1:4 and transfer 10 mL of each dilution to three Nessler tubes. Add 1 mL of Nessler reagent to each one of the tubes with the sample and the standards and compare the color. The intensity of the sample color is equal or lower than the one from the standard solution diluted at 1:3. The manufacturer has the option of using the result obtained in the product before packaging. Not more than 0.2% (w/v).

Residual humidity. It is determined when the product is presented under lyophilized form. Transfer 80 mg of the sample to a weighing bottle previously dried and tared. Keep for three hours in an anhydrous phosphorus pentoxide atmosphere, under pressure not greater than 5 mm of mercury, at temperature of 60°C. The weighing bottle with the sample is cooled for 20 minutes in a dryer with silica gel and weighed immediately. The heating and cooling phase is repeated until obtaining constant weight. The residual humidity value is the average of the weight loss percentage from not less than three sample assessments. The volumetric method for determining water **(5.2.20.1)** can also be used. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test. Use only the membrane filtration method, which must have maximum nominal porosity of 0.45 μm.

Pyrogens (5.5.2.1). Complies with the test. Inject 1 mL/kg and do not reuse the animals used in the test.

ASSAY

For potency determination, proceed as described in the specific monograph. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

The temperature and the validity period are the ones indicated by the serum manufacturer, based on experimental evidence, approved by the national regulatory control authority.

LABELLING

Comply with current legislation.

TINZAPARIN SODIUM
Tinzaparinum natricum

Tinzaparin sodium is a low molecular mass heparin sodium salt obtained by controlled enzyme depolymerization of heparin from the porcine intestinal mucosa, using heparinase from *Flavobacterium heparinum*. Most components have a structure of 2-O-sulfo-4-enepyranosuronic acid in the region of the non-reducing end and one 2N,6-O-disulfo-D-glucosamine in the region of the reducing end of the chain.

Tinzaparin sodium must be in compliance with the monograph *Low molecular mass heparin* with the modifications and additional requirements described below.

The relative mean molecular mass varies from 5500 Da to 7500 Da, with a characteristic value of approximately 6500 Da. The sulfation degree is of 1.8 to 2.5 per disaccharide unit. The potency must not be less than 70 IU or more than 120 IU of anti-factor Xa activity per milligram, calculated in relation to the dried substance. The ratio between the anti-factor Xa activity and the anti-factor IIa activity is between 1.5 and 2.5.

IDENTIFICATION

A. Proceed as described in test **A.** of *Identification* from the monograph *Low molecular mass heparin*, using the standard tinzaparin sodium.

B. Proceed as described in test **B.** of *Identification* from the monograph *Low molecular mass heparin*. The spectrum obtained must be similar to the one from standard tinzaparin.

C. Proceed as described in test **C.** of *Identification* from the monograph *Low molecular mass heparin*. The following requisites apply: the relative mean molecular mass varies from 5500 Da to 7500 Da; the percentage in mass of chains below 2000 Da is not more than 10%; the percentage of chains with molecular mass between 2000 Da and 8000 Da may vary between not more than 60% and 72%; the percentage of chains with molecular mass above 8000 Da may vary between 22% and 36%.

CHARACTERISTICS

Aspect of the preparation. Dissolve 1 g of the sample in 10 mL of water. The preparation is clear **(5.2.25)**.

PURITY TESTS

Specific absorbance. 8.0 to 12.5 in relation to the dried substance, determined at 231 nm. Dissolve 50 mg of the sample in 100 mL of hydrochloric acid 0.01 *M*.

TETANUS TOXOID ADSORBED

Toxoidum tetanicum adsorbatum

Tetanus toxoid is the tetanus anatoxin diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. It is an opalescent, slightly brownish suspension, that does not present lumps or foreign particles.

The tetanus toxin preparation is based on the seed lot system, which is an amount of ampoules with lyophilized *Clostridium tetani*, with uniform composition, obtained from a lyophilized strain of known origin. The culture media used for preparations of the seed lot and the production inoculum must enable the growth of *C. tetani.* The culture medium for preparation of the tetanus toxin must be free from substances capable of inducing toxic and/or allergic reactions in humans. The tetanus toxin is a toxic filtrate obtained from cultivating *C. tetani* in culture medium for toxin preparation and collected aseptically in a single process. At the end of the cultivation and lysis of bacterial cells, the culture purity is checked through microscopic exam or inoculation of sample in adequate culture media. The flocculation limit (Fl/mL) is assessed by Ramon's method.

Flocculation limit – Ramon's Method. Distribute in test tubes variable volumes of standardized tetanus antitoxin. Add to each tube a constant volume of 1 mL of the sample. homogenize and place in water bath at temperature of 45ºC to 50ºC. Constantly observe and record the first tube that presents flocculation and the required time. Determine the Fl/mL of the sample, multiplying the volume of reference antitoxin added to the tube by its concentration in Fl.

The toxin is purified by physical or chemical methods and submitted to Fl/mL and pH controls.

The tetanus anatoxin is obtained by detoxifying the concentrated tetanus toxin, through the addition of chemical agents in adequate PH and temperature conditions. The most commonly used chemical agent is formaldehyde at temperature of 35ºC. pH, Fl/mL and specific toxicity controls are carried out.

Specific toxicity. Do not dilute the anatoxin if it is not concentrated. Dilute the sample in saline solution to 100 Fl/mL. Inoculate 5 mL of the dilution, subcutaneously, in each one of at least five guinea pigs with 250 g to 350 g. Observe the animals for four weeks. Not less than 80% of the animals inoculated must survive during the observation period, without presenting signs of tetanus intoxication. If more than one animal dies of non-specific cause during the test period, the test can be repeated one more time. If more than one animal dies in the second test, the sample does not meet the test.
Purified anatoxin is prepared from an individual collection or from the mixture of individual collections of anatoxin and, after a sterilizing filtration process, to which a preservative agent may be added. The use of phenol is not allowed as it affects the product's antigenic properties. The purified anatoxin is assessed as to the antigen concentration (Fl/mL), sterility and to the following tests.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

pH (5.2.19). Determine the sample pH. The limits must be determined by the history in the product registration.

Immunogenic activity. Proceed as described in *Determination of immunogenic activity*. Not less than 2 IU/mL or 40 IU/individual human dose, according to the method used.

Specific toxicity. Proceed as described previously for tetanus anatoxin, with the sample being diluted to 500 Fl/mL and each guinea pig inoculated with volume of 1 mL.

Antigenic purity. Determine the content of protein nitrogen **(5.3.3.2)** and express the concentration in mg/mL. The antigenic purity is determined by the relation of antigen concentration in Fl/mL and the concentration of protein nitrogen found. The product has an antigenic purity of not less than 1000 Fl/mg of protein nitrogen.

Reversal of toxicity. Dilute the sample to 25 Fl/mL in saline solution and distribute in two vials. Keep one of the vials at temperature of 4ºC to 8ºC and the other between 35°C and 37ºC, for six weeks. At the end of the six weeks, inoculate the content from each vial, subcutaneously, in five guinea pigs weighing 250 g to 350 g, with the inoculum volume being 5 mL per animal. The animals cannot present signs of tetanus intoxication. If more than one animal dies of non-specific cause during the test period, the test can be repeated one more time. If more than one animal dies in the second test, the sample does not meet the test.
Tetanus toxoid is prepared by dilution and adsorption, in aluminum compounds, of a certain quantity of anatoxin. One dose for human use contains not more than 25 Fl. Before packaging, product samples are submitted to sterility, immunogenic activity, pH, residual formaldehyde, thiomersal and aluminum controls.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

A. Add sodium citrate to the vaccine sample until a concentration of 5% to 10% of sodium citrate is obtained. Keep at 37ºC for, approximately, 16 hours and centrifuge. Use the supernatant liquid for identification. Other adequate methods can be used for adjuvant separation. Prepare agar gel at 1% (w/v) in buffered saline solution and distribute on microscope slide, so that it results in a thin layer. Place in oven at 37°C, without drying. Add a volume of 4 mL of agar to the slide and place at temperature of 2°C to 8°C in a humid chamber for one hour. Punch the gel, keeping the same distance between the central and the peripheral holes. Fill the central hole with reference tetanus antitoxin and the peripheral holes with the sample in variable dilutions. As positive control, fill one of the holes with fluid tetanus toxoid. Incubate at 37°C for 24 hours in humid chamber and carry out reading in contrast light. Observe the presence of precipitation line, identity reaction among the components analyzed.

B. Determine the flocculation limit (Fl/mL) by Ramon's method.

C. The *Determination of immunogenic activity* can be used.

CHARACTERISTICS

pH (5.2.19). Determine the vaccine sample pH. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

DETERMINATION OF IMMUNOGENIC ACTIVITY

Use one of the methods described below.

A. By *Determination of antitoxic titer in sera from immunized animals*

Immunization and bleeding of animals: inoculate 0.75 mL (half the total human dose) of the sample, subcutaneously, to each one of the six guinea pigs with 450 g to 550 g. Six weeks after inoculation, collect blood from each animal and extract the serum. Mix equal volume of sera from, at least, four guinea pigs.
Control of paralyzing limit (Pl) from standardized tetanus toxin: distribute in a series of test tubes constant volumes of reference tetanus antitoxin, checked by international standard, so that the volume to be inoculated contains 0.1 IU. Add variable volumes of standardized tetanus toxin and equalize the volumes from all tubes with buffered saline solution with 1% (w/v) of peptone. homogenize and incubate at 37ºC for 60 minutes. Inoculate a constant volume from each dilution, subcutaneously, in ten Swiss albino mice weighing between 17 g and 22 g. Observe the animals as to symptoms of paralysis for the period of 96 hours after inoculation. Calculate the paralyzing dose 50% (Pl/10/50) by an adequate statistical method.

Serum titration: distribute, in a series of test tubes, variable volumes of the sample serum and the reference serum. Add a constant volume of standardized tetanus toxin, so that the volume to be inoculated per animal contains 1 Pl/10/50. Equalize the volumes of all tubes with buffered saline solution with 1% (w/v) of peptone. homogenize and incubate at 37ºC for 60 minutes. Inoculate each mixture, subcutaneously, in at least six Swiss albino mice weighing between 17 g and 22 g. Observe the animals for a period of 96 hours after inoculation and record the number of non-paralyzed animals in each mixture. The values of average effective doses (ED_{50}) of the sample and the reference antitoxin are determined using an adequate statistical method. The confidence limits ($P = 0.95$) must be between 50% and 200% of the estimated potency and the statistical analysis must show linearity and parallelism of the response dose curves. Calculate the immunogenic activity by the formula:

$$IA = \frac{A}{B} \times C$$

where

IA = immunogenic activity in IU/mL;
A = ED_{50} of the reference antitoxin;
B = ED_{50} of the sample;
C = IU/mL of the reference antitoxin.
Not less than 2 IU/mL of serum. The manufacturer has the option of using the result obtained in the product before packaging.

An *in vitro* method, such as immunoenzymatic assay or ToBI (toxin-binding inhibition test), can be used, provided that they are validated against the serum neutralization test described.

B. By *Challenge in mice*. This determination proves the immunogenic activity of the product, by comparison with a reference tetanus toxoid checked by an international standard. Separate nine groups with, at least, 20 mice of 11 g to 14 g to carry out the assay and a group of 12 animals, without inoculating, for challenge toxin control. Make four sample dilutions with saline solution, using a dilution factor 2. Proceed the same way with the reference tetanus toxoid. Immunize, subcutaneously, with a volume of 0.5 mL of each dilution of sample per animal. Twenty eight days after the immunization, dilute the standardized tetanus toxin in buffered saline solution with 1% (w/v) of peptone, so that it contains 200 Pd_{50}/mL (average paralyzing dose) and inoculate each immunized mouse, subcutaneously, with a volume of 0.5 mL of the standardized toxin challenge dose. Observe the animals up to 96 hours after the inoculation and record the number of live animals and with no paralysis in each dilution. In parallel, as challenge dose control, make dilutions 1:50, 1:100 and 1:200 from the toxin solution that contains 200 Pd_{50}/mL, using the same diluent. Inoculate 0.5 mL of each dilution subcutaneously, in the group of 12 separate animals, divided into groups of four animals. Observe the animals up to 96 hours after inoculation and record the number of dead ones and paralyzed ones in each dilution. All challenge control animals, inoculated with the dilution 1:50, should die or present paralysis whereas none of the animals inoculated with the dilution 1:200 should die or present paralysis. Calculate the average effective doses (ED_{50}) of the sample being tested and the reference toxoid, using an adequate statistical method. The response range produced (survival percentage) must be between the highest and the lowest dilution used in the test and standard sample, the confidence limits ($P = 0.95$) must be between 50% and 200% of the estimated potency, and the statistical analysis must show linearity and parallelism of the response dose curves. Calculate the immunogenic activity by the formula:

$$IA = \frac{A}{B} \times C$$

where

IA = immunogenic activity in IU/individual human dose;
A = ED_{50} of the reference toxoid;
B = ED_{50} of the sample;
C = IU/individual human dose of the reference toxoid.

Not less than 40 IU/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

C. By *Challenge in guinea pigs*. This determination proves the immunogenic activity of the product, by comparison with a reference tetanus toxoid checked by an international standard. Separate eight groups with, at least, 16 guinea pigs of 250 g to 350 g to carry out the assay and a group of 12 animals, without inoculating, for challenge toxin control. Make four sample dilutions with saline solution, using a dilution factor 2. Proceed the same way with the reference tetanus toxoid. Immunize, subcutaneously, with a volume of 1 mL of each dilution of sample per animal. Twenty eight days after immunization, dilute the standardized tetanus toxin in buffered saline solution with 1% (w/v) of peptone, so that it contains 100 LD_{50}/mL and inoculate each immunized guinea pig, subcutaneously, with a volume of 1 mL of the standardized toxin challenge dose. Observe the animals up to 96 hours after inoculation and record the number of live animals in each dilution. In parallel, as challenge dose control, make dilutions 1:50, 1:100 and 1:200 from the toxin solution that contains 100 LD_{50}/mL, using the same diluent. Inoculate 1 mL of each dilution subcutaneously, in the group of 12 separate animals, divided into groups of four animals. Observe the animals up to 96 hours after inoculation and record the number of dead ones in each dilution. All challenge control animals inoculated with the dilution 1:50 should die whereas none of the animals inoculated with the dilution 1:200 should die. Calculate the average effective doses (ED_{50}) of the sample and the reference toxoid, using an adequate statistical method. The response range produced (survival percentage) must be between the highest and the lowest dilution used in the test and standard sample, the confidence limits (P = 0.95) must be between 50% and 200% of the estimated potency, and the statistical analysis must show linearity and parallelism of the response dose curves. Calculate the immunogenic activity by the formula:

$$IA = \frac{A}{B} \times C$$

where

IA = immunogenic activity in IU/individual human dose;
A = ED_{50} of the reference toxoid;
B = ED_{50} of the sample;
C = IU/individual human dose of the reference toxoid.

Not less than 40 IU/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA AND TETANUS VACCINE ADSORBED FOR ADULTS

Vaccinum diphtheriae et tetani adsorbatum

diphtheria and tetanus vaccine adsorbed for adults; 09039

The vaccine is a mixture of diphtheria and tetanus anatoxins, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. It is an opalescent, slightly brownish suspension, that does not present lumps or foreign particles.
Diphtheria component: the purified diphtheria anatoxin meets the production and controls specifications described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use*.

Tetanus component: the purified tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

The vaccine is prepared by dilution and adsorption, in aluminum compounds, of determined quantity of purified diphtheria anatoxin and tetanus anatoxin. One dose for human use must contain not more than 2.5 Fl for the diphtheria component and 25 Fl for the tetanus component. Before packaging, product samples are submitted to sterility, immunogenic activity, pH, residual formaldehyde, thiomersal and aluminum controls.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

It complies with the *Identification* described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.*

CHARACTERISTICS

pH (5.2.19). Determine the vaccine sample pH. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

ASSAY

The immunogenic activity of the vaccine is determined by each of the individual components. There are no international reference standards for the combined vaccines, and the activity from each component is expressed in international units, by comparison of calibrated reference standards with reference standards from individual components.

Diphtheria component. Proceed according to *Assay*, using one of the methods described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.*

A. Not less than 0.5 IU/mL.

B. Not less than 2 IU/individual human dose.

The manufacturer has the option of using the result obtained in the product before packaging.
Tetanus component. Proceed according to *Assay*, using one of the methods described in the monograph *Adsorbed tetanus toxoid.*

A. Not less than 2 IU/mL.

B. Not less than 40 IU/individual human dose.

C. Not less than 40 IU/individual human dose.

The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA AND TETANUS VACCINE ADSORBED FOR PEDIATRIC USE

Vaccinum diphtheriae et tetani adsorbatum

diphtheria and tetanus vaccine adsorbed for pediatric use; 09988

The vaccine is a mixture of diphtheria and tetanus anatoxins, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. It is an opalescent, slightly brownish suspension, where there are no lumps or foreign particles.

Diphtheria component: the diphtheria toxin preparation is based on the seed lot system, which is an amount of ampoules with lyophilized *Corynebacterium diphtheriae*, with uniform composition, obtained from a lyophilized strain of known origin. The culture media used for preparations of the seed lot and the production inoculum must enable the growth of *C. diphtheriae.* The culture medium for preparation of the diphtheria toxin must be free from substances capable of inducing toxic and/or allergic reactions in humans. At the end of the cultivation and lysis of bacterial cells, the culture purity is checked through microscopic exam or inoculation of sample in adequate culture media. The diphtheria toxin is a toxic filtrate obtained from cultivating *C. diphtheriae* in culture medium for toxin preparation and collected aseptically in a single process. The flocculation limit (Fl) is assessed using Ramon's method, as described in the monograph *Adsorbed tetanus toxoid*. The toxin is purified by physical or chemical methods and the sample is submitted to Fl/mL and pH controls.

The diphtheria anatoxin is obtained by detoxifying the concentrated diphtheria toxin, through the addition of chemical agents in adequate pH and temperature conditions. The most commonly used chemical agent is formaldehyde at temperature of 35ºC. pH, Fl/mL and specific toxicity controls are carried out.

Specific toxicity

Subcutaneous test: dilute the sample in saline solution to 100 Fl/mL. Inoculate 5 mL of the dilution, subcutaneously, in each one of at least five guinea pigs with 250 g to 350 g. Observe the animals for four weeks. Not less than 80% of the animals inoculated should survive during the observation period, without presenting signs of diphtheria intoxication. If more than one animal dies of non-specific cause during the test period, the test can be repeated one more time. If more than one animal dies in the second test, the sample does not meet the test.
Intradermal test: dilute the sample in saline solution to 100 Lf/mL. Inoculate 0.2 mL of the dilution, intradermally, in a guinea pig shaved in advance. As a control, inoculate the same volume of saline solution in the same animal. After 48 hours of observation, specific erythema must not be formed in the inoculation sites.

Purified anatoxin is prepared from individual collection or from the mixture of individual collections of anatoxins and, after a sterilizing filtration process, a preservative agent can be added. The use of phenol is not allowed as it affects the product's antigenic properties. Product samples are assessed as to the antigen concentration (Fl/mL), sterility and the following controls.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

pH (5.2.19). Determine the sample pH. The limits must be determined by the history in the product registration.

Immunogenic activity. Proceed as described in *Determination of immunogenic activity*. Not less than 2 IU/mL or 30 IU/individual human dose, according to the method used.

Specific toxicity. Proceed with the subcutaneous test as described previously for diphtheria anatoxin, with the sample being diluted to 500 Fl/mL and each guinea pig inoculated with volume of 1 mL.

Antigenic purity. Determine the content of protein nitrogen **(5.3.3.2)** and express the concentration in mg/mL. The antigenic purity is determined by the relation of antigen concentration in Fl/mL and the concentration of protein nitrogen found. The product has an antigenic purity of not less than 1500 Fl/mg of protein nitrogen.

Reversal of toxicity. Proceed as described in the monograph *Adsorbed tetanus toxoid*. The animals cannot present signs of diphtheria intoxication and must present weight gain.

Tetanus component: the tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

The diphtheria and tetanus vaccine adsorbed for pediatric use is prepared by dilution and adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins. One dose for human use contains not more than 30 Fl and 25 Fl for diphtheria and tetanus components, respectively. Before packaging, product samples are submitted to sterility, immunogenic activity, pH, residual formaldehyde, thiomersal and aluminum controls.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

Diphtheria component

A. Add sodium citrate to the vaccine sample until a concentration of 5% to 10% of sodium citrate is obtained. Keep at 37ºC for approximately 16 hours and centrifuge. Use the supernatant liquid for identification. Other adequate methods can be used for adjuvant separation. Prepare agar gel at 1% (w/v) in buffered saline solution and distribute on microscope slide, so that it results in a thin layer. Place in oven at 37°C, without drying. Add a volume of 4 mL of agar to the slide and place at temperature of 2°C to 8°C in a humid chamber for one hour. Punch the gel, keeping the same distance between the central and the peripheral holes. Fill the central hole with reference diphtheria antitoxin and the peripheral holes with the sample in variable dilutions. As positive control, fill one of the holes with fluid diphtheria toxoid. Incubate at 37°C for 24 hours in humid chamber and carry out reading in contrast light. Observe the presence of precipitation line, identity reaction among the components analyzed.

B. Determine the flocculation limit (Fl/mL) by Ramon’s method, as described in the monograph *Adsorbed tetanus toxoid*.

C. The *Determination of immunogenic activity* can be used.

Tetanus component. Proceed as described in *Identification* in the monograph *Adsorbed tetanus toxoid.*

CHARACTERISTICS

pH (5.2.19). Determine the sample pH. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

DETERMINATION OF IMMUNOGENIC ACTIVITY

The vaccine immunogenic activity is determined for each one of the components. There are no international reference standards for the combined vaccines, and the activity from each component is expressed in international units, by comparison with calibrated reference standards against reference standards from the components.

Diphtheria component

Use one of the methods described below.

A. By *Determination of antitoxic titer in sera from immunized animals.*

Immunization and bleeding of animals: inoculate 0.75 mL (half the total human dose) of the sample, subcutaneously, to each one of the six guinea pigs with 450 g to 550 g. Four weeks after inoculation, collect blood from each animal, by cardiac puncture, and extract the serum. Mix equal volume of sera from, at least, four guinea pigs that present weight gain.

Control of death limit (L+/50) from standardized diphtheria toxin: distribute in a series of test tubes constant volumes of reference diphtheria antitoxin, checked by international standard, so that the volume to be inoculated contains 1 IU. Add variable volumes of standardized diphtheria toxin and equalize the volumes from all tubes with buffered saline solution with 1% (w/v) of peptone. homogenize and incubate at 37ºC for 60 minutes. Inoculate a constant volume from each dilution, subcutaneously, in four guinea pigs weighing between 250 g and 350 g. Observe the animals as to mortality for a period of 96 hours after inoculation. Calculate the lethal dose 50% (L+/50) by an adequate statistical method.

Serum titration: distribute in a series of test tubes variable volumes of serum. Add a constant volume of standardized diphtheria toxin, so that the volume to be inoculated per animal contains 1 L+/50 (death limit). Equalize the volumes of all tubes with buffered saline solution with 1% (w/v) of peptone. homogenize and incubate at 37ºC for 60 minutes. Inoculate each mixture, subcutaneously, in at least four guinea pigs weighing 250 g and 350 g. Observe the animals for a period of 96 hours after inoculation and record the number of live animals in each mixture. The values of average effective doses (ED_{50}) of the sample and the reference antitoxin are determined using an adequate statistical method. For the determination to be considered valid, it is necessary that: (a) the response range produced (ED_{50}) is between the highest and the lowest dilution used in the test sample and standard, forming the regression curve that must present a linear relation; (b) the confidence limits (p = 0.95) are between 50% and 200% of the potency calculated; (c) parallelism between the reference curve and the samples. Calculate the immunogenic activity by the formula:

$$IA = \frac{A}{B} \times C$$

where

IA = immunogenic activity in IU/mL;
A = ED_{50} of the reference antitoxin;
B = ED_{50} of the sample;
C = IU/mL of the reference antitoxin.

Not less than 2 IU/mL. The manufacturer has the option of using the result obtained in the product before packaging.

An *in vitro* method, such as immunoenzymatic assay or cytotoxicity in Vero cell, can be used, provided that they are validated against the serum neutralization test described.

B. By *Challenge in guinea pig*. Prove the product's immunogenic activity in test by comparison with reference diphtheria toxoid. Separate eight groups with, at least, 16 guinea pigs weighing between 250 g and 350 g. Make four dilutions of the sample being tested with sodium chloride solution at 0.85% (w/v), using dilution factor 2. Proceed the same way with the reference diphtheria toxoid. Inoculate, subcutaneously, a volume of 1 mL per animal of each dilution. Separate a group of 12 animals without inoculating, for challenge toxin control. After 28 days of inoculation, dilute the standardized diphtheria toxin in buffered saline solution with 1% (w/v) of peptone, so that it contains 100 LD_{50}/mL (lethal dose 50%), and inoculate each immunized guinea pig, subcutaneously, with a volume of 1 mL of the toxin challenge dose. Observe the animals up to 96 hours after inoculation and record the number of live animals in each dilution. In parallel, as challenge dose control, make a dilution 1:100 from the toxin solution that has 100 LD_{50}/mL, using the same diluent. Inoculate 1 mL of the dilution, subcutaneously, in each one of five guinea pigs. Observe the animals up to 96 hours after inoculation and record the number of dead ones in the dilution. Calculate the Effective Doses 50% (ED_{50}) of the sample being tested and the reference toxoid, using an adequate statistical method.

The response range produced (survival percentage) must be between the highest and the lowest dilution used in the test and standard sample, the confidence limits ($P = 0.95$) must be between 50% and 200% of the estimated potency, and the statistical analysis must show linearity and parallelism of the response dose curves. Calculate the immunogenic activity by the formula:

$$IA = \frac{A}{\underline{B}} \times C$$

where

IA = immunogenic activity in IU/individual human dose;
A = ED50 of the reference toxoid;
B = ED50 of the sample;
C = IU/individual human dose of the reference toxoid.

Not less than 30 IU/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Tetanus component. Proceed with the determinations of immunogenic activity, using one of the methods described in the monograph *Adsorbed tetanus toxoid.* Not less than 2 IU/mL (method **A.**). Not less than 40 IU/individual human dose (method **B.**). Not less than 40 IU/individual human dose (method **C.**). The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA, TETANUS AND PERTUSSIS ADSORBED VACCINE

Vaccinum diphtheriae et tetani et pertussis adsorbatum

diphtheria, tetanus and pertussis adsorbed vaccine; 09040

The vaccine is a mixture of diphtheria and tetanus anatoxins and suspension of dead whole cells of *Bordetella pertussis*, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. It is an opalescent, slightly brownish suspension, that does not present lumps or foreign particles.

Diphtheria component: the purified diphtheria anatoxin meets the production and controls specifications described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use*.

Tetanus component: the purified tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component: the pertussis vaccine is a homogeneous suspension of dead whole cells of one or more strains of *B. pertussis* in saline solution. The strains used in vaccine preparation are identified by full background registrations, including their origin, characteristics of isolation, and all tests carried out periodically to check their characteristics. The strains must be lyophilized in the phase I containing at least the agglutinogens 1, 2 and 3 and kept at maximum temperature of 4ºC.

The vaccine production is based on the seed lot system, which must have the same characteristics of the original lot. The culture medium used in the cultivation of *B. pertussis* must allow the maintenance of agglutinogens and of the immunogenic activity. This medium cannot increase the specific toxicity of the strain and cannot have substances capable of inducing toxic and/or allergic reactions to humans. At the end of the cultivation, the bacteria are collected, washed to remove substances derived from the culture medium, and resuspended in isotonic saline solution. Samples of individual collections are assessed as to opacity and bacterial purity. The suspension can be inactivated by heating at 56ºC for a determined time or detoxified by addition of chemical agents in adequate pH, temperature and treatment time conditions. Samples of suspension are assessed as to bacterial inactivation, seeding in appropriate culture medium, purity, identification, sterility, and submitted to the following controls.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

pH (5.2.19). Determine the vaccine sample pH. The limits must be determined by the history in the product registration.

Determination of immunogenic activity. Proceed as described for the end product. Not less than 4 IU/dose.

Presence of agglutinogen. Transfer 50 µL of the sample to three glass slides and add 50 µL of mono-specific serum of agglutinogens 1, 2 and 3 to the samples in each of the slides. homogenize for one minute and allow to stand for three minutes. Observe the agglutination of the sample on the three slides for not more than five minutes. The *B. pertussis* strain must present agglutination with the three specific monovalent sera.

Opacity. Conduct within not more than 15 days after the suspension preparation. Check with a turbidimetric standard approved by the national control authority. This standard is assigned a value of 10^6 opacimetric units, when examined by photometry, using a green filter at a wavelength of 530 nm. Such level of opacity corresponds to approximately 10^9 bacteria/mL. Place 1 mL of the sample in a test tube and add saline solution until the opacity is similar to that of standard. Compare the opacity visually against the reference opacity preparation. The opacity unit (OpU) is determined by the formula:

$$OpU / mL = \frac{final\ volume\ of\ the\ diluted\ sample}{initial\ volume} \times 10$$

For the pertussis component, the bacteria concentration must be not more than 20 OpU/dose.

Specific toxicity. Dilute the sample in saline solution for maximum concentration corresponding to 20 OpU/dose. Use two groups with, at least, 10 Swiss albino mice weighing between 14 and 16 g. Immediately before inoculation, determine the total weight of the animals. Inoculate 0.5 mL of the diluted sample, intraperitoneally, in each mouse from the first group. For the second group, proceed as described, inoculating saline solution with the same quantity of the preservative agent as the inoculum injected in the animals from the test group. Determine the total weight of each of the mice groups on the 3rd and 7th days after inoculation. The product is considered non-toxic if (a) on the 3rd day the total weight of the group is not less than its initial weight; (b) on the 7th day the average weight gain of the groups inoculated with the sample is not less than 60% of the average weight gain from the negative control group, and (c) not more than 10% of the animals inoculated with the sample die.

The diphtheria, tetanus and pertussis adsorbed vaccine is prepared by dilution and adsorption in aluminum compounds of determined quantities of diphtheria and tetanus anatoxins and dead whole cells of *B. pertussis*. One individual human dose can contain not more than 30 Fl and 25 Fl for diphtheria and tetanus components, respectively. For the pertussis component, the bacteria concentration must be not more than 20 OpU/dose. Before packaging, product samples are submitted to sterility, pH, residual formaldehyde, thiomersal, aluminum, specific toxicity controls and determination of immunogenic activity for each component.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

Add sodium citrate to the vaccine sample until a concentration of 5% to 10% of sodium citrate is obtained. Keep at 37ºC for approximately 16 hours and centrifuge. Use the supernatant to identify the diphtheria and tetanus components, using specific antisera. Resuspend the precipitate to identify the pertussis component. Other adequate methods can be used for adjuvant separation.

Diphtheria component. Proceed as described in *Identification* in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.*

Tetanus component. Proceed as described in *Identification* in the monograph *Adsorbed tetanus toxoid.*

Pertussis component

A. Transfer 50 µL of the sample on a glass slide and add the same volume of polyvalent antiserum of *B. pertussis*. homogenize the homogenizeture with circular movements, for one minute, and allow the material to stand for three minutes. Observe the agglutination of the sample for not more than five minutes.

B. The *Determination of immunogenic activity* can be used.

CHARACTERISTICS

pH (5.2.19). Determine the vaccine sample pH. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

Specific toxicity for the pertussis component. It complies with the test described.

DETERMINATION OF IMMUNOGENIC ACTIVITY

The vaccine immunogenic activity is determined for each one of the components. There are no international reference standards for the combined vaccines, and the activity from each component is expressed in international units, by comparison with reference standards checked by reference standards from the components.

Diphtheria component. Conduct the determination of the immunogenic activity, using one of the methods described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.* Not less than 2 IU/mL (method A). Not less than 30 IU/individual human dose (method B). The manufacturer has the option of using the result obtained in the product before packaging.

Tetanus component. Proceed with the determinations of immunogenic activity, using one of the methods described in the monograph *Adsorbed tetanus toxoid.* Not less than 2 IU/mL (method A). Not less than 60 IU/individual human dose (method B). Not less than 40 IU/individual human dose (method C). The manufacturer has the option of using the result obtained in the product before packaging.

Pertussis component. The immunogenic activity is determined by the comparative assessment with the standardized reference vaccine against the international standard for the pertussis vaccine. Use Swiss albino mice weighing between 12 g and 16 g, from a homogeneous group of standardized lineage. The animals must preferably have the same sex. For each dilution of the sample and the reference vaccine, use at least 16 animals. For control of the challenge dose, separate groups of at least 10 mice.

Immunization of animals: make not less than three serial dilutions of the sample and the reference vaccine in buffered saline solution, so that the intermediate dilution is close to a protection of 50% of mice against the lethal effects from the challenge dose of *B. pertussis*. A dilution factor of five (5) is suggested. Inoculate, intraperitoneally, 0.5 mL of the dilutions in each mouse from each immunization group. Keep the animals from control groups without inoculation. The interval between immunization and challenge is of 14 to 17 days.

Challenge: reconstitute an ampoule from the *B. pertussis* strain batch with aqueous solution with casein peptone 1% (w/v) and NaCl 0.6% (p/v), pH 7.0 to 7.2. The challenge strain must be capable of inducing death in mice after 14 days of an intracerebral inoculation, and must be rejected in case of death of more than 20% of animals within the first 48 hours.

Cultivate in test tubes and plates with appropriate medium and incubate between 35°C and 37°C for up to 48 hours. Replicate the culture in plates and tubes with Bordet-Gengou agar or another appropriate medium and incubate between 35°C and 37°C for 24 hours. A second replication must be carried out in the same conditions described and incubated for 18 hours. The cultivations obtained on the plates are used to observe the colonies and identify them by serum agglutination against a specific antiserum for the strain. Alternatively, suspension aliquots for the challenge can be frozen and stored in liquid nitrogen and, after defrosting and dilution, can be used directly as challenge cultivation. Prepare a suspension, using an adequate diluent where the microorganisms remain viable, so that it contains 10 OpU/mL, when compared with the 5th international standard of opacity. Adjust the solution so that each challenge dose contains 100 to 1000 LD_{50} (lethal dose 50%) in 30 µL and inoculate in each immunized mouse, intracerebrally. To obtain an estimate of the LD_{50}, inoculate serial dilutions of the challenge dose, intracerebrally, in each of the control groups. Cultivate dilution of the challenge dose in Bordet-Gengou medium to determine the number of colony-forming units (CFU). The value of the effective dose 50% (ED_{50}) of the sample being tested is determined, using an adequate statistical method. The test is valid if: (a) the response range produced (survival percentage) is between the highest and the lowest dilution used from the test sample and the standard; (b) the confidence limits (P = 0.95) are between 65% and 156% of the estimated potency and the statistical analysis demonstrates linearity and parallelism of the response dose curves; (c) the challenge dose is between 100 and 1000 LD_{50}; and (d) the LD_{50} contains not more than 300 colony-forming units. The immunogenic activity is calculated by the formula:

$$IA = \frac{A}{B} \times C$$

where

IA = immunogenic activity in IU/individual human dose;
A = ED_{50} of the reference pertussis vaccine;
B = ED_{50} of the sample;
C = IU/individual human dose of the reference vaccine.

The potency must be not less than 4 IU/individual human dose and the lowest confidence limit (P=0.95) estimated cannot be less than 2 IU/dose. If the immunogenic activity determined does not meet the potency or the lowest confidence limit, the test can be repeated. The product meets the requisites if the weighted geometric mean from all valid results presents minimum potency and lowest confidence limit (P=0.95) for approval. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA, TETANUS, PERTUSSIS AND HAEMOPHILUS INFLUENZAE B (CONJUGATED) ADSORBED VACCINE

Vaccinum diphtheriae, tetani, pertussis adsorbatum et haemophili stirpe b conjugatum

The diphtheria, tetanus, pertussis and *Haemophilus influenzae* b (conjugated) adsorbed vaccine is a combined vaccine comprised of diphtheria and tetanus anatoxins and suspension of dead whole cells of *Bordetella pertussis* and purified polyribosylribitol phosphate (PRP) from *Haemophilus influenzae* b bonded covalently to a carrier protein, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. The product can be presented as a tetravalent liquid preparation in the same vial or with the lyophilized *Haemophilus influenzae* b component in ampoule-vial, which is reconstituted, immediately before use, by the diphtheria, tetanus and pertussis adsorbed vaccine.

Diphtheria component: the purified diphtheria anatoxin meets the production and controls specifications, described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use*.

Tetanus component: the purified tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component: the pertussis vaccine meets the production and controls specifications, described in the monograph *Diphtheria, tetanus and pertussis adsorbed vaccine*.

Polysaccharide component: meets the production and controls specifications, described in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

In the potency assays on the combined vaccine, a combined vaccine or each of its antigens can be used, as a reference, separately. If this is not possible due to the interaction between the components of the combined vaccine or to a difference in the composition between the mono-competent reference vaccine and the vaccine being tested, a batch of the combined vaccine that has shown effectiveness in clinical trial, or a representative batch, is used as a reference vaccine. When producing a representative batch, it is necessary to strictly follow the production process used for the batch tested in clinical trial. The reference vaccine can be established by an adequate method that does not interfere with the assay procedure.

The bulk finished product vaccines are prepared separately, being one of them by adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis*, and the other with the component *Haemophilus influenzae* b. The product can also be formulated by adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis* and conjugated PRP. An adequate preservative can be added to the product. Before packaging, product samples are submitted to controls on sterility, pH, specific toxicity for pertussis, residual formaldehyde, antimicrobial preservative, aluminum, determination of immunogenic activity for the components diphtheria, tetanus, pertussis and residual polysaccharide.

The products are packaged in adequate containers, labeled and submitted to the required controls.

The vaccines prepared as bulk finished product, as well as packaged, separately, one of them containing the diphtheria, tetanus and pertussis components and the other, the *Haemophilus influenzae* b component, must comply with the respective monographs *Diphtheria, tetanus and pertussis adsorbed vaccine* and *Haemophilus influenzae b (conjugated) vaccine*.

IDENTIFICATION

Dissolve the adsorbed vaccine sample with sodium citrate at pH 9.0 to obtain a solution at 10% (w/v). Keep at 37ºC for approximately 16 hours and centrifuge. Use the supernatant liquid to identify each of the components, diphtheria or tetanus. Resuspend the precipitate to identify the pertussis component. Other adequate methods can be used for adjuvant separation.

Diphtheria component. Meets the *Identification* tests for *Diphtheria component* described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use*.

Tetanus component. Meets the *Identification* tests described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component. Meets the *Identification* tests for *Pertussis component* described in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed*.

***Haemophilus influenzae* b component.** Meets the *Identification* tests described in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

CHARACTERISTICS

pH (5.2.19). Determine the pH in the tetravalent liquid vaccine sample or, in the case of the lyophilized haemophilus component, pH must be determined after reconstitution with the adequate diluent. The limits must be determined by the history in the product registration.

PHYSICOCHEMICAL TESTS

It meets the tests described in *Physicochemical tests* in the monographs *Haemophilus influenzae b (conjugated) vaccine* and *Diphtheria, tetanus and pertussis vaccine adsorbed.*

BIOLOGICAL SAFETY TESTS

It meets the tests described in *Biological safety tests* in the monographs *Haemophilus influenzae b (conjugated) vaccine* and *Diphtheria, tetanus and pertussis vaccine adsorbed.*

ASSAY

Diphtheria component

It complies with the *Assay* described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.* The manufacturer has the option of using the result obtained in the product before packaging.

Tetanus component

It complies with the *Assay* described in the monograph *Adsorbed tetanus toxoid*. The manufacturer has the option of using the result obtained in the product before packaging.

Pertussis component

It complies with the *Assay* described in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed.* The manufacturer has the option of using the result obtained in the product before packaging.

Component ***Haemophilus influenzae b***

It complies with the *Assay* described in the monograph *Haemophilus influenzae b (conjugated) vaccine*. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Proceed as described in the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA, TETANUS, PERTUSSIS, HEPATITIS B (RECOMBINANT) AND HAEMOPHILUS INFLUENZAE B (CONJUGATED) VACCINE ADSORBED

Vaccinum diphtheriae, tetani, pertussis, hepatitidis B adsorbatum et haemophili stirpe b conjugatum

The diphtheria, tetanus, pertussis, hepatitis B (recombinant) and *Haemophilus influenzae* b (conjugated) adsorbed vaccine is a combined vaccine comprised of diphtheria and tetanus anatoxins, suspension of dead whole cells of *Bordetella pertussis*, suspension of purified antigen (HBsAg) from the surface of the hepatitis B virus, purified polyribosylribitol phosphate (PRP) from *Haemophilus influenzae* b bonded covalently to a carrier protein, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. The product can be presented with the *Haemophilus influenzae* b component in a separate ampoule-vial, which is mixed to the other components of the vaccine immediately before use.

Diphtheria component: the purified diphtheria anatoxin meets the production and controls specifications described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric* use.

Tetanus component: the purified tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component: the pertussis vaccine meets the production and controls specifications, described in the monograph *Diphtheria, tetanus and pertussis adsorbed vaccine*.

Hepatitis B component: the hepatitis B vaccine meets the production and controls specifications, described in the monograph *Hepatitis b (recombinant) vaccine*.

Polysaccharide component: meets the production and controls specifications, described in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

If the vaccine is presented with the *Haemophilus influenzae* b component in separate vial-ampoule, the assays carried out on the diphtheria, tetanus, pertussis and hepatitis B components must show consistency in an adequate number of batches of the combined vaccine reconstituted before use. For subsequent routine controls, the tests conducted in these components must be developed without mixing with the *Haemophilus influenzae* b component.

In the potency assays on the combined vaccine, a combined vaccine or each of its antigens can be used, as a reference, separately. If this is not possible due to the interaction between the components of the combined vaccine or to a difference in the composition between the mono-competent reference vaccine and the vaccine being tested, a batch of the combined vaccine that has shown effectiveness in clinical trial, or a representative batch, is used as a reference vaccine. When producing a representative batch, it is necessary to strictly follow the production process used for the batch tested in clinical trial. The reference vaccine can be established by an adequate method that does not interfere with the assay procedure.

The bulk finished product vaccines are prepared separately, being one of them by adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis* and hepatitis B (recombinant) and the other with the *Haemophilus influenzae* b component. The product can also be formulated by the adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis*, conjugated PRP and hepatitis B (recombinant). An adequate antimicrobial preservative can be added to the product. Before packaging, product samples are submitted to controls on sterility, pH, specific toxicity for pertussis, residual formaldehyde, antimicrobial preservative, aluminum, determination of immunogenic activity for the components diphtheria, tetanus, pertussis, hepatitis B and residual polysaccharide.

The products are packaged in adequate containers, labeled and submitted to the required controls.

The vaccines prepared as bulk finished product, as well as packaged, separately, one of them containing the diphtheria, tetanus, pertussis and hepatitis B components and the other, the *Haemophilus influenzae* b component, must comply with the respective monographs *Diphtheria, tetanus and pertussis adsorbed vaccine, Hepatitis B (recombinant) vaccine* and *Haemophilus influenzae b (conjugated) vaccine*.

IDENTIFICATION

Dissolve the adsorbed vaccine sample with sodium citrate at pH 9.0 to obtain a solution at 10% (w/v). Keep at 37°C for approximately 16 hours and centrifuge. Use the supernatant liquid to identify each of the components, diphtheria or tetanus. Resuspend the precipitate to identify the pertussis component. Other adequate methods can be used for adjuvant separation.

Diphtheria component. It complies with the *Identification* described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.*

Tetanus component. It complies with the *Identification* described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component. It complies with the *Identification* described in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed for pediatric use.*

Hepatitis B component. It complies with the *Identification* described in the monograph *Hepatitis B (recombinant) vaccine*.

***Haemophilus influenzae* b component.** Meets the *Identification* tests described in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

CHARACTERISTICS

pH (5.2.19). Determine the pH in the pentavalent liquid vaccine sample or, in the case of the lyophilized haemophilus component, pH must be determined after reconstitution with the adequate diluent. The limits must be determined by the history in the product registration.

PHYSICOCHEMICAL TESTS

Carry out the assays required in *Physicochemical tests*, described in the monographs *Diphtheria, tetanus and pertussis adsorbed vaccine, Hepatitis B (recombinant) vaccine* and *Haemophilus influenzae b (conjugated) vaccine.*

BIOLOGICAL SAFETY TESTS

Carry out the assays required in *Biological safety tests*, described in the monographs *Diphtheria, tetanus and pertussis adsorbed vaccine, Hepatitis B (recombinant) vaccine* and *Haemophilus influenzae b (conjugated) vaccine.*

ASSAY

Diphtheria component

It complies with the *Assay* described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.* The manufacturer has the option of using the result obtained in the product before packaging.

Tetanus component

It complies with the *Assay* described in the monograph *Adsorbed tetanus toxoid*. The manufacturer has the option of using the result obtained in the product before packaging.

Pertussis component

It complies with the *Assay* described in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed.* The manufacturer has the option of using the result obtained in the product before packaging.

Hepatitis B component

It complies with the *Assay* described in the monograph *Hepatitis B (recombinant) vaccine*. The manufacturer has the option of using the result obtained in the product before packaging.

Haemophilus influenzae b **component**

It complies with the *Assay* described in the monograph *Haemophilus influenzae b (conjugated) vaccine*. The manufacturer has the option of using the result obtained in the product before packaging.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

DIPHTHERIA, TETANUS, PERTUSSIS, POLIOMYELITIS 1, 2 AND 3 (INACTIVATED) AND HAEMOPHILUS INFLUENZAE B (CONJUGATED) ADSORBED VACCINE

Vaccinum diphtheriae, tetani, pertussis, poliomyelitidis typus I, II, III adsorbatum et haemophili stirpe b conjugatum

The diphtheria, tetanus, pertussis, poliomyelitis 1, 2 and 3 (inactivated) and *Haemophilus influenzae* b (conjugated) adsorbed vaccine is a combined vaccine comprised of diphtheria and tetanus anatoxins, suspension of dead whole cells of *Bordetella pertussis*, strains of human poliovirus type 1, 2 and 3 cultivated in cells and inactivated by an adequate method, and purified polyribosylribitol phosphate (PRP) from *Haemophilus influenzae* b bonded covalently to a carrier protein, diluted in buffered saline solution and adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. The product can be presented with the *Haemophilus influenzae* b component in a separate ampoule-vial, which is mixed to the other components of the vaccine immediately before use.

Diphtheria component: the purified diphtheria anatoxin meets the production and controls specifications described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use*.

Tetanus component: the purified tetanus anatoxin meets the production and controls specifications described in the monograph *Adsorbed tetanus toxoid*.

Pertussis component: the pertussis vaccine meets the production and controls specifications, described in the monograph *Diphtheria, tetanus and pertussis adsorbed vaccine*.

Poliomyelitis component: the poliomyelitis vaccine meets the production and controls specifications, described in the monograph *Poliomyelitis 1, 2 and 3 (inactivated) vaccine*.

Polysaccharide component: meets the production and controls specifications described in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

The assays conducted in each of the component must maintain consistency in an adequate number of batches of the combined vaccine reconstituted before use. For subsequent routine controls, the tests conducted in these components must be developed without mixing with the *Haemophilus influenzae* b component.

Mono-component reference vaccines can be used in potency assays on the combined vaccine. If this is not possible due to the interaction between the components of the combined vaccine or to a difference in the composition between the mono-competent reference vaccine and the vaccine being tested, a batch of the combined vaccine that has shown effectiveness in clinical trial, or a representative batch, is used as a reference vaccine. When producing a representative batch, it is necessary to strictly follow the production process used for the batch tested in clinical trial. The reference vaccine can be established by an adequate method that does not interfere with the assay procedure.

The bulk finished product vaccines are prepared separately, being one of them by adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis* and poliomyelitis, and the other with the *Haemophilus influenzae b* component. The product can also be formulated by the adsorption, in aluminum compounds, of determined quantities of diphtheria and tetanus anatoxins, dead whole cells of *B. pertussis*, conjugated PRP and poliomyelitis. An adequate antimicrobial preservative can be added to the product. Before packaging, product samples are submitted to controls on sterility, pH, specific toxicity for pertussis, residual formaldehyde, antimicrobial preservative, aluminum, residual polysaccharide and determination of immunogenic activity for the diphtheria, tetanus and pertussis components.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

Dissolve the adsorbed vaccine sample with sodium citrate at pH 9.0 to obtain a solution at 10% (w/v). Keep at 37°C for approximately 16 hours and centrifuge. Use the supernatant liquid to identify each of the components, diphtheria or tetanus. Suspend the precipitate to identify the pertussis component. Other adequate methods can be used for adjuvant separation.

Diphtheria component. Proceed as described in *Identification* in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.*

Tetanus component. Proceed as described in *Identification* in the monograph *Adsorbed tetanus toxoid*.

Pertussis component. Proceed as described in *Identification* in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed.*

Poliomyelitis component. Proceed as described in *Identification* in the monograph *Poliomyelitis 1, 2 and 3 (inactivated) vaccine.*

***Haemophilus influenzae* b component.** Proceed as described in *Identification* in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

CHARACTERISTICS

pH (5.2.19). Determine the pH in the liquid vaccine sample containing all antigens or, in the case of the lyophilized haemophilus component, pH must be determined after reconstitution with the adequate diluent. The limits must be determined by the history in the product registration.

PHYSICOCHEMICAL TESTS

Proceed as described in *Physicochemical tests* in the monographs *Diphtheria, tetanus and pertussis adsorbed vaccine, Poliomyelitis 1, 2 and 3 (inactivated) vaccine* and *Haemophilus influenzae b (conjugated) vaccine.*

BIOLOGICAL SAFETY TESTS

Proceed as described in *Biological safety tests* in the monographs *Diphtheria, tetanus and pertussis adsorbed vaccine, Poliomyelitis 1, 2 and 3 (inactivated) vaccine* and *Haemophilus influenzae b (conjugated) vaccine.*

ASSAY

Diphtheria component

Proceed as described in *Assay* in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.* The manufacturer has the option of using the result obtained in the product before packaging.

Tetanus component

Proceed as described in *Assay* in the monograph *Adsorbed tetanus toxoid*. The manufacturer has the option of using the result obtained in the product before packaging.

Pertussis component

Proceed as described in *Assay* in the monograph *Diphtheria, tetanus and pertussis vaccine adsorbed.* The manufacturer has the option of using the result obtained in the product before packaging.

Poliomyelitis component

Proceed as described in *Assay* in the monograph *Poliomyelitis 1, 2 and 3 (inactivated) vaccine.*

Haemophilus influenzae b component

Proceed as described in *PRP concentration* in the monograph *Haemophilus influenzae b (conjugated) vaccine*.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

BCG VACCINE
Vaccinum BCG

The lyophilized BCG vaccine is a live vaccine obtained from cultivating the Calmette and Guérin bacillus, an attenuated strain of *Mycobacterium bovis,* of known safety and efficacy, to provide protection to humans against tuberculosis. The lyophilized is a dry bacillary mass, with powder consistency, in whitish or pale yellow color which, when reconstituted, is slightly turbid and with homogeneous aspect.

The vaccine production is based on the seed lot system, with the possibility of having not more than eight subcultures from the original strain. The selected strain must preserve its stability and maintain its non-pathogenic character both for humans and for animal testing. This vaccine must be produced by a team in good health conditions that does not work with infectious agents and, in particular, with virulent strains of *Mycobacterium tuberculosis.*

The working seed lot must meet the following requisites.

Sterility (5.5.3.2.1). It complies with the test, except for the presence of micro bacteria.

Identity. Identify the bacteria as *Mycobacterium bovis* BCG, using microbiological and molecular biology methods, such as amplification of nucleic acid.

Virulent micro bacteria. It complies with the test described for the end product.

The bacterium is inoculated in appropriate culture medium, free from substances that may cause toxic and/or allergic reaction to humans. The cultivations and the culture medium from each container are visually examined as to the aspect, presenting a bacterial veil on the surface and clear culture medium. The cultivations are transferred to a new medium and, after growth, are tested for sterility and assessed visually for medium transparency and aspect of the bacterial veil. After filtration, the bacterial veil is re-suspended in appropriate medium and submitted to bacterial breath, opacity and sterility tests. The bacterial suspension is diluted to the appropriate number of doses and, before proceeding to packaging, the product is assessed for the number of colony-forming units and sterility. The product is packaged in ampoules or vial-ampoules of pharmaceutical-class amber glass, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

Observe by microscopy a smear obtained after reconstitution of the vaccine and stained by the Ziehl-Nielsen technique. Only acid-alcohol-resistant bacilli are detected. As a complement, observe the morphology of colonies cultured in the Lowenstein-Jensen medium, used in the *microbiological assay* (colony-forming units). The colonies are rough, predominantly spread and non-pigmented. Alternatively, molecular biology techniques can be used.

CHARACTERISTICS

pH (5.2.19). Determine after the vaccine reconstitution with an adequate diluent. The limits must comply with the history in the product registration.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

Virulent micro bacteria. Reconstitute the content of the ampoules with the recommended diluent, to obtain 50 human doses. Inoculate a volume of 1 mL in each of the six guinea pigs, weighing 250 g to 400 g, subcutaneously, in the abdominal region, on the right side. Keep the animals under observation for 42 days. At the end of the period, weigh, euthanize and conduct a necropsy on the animals. Examine the inoculation site, the regional, inguinal, axillary, mediastinum, lumbar and portal ganglia, and other organs, particularly lungs, liver, spleen and kidneys.

No guinea pig can present evidence of progressive tuberculosis, and not less than 2/3 of the animals must survive at the end of the observation period, with weight gain. Repeat the test if more than 1/3 of the animals die or lose weight. The end product assay can be omitted if a satisfactory result was obtained on the bulk product.

Cutaneous reactivity. Reconstitute a sample and prepare dilutions 1:10 and 1:100, using the diluent recommended. Inoculate, intradermally, 0.1 mL of each one of the dilutions in the left flank of four albino guinea pigs of the same sex, with minimum weight of 350 g each. The animals must present negative tuberculosis reaction, and cannot have undergone treatment that may result in a false negative. Proceed as described for the reference vaccine, inoculating the same animal in the right flank. Observe the animals for four weeks and conduct weekly readings of the diameter of the lesions found in the inoculation sites. At the end of the observation period, calculate, for each corresponding dilution, the average from four readings of the vaccine and the reference vaccine. The vaccine meets the requisite if the reaction produced by the sample is similar to the one from the reference vaccine. The end product assay can be omitted if it was held in the working seed lot and in five consecutive end batches derived from the same seed lot.

MICROBIOLOGICAL ASSAY

Number of colony-forming units (CFU)

Reconstitute five ampoules of the vaccine with the recommended diluent, being careful to add it slowly to avoid foaming. Transfer the content of the ampoules to a single test tube, homogenize and make three dilutions, to obtain an optimum number of colonies around 40, discarding counts greater than 100. Inoculate in Lowenstein-Jensen medium, using five tubes for each of the two most concentrated dilutions and 10 tubes for the most diluted concentration. Seal the tubes and incubate in vertical position at 37°C, for four weeks. Analyze in parallel a sample of the reference vaccine and express the results in CFU/mL. The acceptance range must be according to the history of registration of the product submitted to the national regulatory authority, and the maximum limit must not exceed four times the limit defined as minimum.

If bioluminescence or any other method is used, it must be validated against the method for counting the viable ones.

THERMOSTABILITY

Incubate five vaccine ampoules at the temperature of 37°C for four weeks and proceed according to the *Microbiological assay*. Compare the results obtained with the ones from samples stored at the temperature of 2°C to 8°C. The number of CFU/mL cannot be below 20% of the CFU/mL from the vaccine stored between 2°C and 8°C.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MUMPS (ATTENUATED) VACCINE

Vaccinum parotiditis vivum

mumps (attenuated) vaccine; 09037

The mumps (attenuated) vaccine is composed of live attenuated viruses, presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and can demonstrate coloration due to the presence of pH indicator.

The vaccine production is based on the seed lot system and the strain of virus used, or five consecutive batches of the vaccine cannot induce neuropathogenesis in monkeys susceptible to the mumps virus. Additionally, the virus strain must demonstrate adequate immunogenicity and safety for humans. The virus replication is carried out in a culture of susceptible cells and the virus suspension is identified and controlled as to sterility. Stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added after clarification of the virus suspension by an adequate method to remove cell residues. Before packaging and lyophilization, the product is analyzed for sterility, concentration of viruses and proteins derived from animal serum used.

The vaccine is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

Further information related to production criteria and their controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Reconstitute the vaccine with an appropriate diluent and add an equal volume of serum, containing neutralizing antibodies for the mumps virus. Incubate at 36°C for one hour. After incubation, inoculate the mixture in culture of susceptible cells and keep at temperature of 36°C for 10 days. Use as control a cell culture inoculated with the vaccinal virus and another not inoculated, which present, at the end of the test, presence and absence of cytopathic effect, respectively. The absence of CPE in the cell culture identifies the vaccinal virus.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 2%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Proceed to the determination protected from direct light. Dilute two samples of vaccine and one sample of reference vaccine at intervals of not less than 1.0 $\log_{10}$, in adequate culture medium.

Inoculate each dilution in not less than 10 wells of the microplate with Vero cells in suspension and incubate at temperature of 36°C for 10 days. Observe the cell cultures for the presence or absence of CPE and calculate the vaccine titer by a proven statistical method. The vaccine potency is the value of the geometric mean of the vials analyzed, expressed in $CCID_{50}$ (cell culture infecting dose 50%) per dose. For the determination to be considered valid, it is necessary that: (a) at the end of the assay the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ $CCID_{50}$; (c) the variation of the reference vaccine titer is not more than 0.5 $\log_{10}$ $CCID_{50}$ of its mean titer; (d) the CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The vaccine potency is not less than 103.7 $CCID_{50}$/dose. If it does not meet the requisite, repeat the determination. The vaccine potency is the geometric mean of the two assays carried out.

The plaque forming units method (PFU) can also be used. The potency value for product approval must be correlated with the one from $CCID_{50}$.

THERMOSTABILITY

The test is carried out in parallel with *Assay*. Incubate a vaccine sample at 37°C, for seven days, and analyze according to the method described for the product potency. The vaccine cannot lose more than 1.0 $\log_{10}$ $CCID_{50}$/dose, in relation to the titer of the vaccine stored in adequate temperature conditions. Also, it cannot have a titer lower than the product's potency requisite.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

YELLOW FEVER (ATTENUATED) VACCINE

Vaccinum febris flavae vivum

The vaccine against yellow fever is composed of live attenuated viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of homogeneous suspension, and can demonstrate coloration due to the presence of pH indicator.

The vaccine production is based on the primary seed lot system of the 17D strain, from which, through passages in SPF (specific pathogen free) embryonated chicken eggs, the secondary seed lot is originated.

Each primary seed lot of virus must be submitted to identification tests, adventitious agents (bacterial and fungal sterility; mycoplasmas and mycobacteria), avian leukosis virus and inoculation in monkeys to check viscerotropism, immunogenicity and neurotropism.

Each secondary seed lot of virus must be submitted to identification tests, adventitious agents (inoculation in guinea pigs and mice, bacterial and fungal sterility, mycoplasmas and mycobacteria, cell cultivation and avian viruses), avian leukosis virus and inoculation in monkeys to check viscerotropism, immunogenicity and neurotropism.

The virus replication is carried out in embryonated chicken eggs, free from specific pathogens, or in susceptible cell culture. If the vaccine production occurs in embryonated eggs, 2% and not less than 20 eggs not infected with the vaccine strain must demonstrate absence of pathogens specific for avian species.

The virus suspension is clarified by an adequate method to remove cell residues, and stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added. Protein of human origin cannot be added to any production step. The virus suspension or the mixture of individual virus suspensions are tested for identification, bacterial and fungal sterility, mycoplasmas, mycobacteria and viral concentration.

Tests in cell culture for other adventitious agents. Inoculate 5 mL of the sample from control egg extracts in monkey kidney cell cultures and in chicken embryo fibroblasts. Incubate the cells at temperature between 35°C and 37°C and observe for 14 days. The presence of any adventitious agents must not be evidenced and not les than 80% of the cell cultures must remain viable.

Avian viruses. Inoculate 0.1 mL of extracts from control eggs via allantoic membrane in each one of the 10 SPF embryonated eggs with 9 to 10 days. Proceed likewise, inoculating in the yolk sac 10 SPF embryonated eggs with five to seven days. At the end of seven days of incubation, not less than 80% of the inoculated eggs must remain viable, and hemagglutinating agents and/or typical macroscopic pathologies must not be evidenced in embryos and in chorioallantoic membranes.

After the formulation, the bulk finished product is analyzed for sterility, virus concentration and protein nitrogen.

Content of protein nitrogen (5.3.3.2). Not more than 0.25 mg per dose, before adding any stabilizer.

The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

Further information related to production criteria and their controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

The vaccine, when neutralized with serum containing specific antibody for the yellow fever virus, inhibits the formation of plaque forming units (PFU) in susceptible cells as described in *Assay*. Molecular methods, such as sequencing and amplification of nucleic acid, can be used.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. The maximum limit must ensure that the product maintains its stability, according to the registration submitted to the national regulatory authority.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Complies with the test. Not more than 5 EU per human dose.

Residual ovalbumin. Determine the ovalbumin content by adequate *Immunochemical method* **(5.6)**, using as reference a standard preparation of ovalbumin. The vaccine is considered satisfactory if the content of residual ovalbumin is less than or equal to 5 µg/dose.

ASSAY

A reference vaccine calibrated in International Units (IU) must be used for determining the titer of the viral inoculum and in the vaccine potency assay.

Not less than three vials of lyophilized vaccine and one of reference vaccine are submitted to the method of plaque forming units (PFU). Dilute the vaccine applying factor 4 and inoculate at least two dilutions in minimum three wells in a six-well plate with a single layer of Vero cells previously cultured. The concentration of the cell lineage may vary from 100 000 to 300 000 cells per mL, according to day of use. After adsorption for a period of up to 90 minutes at temperature between 35°C and 37°C, in an atmosphere of CO_2 at 5%, add a culture medium with agarose or carboxymethylcellulose in adequate concentration. Incubate the plates for five to seven days, at temperature between 35°C and 37°C, in an atmosphere of CO_2 at 5%. After the incubation period, remove the growth medium, fix the cells with formaldehyde, and color with a vital stain.

Calculate the average number of plaques from the vials of the vaccine being tested and the reference vaccine, through proven statistical methods. Compare the viral concentration of the vaccine being tested with the one from the reference vaccine and express the result in International Units (IU) per dose. The minimum potency must be 3.0 $\log_{10}$ UI per dose. For the determination to be considered valid, it is necessary that: (a) there is an unchanged single layer in the cell culture; (b) the variation of potency among the three vaccine samples is not more than 0.3 $\log_{10}$ UI; (c) the potency of the reference vaccine varies not more than 0.5 $\log_{10}$ IU from the established titer; and (d) the number of

PFU is decreasing in relation to increasing dilutions. The assay must be repeated if the requisites are not met. Other assay methods can be used, provided they are justified and approved by national regulatory authorities. However, if the vaccine is assessed by the method described above, it must meet the requirements already established.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate not less than two vials of vaccine for 14 days between 36°C and 38°C and analyze as described in *Assay.* The vaccine can lose not more than1 $\log_{10}$ in relation to the titer determined in the sample stored in adequate temperature conditions. Additionally, it must have a titer at least equal to the one specified for the product potency. Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

HAEMOPHILUS INFLUENZAE B (CONJUGATED) VACCINE

Vaccinum haemophili stirpe b conjugatum

The *Haemophilus influenzae* b (conjugated) vaccine is a liquid or lyophilized preparation of capsular polysaccharide, obtained from a strain of *Haemophilus influenzae* type b, covalently bonded to a carrier protein.

The polysaccharide polyribosyl-ribitol-5-phosphate (PRP) is a polymer composed of alternate units of ribose and ribitol, covalently grouped by a phosphate through phosphate diester bonds. The carrier protein, when conjugated to the polysaccharide, is capable of inducing a T-cell dependent immune response.

The production of the type B polysaccharide is based on the seed lot system, which is an amount of ampoules with lyophilized *H. influenzae* type b, with uniform composition, obtained from a lyophilized strain of known origin. The culture media used to preserve the viability of the lyophilized, or frozen, strain cannot contain animal-origin proteins. PRP produced by seed lot should be characterized by nuclear magnetic resonance spectrometry.

The microorganism *H. influenzae* type b is cultivated in an adequate liquid medium that does not contain polysaccharides with high molecular mass. If any component of the medium contains blood-originated substances, the process must be validated to prove that they are not detected after purification. At the end of the cultivation, the culture purity is checked through microscopic exam or inoculation of sample in adequate cultivation media and culture agglutination with specific antiserum. The culture can be inactivated. PRP is separated from the cultivation medium and purified by an adequate method.

HAEMOPHILUS INFLUENZAE POLYSACCHARIDE (PRP)

The purified polysaccharide can only be used in the conjugate preparation if it meets the following requisites.

Identification. PRP is identified by an *Immunochemical method* **(5.6)** or another validated method, such as nuclear magnetic resonance spectrometry.

Humidity. The humidity content of the polysaccharide is determined by thermogravimetric analysis on halogen lamp balance. Weight loss is determined on a sample dried at 60°C for 60 minutes. Transfer 120 mg of the sample to the chamber, set the humidity analyzer and, when analysis is completed, transfer the sample quantitatively to a 10 mL volumetric flask and add ultra-purified water.

Distribution by molecular size. It corresponds to the percentage of PRP, eluted before a given distribution coefficient (Kd) or within a range of Kd values. The distribution of PRP molecular dimensions can be determined by size-exclusion *High Performance Liquid Chromatography* **(5.2.17.4)**. The method is conducted using a filtration gel column with 300 x 7.8 mm for 10 µm particles, with *Mobile phase* flow of 0.3 mL/minute. The columns are kept at constant temperature of 25°C. The elution is monitored using a refractometric detector for PRP analysis.

Mobile phase: prepare a sodium chloride solution 0.2 *M* and tromethamine 0.01 *M*. homogenize and, if necessary, adjust to pH 7.0. Filter in a 0.45 µm membrane filter before use.

PRP solution: prepare a PRP solution at 4 mg/mL in *Mobile phase*.

Inject 100 µL of samples from the polysaccharide solution every 60 minutes.

For data analysis, it is necessary to determine the total exclusion volume (V_0) and the total inclusion volume (Vt). V_0 and Vt are determined, respectively, with dextran with molecular mass near Kd = 0.3 and sodium azide.
Polysaccharides have fraction with high molecular mass eluting in the dead volume. The elution volume of these polysaccharides with high molecular mass can also be used to determine the dead volume of the column. In the determination of the molecular mass distribution, the Kd value is determined by the formula:

$$Kd = \frac{Ve - Vo}{Vt - Vo} \qquad \text{or} \qquad Kd = \frac{Rt\,(\,Ve\,) - Rt\,(\,Vo\,)}{Rt(Vt) - Rt(Vo)}$$

where

Rt (Ve) = retention time of the polysaccharide analyzed;
Rt (Vo) = Vo retention time;
Rt (Vt) = Vt retention time.

To calculate the quantity of polysaccharide eluted at a Kd ≤ 0.30, the elution volume corresponding to a Kd = 0.30 is determined according to the formula:

$$Ve = [3.0(Vt - Vo)] + Vo$$

With the Ve value, the percentage of polysaccharide eluted at Kd ≤ 0.30 is, then, determined, tagging this value in the chromatogram and integrating the peak area up to this point.

An acceptable value is established, specifically, for the product and each PRP run must comply with this limit. The limits for products approved, using the stationary phases indicated, are recorded for information on **Table 1**.

The *Liquid chromatography* **(5.2.17.4)** method of detection by light scattering can also be used.

Other validated methods, such as determination of polymerization degree or average molecular weight and dispersion of molecular masses, can replace the test for *Distribution by molecular size* described.

PRP Concentration. PRP concentration is determined by *Immunochemical method* **(5.6)**, *Anion exchange liquid chromatography with pulsed amperometric detection* **(5.2.17.3)** or Ribose by spectrophotometry (Orcinol). If the ribose content is used, proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**.

Standard D-ribose solution: dilute *D*-ribose in distilled water to obtain a solution at 25 µg/mL. Distribute and store the aliquots at -20°C.

Solution A: weigh, accurately, approximately 50 mg of iron chloride hexahydrate and dissolve in 100 mL of concentrated hydrochloric acid.

Orcinol reagent: weigh 375 mg of orcinol monohydrate and dissolve in 5 mL of ethyl alcohol at 96% (v/v). Add to the *Solution A* in the ratio 1:20 and homogenize.

The Hib polysaccharide is a polymer of ribosylribitol-5-phosphate and can be quantified by measuring the ribose content. The determination of the ribose content is based on the spectrophotometric measurement of absorbance of a complex with green color, formed by the reaction between the *Orcinol reagent* and the ribose in the polymer subunits. Within the analysis range, the absorbance is proportional to the ribose concentration. For the analytical curve, pipet, for each of the 5 test tubes, 0 µL, 100 µL, 200 µL, 300 µL and 400 µL of *D-ribose standard solution*. Add distilled water to each of the tubes up to 400 µL. For the analysis, dissolve the purified polysaccharide to 0.05 mg/mL in distilled water and pipet 400 µL of the sample solution to the tubes. Add to each tube 800 µL of *Orcinol reagent* and place them at 100°C for 20 minutes. Allow to cool at room temperature and read the absorbance at 669 nm. The ribose contents from the samples obtained from the analytical curve are expressed in micrograms of ribose per milliliter *versus* the mass of dry polysaccharide. Calculate the percentage (w/w) of D-ribose according to the formula:

$$\%\text{D - ribose} = \frac{\text{mass of D - ribose}(\mu\text{g}) \text{ obtained from the standard curve}}{\text{mass of polysaccharide } (\mu\text{g})} \text{x}100$$

The minimum limit must comply with the registration of the product approved by the national regulatory authority.

Phosphorus. Proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**. The method can be based on the spectrophotometric measure of absorbance of the color blue formed by the reduction of a phosphomolybdic complex by ascorbic acid.

Standard solution: dissolve 109.7 mg of potassium phosphate monobasic in 250 mL of distilled water. Dilute 5 mL of the previous solution to 100 mL with distilled water.

Solution A: weigh 2.5 g of ammonium molybdate and add 100 mL of distilled water.

Solution B: at the moment of use, weigh 10 g of ascorbic acid and add 100 mL of distilled water.

Solution C (Phosphomolybdic complex and ascorbic acid): at the moment of use, mix the solutions in the following order: 1 volume of sulfuric acid 3 *M*, 1 volume of *Solution A*, 2 volumes of distilled water and 1 volume of *Solution B*.

Mineralization reagent: 5 mL of concentrated sulfuric acid and 5 mL of perchloric acid at 70% (w/w).

Dissolve 25 mg of polysaccharide dissolved in distilled water, transfer quantitatively to a 50 mL volumetric flask, complete the volume with distilled water and homogenize. For mineralization, pipet 100 µL of this solution, transfer to a test tube and add 100 µL of *Mineralization Reagent*. The test is carried out in triplicate. Heat at 250°C until there is complete discoloration (four hours). The blank (1 mL of distilled water) and the standard solutions (0.5 mL, 1.0 mL, 1.5 mL and 2.0 mL of *Standard solution*) are treated identically. For coloration development, after each tube cools add 4 mL of *Solution C*. Heat the content of the tubes at 37°C for 120 minutes to develop a blue coloring of the phosphomolybdic complex. Measure the absorbance of the sample and standard solutions at 825 nm using the blank as reference cell. The phosphorus content from the sample is determined by using a calibration curve established from the values obtained for the standard solutions. Calculate the phosphorus percentage through the formula:

$$\%\text{ of phosphorus} = \frac{\text{concentration of the sample obtained in the standard curve } (\mu\text{g/tube})}{\quad} \text{x}100$$

0.1× sample concentration (µg of dry polysaccharide/mL)

The limits must comply with the registration of the product approved by the national regulatory authority.

Protein. Proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**. The assay suggested is based on the Lowry method, where the blue color from copper chelate develops in the presence of proteins.

Standard stock solution of bovine serum albumin: weigh 20 mg of bovine albumin (BSA) and complete to 100 mL with distilled water.

Alkaline copper solution: mix 0.5 mL of sodium tartrate and potassium at 2% (w/v) in distilled water, with 0.5 mL of copper sulfate pentahydrate at 1% (w/v) in distilled water. Adjust to 50 mL with sodium carbonate at 2% (w/v) in sodium hydroxide 0.1 *M*.

Diluted Folin reagent: mix 50 mL of phosphomolybdotungstic reagent (Folin-Ciocalteu-phenol reagent) with 50 mL of distilled water. This solution must be prepared at the moment of use.

In test tubes, add 0 µL, 10 µL, 25 µL, 50 µL 100 µL and 200 µL of the *Standard stock solution of bovine serum albumin*. Adjust the volume in each tube to 200 µL with distilled water. If necessary, dilute the samples with approximately 10 mg/mL of dried polysaccharide and add 200 µL to each tube. Add 200 µL of distilled water as blank solution. Add to the standard, blank and sample solution tubes in the following order: 1 mL of *Alkaline copper solution*, mix and allow to react for 10 minutes; 100 µL of *Diluted Folin reagent*, mixing immediately. After 30 minutes, the solutions are centrifuged and the absorbances of the standard solutions and the sample solutions are measured at 750 nm. The protein concentration in the sample is expressed as micrograms per milliliter. Calculate the percentage of protein according to the formula:

$$\text{\% of protein} = \frac{\text{concentration obtained from the standard curve (µg/tube)}}{\text{sample concentration (µg of dry polysaccharide/mL)}} \text{x100}$$

Not more than 1% (w/w), calculated in relation to the dried basis.

Nucleic acid. Proceed as described in *Ultraviolet spectrophotometry* **(5.2.14)**. The residual acid nucleic from a sample containing not less than 1 mg/mL of dried polysaccharide is measured by ultraviolet absorption spectrophotometry at 260 nm. The absorbance of a solution of aqueous nucleic acid at 10 g/L in a spectrophotometric cell with 1 cm of width at 260 nm is of 200.

$$\text{\% of nucleic acid} = \frac{\text{sample reading} \times 50}{\text{mass of dried polysaccharide (mg)}} \text{x100}$$

Not more than 1% (w/w), calculated in relation to the dried basis.

Residual reagents. When relevant, tests must be carried out to determine residues from reagents used during inactivation and purification. An acceptable value for each reagent is established specifically for the product and each PRP run must comply with this limit.
If the methods for removing a residual reagent have been validated, PRP test can be omitted.

Bacterial endotoxins (5.5.2.2). Not more than 10 EU/µg of PRP.

CARRIER PROTEIN

The carrier protein, when conjugated to PRP, must be capable to induce an adequate immunogenic activity. The proteins are produced through culture of the respective microorganisms, where the bacterial purity is checked. The cultivation can be inactivated and the protein is purified by adequate methods. The proteins approved and their respective conjugation methods are informed on **Table 1**.

Diphtheria component. The diphtheria anatoxin is produced as described in the monograph *Diphtheria and tetanus vaccine adsorbed for pediatric use.* It meets the requirements for purified diphtheria anatoxin, except for the sterility test, which is not required.

Tetanus component. The tetanus anatoxin is produced as described in the monograph *Adsorbed tetanus toxoid*. It meets the requirements for purified tetanus anatoxin, except for the sterility test, which is not required.

Diphtheria protein CRM 197. It contains not less than 90% of diphtheria protein CRM 197, determined by an adequate method. Some tests must be carried out, for validation or routinely, to demonstrate absence of toxicity in the product.

The protein to be used in the conjugation must meet the following requirements.

In case of using the protein complex from the external membrane of *Neisseria meningitidis* group B (OMP) as carrier protein, proceed to the test of *Pyrogens* **(5.5.2.1).** Inject in each rabbit 0.25 µg of OMP per kilogram of body weight.

Table 1 – Product characteristics and specifications for PRP and carrier protein.

Carrier			***Haemophilus Polysaccharide***		***Conjugation***	
Type	***Purity***	***Nominal quantity per dose***	***Type of PRP***	***Nominal quantity per dose***	***Method of conjugation***	***Procedure***
Diphtheria Anatoxin	More than 1500 Lf /mg of nitrogen	18 µg	PRP of reduced weight Kd:0.6-0.7	25 µg	PRP Activation by cyanogen bromide	Activated diphtheria anatoxin (DAH$^+$), PRP activation by cyanogen bromide
Tetanus Anatoxin	More than 1500 Lf /mg of nitrogen	20 µg	PRP ≥ 50% Kd ≤ 0.30	10 µg	Mediated by carbodiimide	PRP activatedHA (PRPcov.-HA) + Tetanus Anatoxin + EDAC
Diphtheria Protein CRM 197	More than 90% of diphtheria protein	25 µg	PRP of reduced weight Dp=15-35 or 1035	10 µg	Reductive amination (1-step method) or activation	Direct conjugation of PRP with the protein CRM 197
					by N-hydroxysuccinimide	cyanoborohydride activation of

External protein membrane from group B meningococci (OMP)	Protein vesicles from external membrane: ≤ 8% of lipopolysaccharide	125 µg or 250 µg	PRP of reduced weight Kd ≤ 0.6, using cross-linked agarose for chromatography R or $M_w > 50 \times 10^3$	7.5 or 15 µg	Thioether bond	PRP Activation by CDI PRP-IM + BuA2 + BrAc = PRP-BuA2BrAc + thioactivated OMP

PRP is chemically modified to allow the conjugation, being partially depolymerized before or after the procedure. Before the conjugation, active functional groups can be introduced to the protein or the PRP. To determine the consistency, the derivation extension is monitored in this production step. The conjugate is obtained by the covalent bond of PRP with protein. Non-reactive residual functional groups, but with potential reactogenicity, may be present after the conjugation process. The process must be validated to prove that reactive functional groups are not present after production. The conjugate is then purified for removal of reagents and submitted to the following related controls. The limits applied to some of these controls are listed on **Table 2**. For a lyophilized vaccine, which is submitted to the lyophilization process that can affect the component to be analyzed, some tests may be carried out on the final batch.

PRP Concentration. PRP concentration is determined by *Immunochemical method* **(5.6)**, *Anion exchange liquid chromatography with pulsed amperometric detection* **(5.2.17.3)** or *Ribose*. When the polysaccharide content is determined by the ribose concentration, proceed as described in *PRP Concentration,* in *Haemophilus influenzae Polysaccharide (PRP)*. The polysaccharide content is calculated multiplying the ribose content, expressed in microgram per milliliter, by the factor 2.488.

Free protein. The concentration can be determined directly by an adequate method or by derivation, through calculation of results from other tests. The value must be within the limits approved for the product.

Protein. Proceed as described in *Protein*, in *Haemophilus influenzae Polysaccharide (PRP)*. The result is calculated in micrograms per milliliter.

Polysaccharide/protein ratio. Quotient between polysaccharide concentration and protein concentration. For each conjugate, the ratio must comply with the range stated in the registration of the product approved by the national regulatory authority.

Residual reagents. The removal of residual reagents, such as: cyanide, EDAC (ethyl-dimethyl-aminopropyl-carbodiimide) and phenol, is confirmed by adequate tests or by process validation.

Distribution by molecular size. Proceed as described on *Distribution by molecular size*, in *Haemophilus influenzae Polysaccharide (PRP)*.

To calculate the quantity of conjugate eluted at a Kd ≤ 0.20, the elution volume corresponding to a Kd = 0.20 is determined according to the formula:

$$Ve = [0.2(Vt - Vo)] + Vo$$

With the value of Ve, the percentage of polysaccharide eluted at Kd ≥ 0.20 is, then, determined, tagging this value in the chromatogram and integrating the peak area up to this point.

For the conjugate, the acceptable value must comply with the registration of the product approved by the national regulatory authority.

Sterility (5.5.3.2.1). Complies with the test. Use for each cultivation medium 10 mL or the equivalent to 100 doses, whichever is lowest.

Table 2 – **Requisites for the bulk conjugate.**

Test	***Carrier protein***			
	Diphtheria anatoxin	***Tetanus anatoxin***	***Diphtheria protein CRM 197***	***External protein membrane from Group B meningococcus (OMP)***
Free PRP	< 37%	< 20%	< 25%	< 15%
Free protein	< 4%	< 1%	< 1% or < 2%, depending on the conjugation method	Not applicable
Ratio PRP/ Protein	1.25 – 1.8	0.30 – 0.6	0.3 – 0.7	0.05 – 0.1
Molecular mass (Kd):		≥ 80% to Kd≤ 0,20		
cross-linked agarose for chromatography	95% < 0.75	60% < 0.2	50% 0.3 – 0.6	85% < 0.3
cross-linked agarose for chromatography	0.6 – 0.7	85% < 0.5		

In the preparation of vaccine, bulk finished product, an adjuvant, an antimicrobial preservative and a stabilizer may be added, before the final dilution with adequate diluent. Before packaging, product samples are submitted to the following controls.

Thiomersal. When applicable, proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm.

Sterility (5.5.3.2.1). Complies with the test.

The product is packaged in adequate containers and, if this is the case, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

The test must be carried out by an approved *Immunochemical method* **(5.6)**, using specific antibodies for the purified polysaccharide.

CHARACTERISTICS

pH (5.2.19). Determine the pH in the vaccine sample or, in the case of the lyophilized component, pH must be determined after reconstitution with the adequate diluent. The limits must be determined by the history in the product registration.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3.0%.

Polysaccharide Concentration. PRP concentration is determined by *Immunochemical method* **(5.6)**, *Anion exchange liquid chromatography with pulsed amperometric detection* **(5.2.17.3)** or Ribose by spectrophotometry (Orcinol). If the ribose content is used, proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**. For lyophilized vaccine, dissolve the content of 20 vials in approximately 4 mL of distilled water and remove lactose by dialysis. Dilute the residual dialysis liquid at 10 mL with distilled water and determine the ribose content from the vaccine as described in *PRP Concentration,* in *Haemophilus influenzae Polysaccharide (PRP)*. The PRP content of the vaccine is calculated by multiplying the ribose content, expressed as microgram per milliliter, by 2.488 and is not less than 80% of the concentration stated on the label.

Distribution by molecular size. It is the PRP percentage, eluted before a given Kd value or within a range of Kd values, determined by size-exclusion *High Performance Liquid Chromatography* **(5.2.17.4)**, as described in *Distribution by molecular size*, for the *Haemophilus influenzae Polysaccharide (PRP)*. For one batch of vaccine, a sample of 5 doses of vaccine is diluted in 2.5 mL of *Mobile phase*. An acceptable value is established, specifically, for the product and each batch of vaccine must comply with this limit. The limits for products approved, using the stationary phases indicated, are listed for information on **Table 2**.

Free polysaccharide. Not more than 20%. The concentration of free PRP is determined after removing the conjugate by formation of a PRP-carrier protein-antibody complex or by other validated methods. Check on the supernatant, by *Immunochemical method* **(5.6)**, the content of Hib polysaccharide and the absence of conjugate. Calculate the percentage of free polysaccharides in the vaccine according to the formula:

$$\%\text{ of free PRP} = \frac{\text{supernatant PRP content}}{\text{vaccine total content of PRP}} \times 100$$

Thiomersal. When applicable, proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Complies with the test. Not more than 25 EU/dose. If the vaccine contains any component that may interfere with the assay, the *Pyrogen* **(5.5.2.1)** test must be carried out.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

HEPATITIS B (RECOMBINANT) VACCINE
Vaccinum hepatitidis B ADN recombinatum

The recombinant vaccine against hepatitis B is a suspension of purified antigen (HBsAg) from the surface of the hepatitis B virus, adsorbed by aluminum hydroxide or aluminum phosphate, to which a preservative may be added. The S gene or the combination of S and pre-S2 genes or of S, pre-S2 and pre-S1 genes is also present. It has the aspect of opalescent suspension that does not present lumps or foreign particles.

The vaccine is produced by expression of the coded virus gene for the antigen on the surface of the hepatitis B virus (HBsAg) in a recombinant strain of yeasts or in culture of susceptible cells. The antigen produced complies with the sterility, plasmid retention and antigen consistency tests. In the case of using mammal cell cultures, the antigen produced must demonstrate absence of mycoplasmas and viruses. Additionally, the cells (host cell in combination with the antigen expression vector) used in the production must derive from a cell bank approved by the national regulatory authority.

The recombinant surface agent (HBsAg) is purified by several physicochemical methods and formulated in aluminum hydroxide or aluminum phosphate gel. The controls mentioned below are pre-requisites for formulating the vaccine.

Residual DNA. Not more than 100 pg/individual human dose.

Proteins. Determined by adequate method.

Antigen concentration. Assessed by validated immunochemical method.

Identification. Assessed by validated immunochemical method.

Purity. Determined by comparison with the reference vaccine using an adequate method such as liquid chromatography or SDS-PAGE. It presents not less than 95% of proteins from the hepatitis B virus surface antigen.

Inorganic ions. Residues of inorganic ions, deriving from salts used in the production process, are determined by adequate methods.

Sterility. Comply with the monograph *Vaccines for human use*.

Animal serum. If animal-origin serum is used in the production processes, the serum residue is not greater than 1 µL/L of vaccine.

Other components. Proteins, lipids, nucleic acid and carbohydrates are also determined.

Before packaging, the product is submitted to adjuvant, preservative and sterility controls.

The vaccine is packaged in adequate containers, labeled and submitted to the required controls.

Further information related to production and controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Assay can be used.

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Proceed as described in the monograph *Vaccines for human use*.

Pyrogens (5.5.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Not more than 10 EU/mL.

ASSAY

Prepare not less than three dilutions of the vaccine and of a reference vaccine in isotonic sodium chloride solution, containing the aluminum adjuvant used in the vaccine. Each dilution is inoculated intraperitoneally, in at least 10 BALB/c mice of H-2q or H-2d haplotype. A group of animals is inoculated only with the diluent. The animals used must be of the same sex. Four to six weeks after the inoculation, anesthetize and bleed all animals. Separate individually the sera and determine the presence of antibodies to hepatitis B virus by immunoenzymatic method. Record the number of animals that demonstrate seroconversion in each dilution and calculate the ED_{50} (effective dose 50%), as well as the relative potency, by an adequate statistical method. The assay is considered valid if (a) the ED_{50} found is between the highest and the lowest concentration of the vaccine inoculated in the mice; (b) the statistical analysis does not demonstrate deviation of linearity and parallelism; (c) the confidence limit of the relative potency is between 30% and 300%.

The upper confidence limit of the relative potency is not less than 1.

Validated *in vitro* methods, such as immunoenzymatic assay and radioimmunoassay, using specific monoclonal antibodies for HBsAg antigen, can also be used.

PACKAGING AND STORAGE

Proceed as described in the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

INFLUENZA (SURFACE ANTIGEN, INACTIVATED) VACCINE

Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum

The influenza (surface antigen, inactivated) vaccine is a suspension of flu virus antigens comprised of different strains of the *influenza* virus, fractioned and purified, whose composition is updated every year. It is presented as an homogeneous and slightly opalescent aqueous suspension if an adjuvant is added.

The World Health Organization (WHO) assesses epidemiologic evidence of influenza worldwide and recommends the strains that must compose the vaccine.

The vaccine production is based on the primary seed lot system, from which the secondary or working seed lot is originated through passages in SPF (specific pathogenic free) embryonated chicken eggs or in susceptible cells. The working lot must be assessed as to sterility and mycoplasmas. Additionally, the virus strain must demonstrate adequate immunogenicity and safety for humans.

The virus replication can be carried out by inoculation in the allantoic cavity of embryonated chicken eggs, free from specific pathogens, or in culture of susceptible cells, deriving from a proven healthy cultivation. If the vaccine production occurs in embryonated eggs, 2% and not less than 20 eggs are separated for control. At the end of the vaccine production, the eggs not infected with the vaccine strain must demonstrate absence of pathogens specific for avian species.

Allantoic fluid is collected from the eggs after a period of incubation at a temperature that favors the replication of each virus strain. The suspensions of each type of virus are collected and processed separately. Then, they are treated by a process with known efficacy that allows to inactivate the virus without changing its immunogenicity.

The virus particles are purified by centrifugation or another established process and fragmented in subunits through approved procedures. Later, they must be submitted to complementary purification and the resulting monovalent suspension can contain an adequate antimicrobial agent.

The monovalent suspensions are submitted to the controls required before the bulk product is prepared.

Virus inactivation. Proceed as described on *Virus inactivation*, in *Biological safety assays*.

Hemagglutinin antigen. The determination of the hemagglutinin content is based on the *in vitro* antigen-antibody reaction by *Simple radial immunodiffusion* **(5.6)**.

Neuraminidase antigen. Detect the type of neuraminidase antigen by enzymatic or immunologic methods.

Sterility (5.5.3.2.1). Complies with the test.

Fragmentation and purification agents. Determine the residual concentration of fragmentation and purification agents used, through an adequate and validated method. The maximum limit must be approved by the national regulatory authority in the product registration.

Purity. Use *Electrophoresis in polyacrylamide gel*, as described in *Electrophoresis* **(5.2.22)**, or another method approved. Hemagglutinin and neuraminidase antigens must be predominantly detected.

Established quantities of monovalent products are mixed to originate the bulk finished product, which can only be packaged when approved in quality control tests.

Sterility (5.5.3.2.1). Complies with the test.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*.

The product is packaged in adequate containers, labeled and submitted to the required controls.

IDENTIFICATION

Proceed as described in *Simple radial immunodiffusion* **(5.6)** or in another *Immunochemical method* **(5.6)**, using specific sera from virus components.

CHARACTERISTICS

pH (5.2.19). Complies with the test. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Total protein (5.3.3.2). Complies with the test. Not more than 40 µg per virus strain and 120 µg per human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. The manufacturer has the option of using the result obtained in the product before packaging.

Inactivation agent. Determine the residual concentration of the virus inactivation agent through an adequate and validated method. If formaldehyde or beta-propiolactone solutions are used, the maximum limits must be, respectively, 200 ppm and 0.1% (v/v). The manufacturer has the option of using the result obtained in the product before packaging.

Adjuvant. Determine the substance content used as adjuvant by a method that is validated and approved by the national regulatory authority. The concentration must be in the range proven to be effective in clinical trials. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Complies with the test. Not more than 100 EU per human dose.

Ovalbumin. Determine the ovalbumin content by adequate *Immunochemical method* **(5.6)**, using as reference a standard preparation of ovalbumin. Not more than1 µg per human dose. The producer has the option of using the result obtained in the product before packaging.

Virus inactivation. Inoculate 0.2 mL of the sample in the allantoic cavity of each of at least 10 embryonated chicken eggs. After incubation at temperature of 33-37°C for three days, not less than 80% of the embryos must survive. Collect from each egg with surviving embryo approximately 1.0 mL of allantoic fluid and mix them. Inoculate 0.2 mL of the mixture in each of the 10 embryonated eggs and incubate at 33-37°C for three days. Collect approximately 1 mL of allantoic fluid from each egg and carry out individually a hemagglutination test to detect hemagglutinins resulting from virus growth. There must not be positive reaction in the allantoic fluid in any of the dilution series.

ASSAY

Hemagglutinin content

The determination of the hemagglutinin content is based on the *in vitro* antigen-antibody reaction by *Simple radial immunodiffusion* **(5.6)**.

Prepare an agar gel at 1% (w/v) with anti-hemagglutinin serum specific for the strain being tested and distribute 27 mL on a glass plate or on plastic film with hydrophilic surface of 12 cm x 12 cm. After not less than two hours at temperature of 2°C to 8°C in humid chamber, punch the gel 49 equidistant holes with 4 mm of diameter.

Treat the sample being tested and the reference hemagglutinin with Zwitterionic detergent, under agitation, for 30 minutes. Prepare a series of three dilutions, in triplicate, of the reference hemagglutinin and the sample being tested. Fill the holes randomly with 20 µL of the antigen solutions and the sample being tested. Incubate in a humid chamber at temperature of 20°C to 25°C for 16 to 20 hours, on a perfectly leveled surface. Wash with sodium chloride solution at 0.9% (w/v) and dry the plates. Proceed to staining with Coomassie blue at 0.15% (w/v) or with another adequate colorant. Measure the perpendicular diameters of the precipitation halos for each of the holes with the aid of a graded magnifier or another adequate method. Calculate the content of hemagglutinin by comparison between the curves of average diameter for each dilution of the sample being tested and the reference antigen, using a multiple regression method or another adequate statistical method. Not less than 15 µg.

The test will be considered valid if the confidence interval (P = 0.95) is between 80 and 125% of the hemagglutinin concentration estimated for each strain. The lower limit of the confidence interval (P = 0.95) is not less than 80% of the nominal concentration declared for each strain.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

INFLUENZA (FRAGMENTED, INACTIVATED) VACCINE

Vaccinum influenzae inactivatum ex virorum fragmentis praeparatum

The influenza (fragmented, inactivated) vaccine is a suspension of flu virus antigens composed of different strains of the *influenza* virus, fractioned and purified, whose composition is updated every year. It is presented as an homogeneous and slightly opalescent aqueous suspension if an adjuvant is added.

The World Health Organization (WHO) assesses epidemiologic evidence of influenza worldwide and recommends the strains that must compose the vaccine.

The vaccine production is based on the primary seed lot system, from which the secondary or working seed lot is originated through passages in SPF (specific pathogenic free) embryonated chicken eggs or in susceptible cells. The working lot must be assessed as to sterility and mycoplasmas. Additionally, the virus strain must demonstrate adequate immunogenicity and safety for humans.

The virus replication can be carried out by inoculation in the allantoic cavity of embryonated chicken eggs, free from specific pathogens, or in culture of susceptible cells, deriving from a proven healthy cultivation. If the vaccine production occurs in embryonated eggs, 2% and not less than 20 eggs are separated for control. At the end of the vaccine production, the eggs not infected with the vaccine strain must demonstrate absence of pathogens specific for avian species.

Allantoic fluid is collected from the eggs after a period of incubation at a temperature that favors the replication of each virus strain. The suspensions of each type of virus are collected and processed separately. Then, they are treated by a process with known efficacy that allows to inactivate the virus without changing its immunogenicity. The virus is purified by centrifugation or by another process established and, later, fragmented through adequate surfactant agents. The fragmented particles are added to a suspension that can contain an adequate antimicrobial agent.

The monovalent suspensions are submitted to the controls required before the bulk product is prepared.

Virus inactivation. Proceed as described in *Virus inactivation* in *Biological safety tests*.

Hemagglutinin antigen. The determination of the hemagglutinin content is based on the *in vitro* antigen-antibody reaction by *Simple radial immunodiffusion* **(5.6)**.

Neuraminidase antigen. Detect the type of neuraminidase antigen by enzymatic or immunologic methods.

Sterility (5.5.3.2.1). Complies with the test.

Fragmentation and purification agents. Determine the residual concentration of fragmentation and purification agents used, through an adequate and validated method. The maximum limit must be approved by the national regulatory authority in the product registration.

Established quantities of monovalent products are mixed to originate the bulk finished product, which can only be packaged when approved in quality control tests.

Sterility (5.5.3.2.1). Complies with the test.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*.

The product is packaged in adequate containers, labeled and submitted to the required controls.

Further information related to production criteria and controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Proceed to the *Simple radial immunodiffusion* assay or another *Immunochemical method* **(5.6)**, using specific sera from virus components.

CHARACTERISTICS

pH (5.2.19). Complies with the test. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Total protein (5.3.3.2). Complies with the test. Not more than 100 µg per virus strain and 300 µg per human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. The limit must be determined by the history of efficacy in the product registration and cannot exceed 115% of the value declared in the vaccine composition. The manufacturer has the option of using the result obtained in the product before packaging.

Inactivation agent. Determine the residual concentration of the virus inactivation agent through an adequate and validated method. If formaldehyde or beta-propiolactone solutions are used, the maximum limits must be, respectively, 200 ppm and 0.1% (v/v). The manufacturer has the option of using the result obtained in the product before packaging.

Adjuvant. Determine the content of the substance used as adjuvant by a method that is validated and approved by the National Regulatory Authority. The concentration must be in the range demonstrated to be effective in clinical trials. The manufacturer has the option of using the result obtained in the product before packaging.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Complies with the test. Not more than 100 EU per human dose.

Ovalbumin. Determine the ovalbumin content by adequate *Immunochemical method* **(5.6)**, using as reference a standard preparation of ovalbumin. Not more than1 µg per human dose. The producer has the option of using the result obtained in the product before packaging.

Virus inactivation. Inoculate 0.2 mL of the sample in the allantoic cavity of each of not less than 10 embryonated chicken eggs. After incubation at temperature of 33-37°C for 3 days, not less than 80% of the embryos must survive. Collect from each egg with surviving embryo approximately 1.0 mL of allantoic fluid and mix them. Inoculate 0.2 mL of the mix in each of the 10 embryonated eggs and incubate at 33-37°C for 3 days. Collect approximately 1 mL of allantoic fluid from each egg and carry out individually a hemagglutination test to detect hemagglutinins resulting from virus growth. There must not be positive reaction in the allantoic fluid in any of the dilution series.

ASSAY

Hemagglutinin content. The determination of the hemagglutinin content is based on the *in vitro* antigen-antibody reaction by *Simple radial immunodiffusion* **(5.6)**.

Prepare an agar gel at 1% (w/v) with anti-hemagglutinin serum specific for the strain being tested and distribute 27 mL on a glass plate or on plastic film with hydrophilic surface of 12 cm x 12 cm. After not less than two hours at temperature of 2°C to 8°C in humid chamber, punch the gel 49 equidistant holes with 4 mm of diameter.

Treat the sample being tested and the reference hemagglutinin with Zwitterionic detergent, under agitation, for 30 minutes. Prepare a series of three dilutions, in triplicate, of the reference hemagglutinin and the sample being tested. Fill the holes randomly with 20 µL of the antigen solutions and the sample being tested. Incubate in a humid chamber at temperature of 20°C to 25°C for 16 to 20 hours, on a perfectly leveled surface. Wash with sodium chloride solution at 0.9% (w/v) and dry the plates. Proceed to staining with Coomassie blue at 0.15% (w/v) or with another adequate colorant. Measure the perpendicular diameters of the precipitation halos for each of the holes with the aid of a graded magnifier or another adequate method. Calculate the hemagglutinin content by comparison between the curves of average diameter for each dilution of the sample being tested and the reference antigen, using a multiple regression method or another adequate statistical method. Not less than 15 µg.

The test will be considered valid if the confidence interval (P = 0.95) is between 80 and 125% of the hemagglutinin concentration estimated for each strain. The lower limit of the confidence interval (P = 0.95) is not less than 80% of the nominal concentration declared for each strain.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MENINGOCOCCAL ACWY (POLYSACCHARIDE) VACCINE

Vaccinum meningococcale polysaccharidicum

The meningococcal ACWY (polysaccharide) vaccine is a lyophilized preparation of one or more purified capsular polysaccharides, obtained from one or more strains of *Neisseria meningitidis* groups A, C, Y and W_{135}, capable of producing polysaccharides.

The group A meningococcal polysaccharide consists of repeated, partially acetylated units of N-acetyl mannosamine bonded by phosphodiester 1α□6 bonds.

The group C meningococcal polysaccharide consists of repeated, partially acetylated units of N-acetylneuraminic acid, bonded by glycoside 2α□9 bonds.

The group Y meningococcal polysaccharide consists of alternate units of sialic acid and glucose, partially O-acetylated, bonded by glycoside 2α□6 and 1α□4 bonds.

The group W_{135} meningococcal polysaccharide consists of alternate units of sialic acid and galactose, partially O-acetylated, bonded by glycoside 2α□6 and 1α□4 bonds.

The component or components must be established on the vaccine label, along with calcium ions and the residual humidity, representing more than 90% of the product mass.

The production of meningococcal polysaccharides is based on the seed lot system, which is an amount of ampoules kept at -70°C containing *N. meningitidis*, with uniform composition, obtained from a lyophilized strain of known origin. The information related to the strain must include biochemical and serologic characteristics. The solid and liquid culture media used, respectively, to preserve the strain viability and in production cultivations must be free from animal-origin proteins. The culture purity must be assessed through colony morphology, microscopic exam, sample inoculation in adequate cultivation media and culture agglutination with specific antiserum.

The production method must result in meningococcal polysaccharide vaccines with immunogenic capacity and free from toxicity.

The polysaccharides are purified through procedures that are effective in removing nucleic acids, proteins and lipopolysaccharides.

The final purification step consists of precipitation of polysaccharides by ethyl alcohol, drying and storage at -20°C. The loss in the drying process is determined by thermogravimetry and the value obtain is used to calculate the results from other physicochemical tests with reference to the dried basis.

Only the purified polysaccharides that meet the following requirements can be used in the preparation of the bulk vaccine product.

Identification. The identification test must be conducted by an approved *Immunochemical method* **(5.6)**, using specific antibodies for each group of polysaccharides.

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 20%.

Protein. Proceed as described in *Visible spectrophotometry* **(5.2.14)**.

Standard stock solution of BSA 0.02% (bovine serum albumin): weigh 20 mg of bovine serum albumin and transfer to a 100 mL volumetric flask. Complete the volume with distilled water.

Solution A: dissolve 2 g of sodium carbonate in sodium hydroxide 0.1 *M*, transfer, quantitatively, to a 100 mL volumetric flask, complete the volume with sodium hydroxide 0.1 *M* and homogenize.

Solution B: dissolve 1 g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) in distilled water, transfer, quantitatively, to a 100 mL volumetric flask, complete the volume with distilled water and homogenize.

Solution C: dissolve 2 g of sodium/potassium tartrate in distilled water, transfer quantitatively to a 100 mL volumetric flask, complete the volume with distilled water and homogenize. Prepare this solution at the moment of using.

Alkaline copper solution: homogenize with agitation 0.5 mL of *Solution B* and 0.5 mL of *Solution C*, complete to 50 mL with *Solution A* and homogenize.

Pipet to test tubes the volumes of *Standard stock solution of BSA 0.02% (bovine serum albumin)* and distilled water, described on **Table 1**, to plot the analytical curve:

Table 1 – Values of concentration and volume of standard stock solution of BSA and volume of distilled water used in the test.

BSA concentration (µg/mL)	*Stock BSA volume (µL)*	*Distilled water volume (µL)*
10	10	190
25	25	175
50	50	150
100	100	100
150	150	50
200	200	---

Dilute the sample to approximately 10 mg of dry polysaccharide per milliliter. Take three aliquots of 200 µL from the sample solution. Prepare the blank test with 200 µL of distilled water. Add to the blank, standards and samples 1 mL of *Alkaline copper solution*. homogenize and wait 10 minutes. Add 100 µL of phosphomolybdotungstic reagent (Folin-Ciocalteu-phenol reagent) 2 *M* diluted in distilled water in the ratio 1:1. homogenize immediately and wait 30 minutes. If there is turbidity, centrifuge the samples and conduct the reading of absorbances at the wavelength of 750 nm. Plot the analytical curve, BSA concentration (µg/mL) *versus* absorbance readings, with the results obtained in different dilutions and calculate, accordingly, the protein concentration in the sample in micrograms per milliliter.

Calculate the percentage of residual protein, according to the formula:

$$\% \text{ protein} = \frac{Cp}{Ca} \times 100$$

where

Cp = concentration obtained from the analytical curve (µg/mL)
Ca = concentration of the sample (µg of dry polysaccharide/mL)

Not more than 1% (w/w) of protein in the polysaccharide, calculated in relation to the dried basis.

Residual phenol. Dilute the sample to approximately 10 mg of dry polysaccharide per milliliter. Transfer to a test tube one drop of this solution and, in another tube, carry out a positive control transferring one drop of the *Standard phenol solution*. Transfer to the tubes the following reagents: five drops of distilled water; one drop of *Alkaline buffer pH 9.8*; one drop of *4-aminoantipyrine solution*; one drop of the *Potassium ferricyanide solution* and one drop of *Monopotassium phosphate M.* No blood red coloring should develop. Phenol should not be detected.

Solution A: weigh 0.05 g of phenol (frozen) and complete the volume to 1000 mL with distilled water.

Standard phenol solution: take an aliquot of 20 mL of stock *Solution A* and complete the volume to 1000 mL with distilled water.

Alkaline buffer pH 9.8: weigh 6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate, dissolve in water, transfer, quantitatively, to a 1000 mL volumetric flask, complete the volume and homogenize. The pH must be 9.8. Keep in a refrigerator protected from light.

4-aminoantipyrine solution: weigh 3 g of 4-aminoantipyrine, dissolve in water, transfer, quantitatively, to a 1000 mL volumetric flask, add distilled water until completing the volume and homogenize. The solution must be prepared at the moment of use.

Potassium ferricyanide solution: weigh 12 g of potassium ferricyanide, dissolve in water, transfer, quantitatively, to a 1000 mL volumetric flask, add distilled water until completing the volume and homogenize. Keep in a refrigerator protected from light.

Potassium phosphate monobasic M solution: weigh 136 g of potassium phosphate monobasic, dissolve in distilled water, transfer, quantitatively, to a 1000 mL volumetric flask, add distilled water until completing the volume and homogenize.

Nucleic acid. Proceed as described in *Ultraviolet spectrophotometry* **(5.2.14)**. Dilute the sample in a 10 mL volumetric flask to a concentration of approximately 10 mg of dried polysaccharide per milliliter. Measure the absorbance of the resulting solution at 260 nm, using distilled water for zero adjustment. Calculate the percentage of nucleic acid according to the formula:

$$\%NuAc = \frac{La}{m} \times 50$$

where

%NuAc = % of nucleic acids;
La = reading of sample;
m = mass of dried polysaccharide (mg).

Not more than 1% (w/w) of polysaccharide, calculated in relation to the dried basis.

Distribution by molecular size. Proceed as described in size-exclusion *High efficiency liquid chromatography* **(5.2.17.4)**. Weigh 5 mg of polysaccharide, transfer to a 1 mL polypropylene microtube and dissolve in 1 mL of *Mobile phase*. Calibrate the liquid chromatography system through passage of *Mobile phase* by a filtration gel column of hydrophilic polymeric matrix with 300 x 7.8

mm for 10 µm particles, at a flow speed of 0.8 mL/minute, until obtaining a stable line at constant temperature of 40°C. Inject 50 µL of the V_0 and V_t *Markers* from the column and samples. Check the retention times of the *Markers* and calculate the V_e from the sample, according to the formula:

$$V_e = Kd \times (V_t - V_0) + V_0$$

where
V_e = elution volume;
Kd = distribution coefficient;
V_t = elution volume of the V_t marker;
V_0 = elution volume of the V_0 marker.

Calculate the area corresponding to the elution volume (V_e) using Kd equal to 0.5. Not less than 65% of the group A polysaccharide, 75% of the group C polysaccharide and 80% of the groups Y and W_{135} polysaccharides, respectively, must be eluted before reaching Kd equal to 0.5.

Mobile phase: mix 1220 mL of the *Solution A* with 780 mL of the *Solution B*, check the pH and adjust to 7.0. Add acetonitrile so that it is present at a final concentration of 15% (v/v).

Solution A: weigh 39.749 g of sodium phosphate dibasic and complete the volume to 1400 mL with distilled water.

Solution B: weigh 22.078 g of sodium phosphate monobasic monohydrate and complete the volume to 800 mL.

Markers: V_0 = Pullulan standard 1.6 x 106 and V_t = sucrose. Weigh 5 mg of marker, transfer to a 1 mL polypropylene microtube, dissolving in 1 mL of *Mobile phase*.

***O*-acetyl groups.** The test is carried out only on polysaccharides A and C. Proceed as described in *Visible spectrophotometry* **(5.2.14)**. For polysaccharide A, dilute the sample to approximately 10 mg of dried polysaccharide per milliliter and prepare a diluted sample solution (1:20). For polysaccharide C, dilute the sample to approximately 10 mg of dried polysaccharide per milliliter and prepare a diluted sample solution (1:10). Follow **Table 2** adding the following volumes in the respective tubes.

Table 2 – Preparations of blank, standard and sample solutions.

Substances	***Distilled water (µL)***	***Standard working solution of acetylcholine 600 µg/mL (µL)***	***Polysaccharide A: sample 1:20 (µL)***	***Polysaccharide C: sample 1:10 (µL)***
Blank	1000	---	---	---
Samples	---	---	1000	---
	---	---	---	1000
P1 (30 ppm)	950	50	---	---
P2 (120 ppm)	800	200	---	---
P3 (240 ppm)	600	400	---	---
P4 (480 ppm)	200	800	---	---
P5 (600 ppm)	---	1000	---	---

Alkaline hydroxylamine solution: mix in equal volumes *Solutions A and B* at the moment of use.

Solution A: weigh 13,9 g of hydroxylamine hydrochloride, dissolve in water, transfer quantitatively to a 100 mL volumetric flask, complete the volume with distilled water and homogenize. Keep the solution at 4°C.

Solution B: dissolve 140 g of sodium hydroxide, dissolve in distilled water, transfer quantitatively to a 1000 mL volumetric flask, complete the volume with distilled water and homogenize.

Solution C: dissolve 1.5 g of acetylcholine chloride, dissolve in distilled water, transfer quantitatively to a 100 mL volumetric flask, complete the volume and homogenize.

Hydrochloric acid 1:3: Transfer, to a graded vial, 400 mL of distilled water and slowly add 200 mL of concentrated hydrochloric acid.

Ferrous chloride solution 0.37 M: weigh 50 g of ferrous chloride hexahydrate, dissolve with hydrochloric acid 0.1 *M*, transfer to a 500 mL volumetric flask, complete the volume with hydrochloric acid 0.1 *M* and homogenize.

Standard working solutions of acetylcholine (600 µg/mL): dilute *Solution C* in the ratio 1:25 in distilled water.

Procedure: add 2 mL of *Alkaline hydroxylamine solution*. Wait two minutes, add 1 mL of *Hydrochloric acid 1:3* and 1 mL of *Ferrous chloride solution 0.37 M*. Measure the absorbances of the resulting solutions at 540 nm and plot the analytical curve.

Calculate the *O*-acetyl concentration according to the formula:

$$O\text{–acetyl} = \frac{La}{m} \times 200$$

where

O-acetyl = concentration of *O*-acetyl groups (µmol/mg);
La = reading of sample (µmol/mL);
m = mass of dried polysaccharide (mg).

The concentration per gram of polysaccharide calculated in relation to the dried basis must meet the limits of 2.0 mmol/g (w/w) for A, 1.5 mmol/g (w/w) for group C, and 0.3 mmol/g (w/w) for groups Y and W135.

Phosphorus. Proceed to the test on polysaccharide A, as described in *Visible spectrophotometry* **(5.2.14)**. Dilute the sample to approximately 10 mg of dry polysaccharide per milliliter. Prepare a diluted sample solution (1:20). Carry out the sample test in triplicate. Add to the blank test tube 0.1 mL of distilled water and, to the sample test tubes, 0.1 mL of the diluted sample (1:20). Add 0.1 mL of *Mineralization reagent* to the blank and the samples. Place the tubes in a thermoblock at 250°C for four hours. Allow to cool and then add to each tube 3.9 mL of distilled water. Pipet to test tubes the following volumes of the *Standard phosphorus solution 5 µg/mL* and distilled water, to plot the analytical curve, according to **Table 3**.

Table 3 – Preparations of standard phosphorus solutions to plot the analytical curve.

Phosphorus concentration (µg/tube)	***Volume of the standard phosphorus solution 5 µg/mL (mL)***	***Distilled water volume (mL)***
0	0	4.0
1.0	0.2	3.8
2.0	0.4	3.6
4.0	0.8	3.2
8.0	1.6	2.4

Add 4 mL of *Staining reagent* to each tube of analytical curve and samples. homogenize and incubate the set of tubes in water bath at 37°C for two hours. Allow to cool. Measure the absorbances of the resulting solutions at 825 nm. Plot the analytical curve in micrograms of phosphorus per tube *versus* absorbance.

*Mineralization reagen*t: transfer to a 10 mL volumetric flask 5 mL of concentrated sulfuric acid and add, very slowly, to the central area of the flask perchloric acid at 70% (w/w), allow to cool, complete the volume and homogenize. Store the solution at 4°C.

Staining reagent: at the moment of using the solution, add to a cylinder 24 mL of distilled water and add 12 mL of sulfuric acid 3 *M*, 12 mL of ammonium molybdate solution at 2.5% (w/v), and 12 mL of ascorbic acid solution 10%. homogenize and allow to stand.

Standard phosphorus solution at 5 µg/mL: this solution must be prepared by dilution at (1:20) of the *Standard phosphorus solution 100 µg/mL*.

Standard phosphorus solution 100 µg/mL: dissolve 109.7 mg of potassium phosphate monobasic, dried in advance, in 100 mL of distilled water. Transfer to a 250 mL volumetric flask and complete the volume up to graduation mark.

Calculate the phosphorus percentage in the samples, according to the following formula:

$$\%\ \textit{phosphorus} = \frac{\mathrm{Pc}}{\mathrm{m}} \times 200$$

where

Pc = phosphorus content found in the curve (µg);
m = dried mass of the sample (mg).

Not less than 8% (w/w) of the group A polysaccharide, calculated in relation to the dried weight.

Sialic acid. The test is carried out for polysaccharides C, W and Y. Proceed as described in *Visible spectrophotometry* **(5.2.14)**. Dilute the sample to approximately 10 mg of dry polysaccharide per milliliter. Prepare a solution of the diluted sample (1:100). Transfer to eight test tubes, respectively, 2 mL, 1.5 mL, 1.9 mL, 1.8 mL, 1.6 mL, 1.2 mL, 0.5 mL and 0 mL of distilled water. Consider the first tube as blank and add to the second tube 0.5 mL of the diluted polysaccharide sample (1:100), and add to each one of the six remaining tubes, respectively, 0.1 mL, 0.2 mL, 0.4 mL, 0.8 mL, 1.5 mL and 2 mL of *Stock solution of Nacetil neuraminic acid (NANA)* in the concentration of 80 µg/mL and shake. Add to each tube (blank, standard and sample) 2 mL of *Staining reagent*. Shake and put the set of tubes in water bath at 100°C for 15 minutes. After cooling the tubes in ice bath for 10 minutes, add to each tube 4 mL of *Organic phase* and shake for 30 seconds. Measure the absorbance of the resulting solutions at 585 nm, using blank for zero adjustment. Only the upper part (blue color) must be submitted to reading. Plot the analytical curve from the data obtained.

Resorcinol solution 4%: dissolve 2 g of resorcinol in distilled water, transfer to a 50 mL volumetric flask, complete the volume with distilled water and homogenize. This solution is stored at 4°C for one week. If a rosy color appears, the solution must be discarded.

Copper sulfate solution 0.1 M: dissolve 2.497 g of copper sulfate in volumetric flask containing 2/3 of distilled water and complete the volume to 100 mL with distilled water. The solution must be stored at room temperature.

Organic phase: transfer to a 100 mL volumetric flask 15 mL of butyl alcohol, complete the volume with butyl acetate and homogenize.

N-acetyl neuraminic (NANA) stock solution: transfer to a 100 mL volumetric flask 40 mg of *N*-acetyl neuraminic acid, complete the volume with distilled water and homogenize. Distribute 2 mL of this solution in vials with small volume and freeze at -20°C.

Working solution at 80 µg/mL: transfer to a 10 mL volumetric flask 2 mL of the *N-acetyl neuraminic acid (NANA) stock solution*, complete the volume with distilled water and homogenize.

Staining reagent: at the moment of use, transfer to a 50 mL volumetric flask 40 mL of concentrated hydrochloric acid, 2.5 mL of *Resorcinol solution 4%* and 0.25 mL of *Copper sulfate solution 0.1 M*. Complete to 50 mL with distilled water and homogenize.

Calculate the percentage of sialic acid according to the formula:

$$\%SiAc = \frac{Tc \times 291.80 \times 100}{m \times 0.5 \times 309.28}$$

where

%SiAc = sialic acid percentage;
Tc = content found on the curve;
m = dried mass of the sample (mg).

Not less than 80% (w/w) of the group C polysaccharide, when *N*-acetyl neuraminic acid is used to prepare the reference solution. Not less than 56% (w/w) of groups Y and W_{135} polysaccharides. The reference solutions used are *N*-acetyl neuraminic acid and glucose for the group Y, and *N*-acetyl neuraminic acid and galactose for the group W_{135}.

Calcium. Dilute the sample to approximately 10 mg of dry polysaccharide per milliliter. In an Erlenmeyer flask, put exactly 1 mL of sample, 25 mL of distilled water, indicator (calcon) and 2 mL of *Sodium hydroxide solution 10%*. Titer with *EDTA solution 0.005 M* until the indicator changes color. Carry out in parallel a blank test.

EDTA solution 0.005 M: dissolve 925 mg of edetate disodium in distilled water, transfer quantitatively to a 500 mL volumetric flask, complete the volume with distilled water and homogenize.

Hydroxide solution 10%: pipet 20 mL of a sodium hydroxide solution at 50% (w/v), transfer to a 100 mL volumetric flask, complete the volume with distilled water and homogenize. Calculate the calcium concentration by the formula:

$$\%Calcium = \frac{V \times N \times f \times 0.02 \times 100}{polysaccharide\ mass\ (g)}$$

where

V = EDTA volume spent (mL);
N = EDTA normality;
f = EDTA correction factor;
0.02 = calcium mEq.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Use for each cultivation medium 10 mL or the equivalent to 100 doses, whichever is lowest. Complies with the test.

Pyrogens (5.5.2.1). Dilute the polysaccharide to 0.025 µg/mL and inject 1 mL/kg. Do not reuse the animals used in the test. Complies with the test.

In the preparation of the bulk vaccine product, an adjuvant, a preservative and a stabilizer may be added before the final dilution with adequate diluent. Before packaging, the product samples must be submitted to Sterility test. If it complies with the requisite, the product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

The identification test must be carried out by an approved *Immunochemical method* **(5.6)**, using specific antibodies for each group of polysaccharides.

CHARACTERISTICS

Aspect. The lyophilized vaccines must be presented at the form of a white mobile pastille. After reconstitution with the diluent, the vaccine must be presented as a colorless solution.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

Distribution by molecular size. Proceed as described in *Distribution by molecular size* for purified polysaccharides, by size exclusion.

For a vaccine groups A and C, 65% and 75%, respectively, of the groups A and C are eluted before Kd (distribution coefficient) equal to 0.50.

For a tetravalent vaccine (groups A, C, Y and W135), when chromatography and an immunochemical method are applied, the Kd for a main peak must be below or equal to 0.70 for the groups A and C, 0.57 for group Y and 0.68 for group W135.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Pyrogens (5.5.2.1). Dilute the vaccines to contain in each mL 0.025 µg of polysaccharide for a monovalent vaccine; 0.050 µg of polysaccharide for a divalent vaccine; and 0.10 µg for a tetravalent vaccine. Inject 1 mL/kg. Do not reuse the animals used in the test. Complies with the test.

ASSAY

Concentration of polysaccharides serogroups A, C and W_{135}.

To carry out the test, a capillary electrophoresis equipment must be used, containing micro-processed digital electronic stabilizer, UV with wavelength of 200 nm, deuterium lamp and automatic sampler, coupled to a computerized system with software for results acquisition and processing. Use 10.0 KV of voltage, current in the range of 4060 µA, 0.5 W of power and injection pressure of 50 mbar.

Calibrate the system to conduct the analysis by storing the capillary with sodium hydroxide *M* for 20 minutes, with sodium hydroxide 0.1 *M* for 10 minutes, and with ultra-purified water for five minutes.

Sodium phosphate dibasic solution 0.2 M: weigh 100 mL, 2.8 g of sodium phosphate disodium on analytical balance, dissolve in approximately 70.0 mL of ultra-purified water in ultrasonic bath until full dissolution, and adjust pH with hydrochloric acid solution 0.1 *M* until pH 7.0. Transfer the solution to a 100 mL volumetric flask, add ultra-purified water until completing the volume and homogenize.

Sodium tetraborate solution 0.2 M: weigh in a 100 mL vial 4.024 g of disodium tetraborate on analytical balance, dissolve in approximately 70 mL of ultra-purified water in ultrasound with heating (temperature range: 40-45°C) for approximately 30 minutes until full dissolution of crystals. Transfer the solution to a 100 mL volumetric flask, add ultra-purified water until completing the volume. homogenize the solution.

Phosphate-borate buffer: take an aliquot of 4 mL from the *Sodium phosphate dibasic solution 0.2 M*, add with 1 mL of *Sodium tetraborate solution 0.2 M* and 10 mL of ultra-purified water. Ratio of 4:1:10. The final pH of this solution is 9.0. The buffer solution must be filtered in a 0.22 µm pore size filter before being used.

Sodium hydroxide solution M: weigh, accurately, 4 g of sodium hydroxide and dissolve in approximately 70 mL of ultra-purified water. Transfer, quantitatively, the solution to a 100 mL volumetric flask, add ultra-purified water until completing the volume and homogenize. The solution must be filtered in a 0.22 µm pore size filter before being used.

Sodium hydroxide solution 0.1 M: dilute 10 times the *Sodium hydroxide solution M*. The solution must be filtered in a 0.22 µm pore size filter before being used.

Prepare standards of polysaccharides A, C and W_{135} to be used in the calibration curve, at the concentration of 500 µg/mL. Reconstitute the meningococcal vaccine with *Phosphate-borate buffer* and filter in a 0.22 µm pore size filter before transferring to the equipment sampling vials.

The standard solutions used as points of the calibration curve must be prepared in test tubes (5 tubes = P1, P2, P3, P4 and P5), adding, respectively, 450 µL, 600 µL, 750 µL, 900 µL and 1050 µL of the solution containing 500 µg/mL of polysaccharide from each serogroup and, also respectively, 1050 µL, 900 µL, 750 µL, 600 µL and 450 µL of *Phosphate-borate buffer*. (Note: filter all points of the curve in a 0.22 µm pore size filter before transferring to the equipment sampling vials).

Plot the calibration curve for each polysaccharide serogroup by linear regression (concentration of points of the curve *versus* areas of signals obtained) for the standards of serogroups W_{135}, C and A.

Calculate the sample concentration from results of the areas plotted in each standard curve from each polysaccharide (W_{135}, C and A) according to the straight line formula: y = ax + b.

$$\text{Polysaccharide concentration (µg/mL)} = \left(\frac{y-b}{a}\right)$$

$$\text{Polysaccharide concentration (µg/dose)} = \left(\frac{y-b}{a}\right) \times 2.5$$

where

y = area related to each polysaccharide;
a = angular coefficient;
b = linear coefficient;
2.5 = buffer volume used for lyophile reconstitution.

The following methods can also be used:

For a vaccine from groups A and C, check the phosphorus and sialic acid concentration as already described.

For a tetravalent vaccine, use an adequate immunochemical method with a reference standard for each of the groups A, C, Y and W_{135}.

Each polysaccharide must be included in the vaccine in the range of 70% to 130% of the value established on the label.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MENINGOCOCCAL C (CONJUGATED) VACCINE

Vaccinum meningococcale classis C conjugatum

The meningococcal group C (conjugated) vaccine is a liquid or lyophilized preparation of capsular polysaccharide, obtained from a strain of *Neisseria meningitidis* group C, covalently bonded to a carrier protein.

The meningococcal group C polysaccharide consists of repeated units of sialic acids, partially *O*-acetylated or *O*-deacetylated, bonded with glycoside bridges 2α□9. The carrier protein, when conjugated to the meningococcal group C polysaccharide, is capable of inducing a T-cell dependent immune response. The vaccine may contain an adjuvant.

The production of meningococcal group C polysaccharide is based on the seed lot system, which is an amount of ampoules with lyophilized *N. meningitidis* group C, with uniform composition, obtained from a lyophilized strain of known origin. The culture media used to preserve the viability of the lyophilized strain cannot contain animal-origin proteins. The culture purity must be assessed through colony morphology, microscopic exam, sample inoculation in adequate cultivation media and culture agglutination with specific antiserum.

The group C *N. meningitidis* is cultivated in adequate liquid medium that does not contain high molecular weight polysaccharides. The culture can be inactivated by heat and filtered before the polysaccharide precipitation. The precipitate is purified by adequate methods for removal of nucleic acids, proteins and lipopolysaccharides; the final step of purification must consist of a precipitation with ethyl alcohol, and a *O*-deacetylation step can also be included. Volatile substances in the purified polysaccharide, including water, are determined by thermogravimetry or another adequate method. The value is used for calculating results from other tests with reference to the dried substance.

Only the group C meningococcal polysaccharides that meet the following requirements can be used in the formulation of the bulk vaccine product.

Identification. The meningococcal group C polysaccharide is identified by an *Immunochemical method* **(5.6)** or another adequate method, such as 1H nuclear magnetic resonance spectrometry.

Protein. Proceed as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine*. Not more than 1.0% of protein in the dry substance.

Nucleic acid. Proceed as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine*. Not more than 1.0% per gram of polysaccharide, calculated with reference to the dry substance.

Distribution by molecular size. The percentage of polysaccharide eluted before a given Kd (distribution coefficient) value or within a Kd value range is determined by size-exclusion *High-efficiency liquid chromatography* **(5.2.17.4)**. An acceptable value is established specifically for the product and each lot of the meningococcal group C polysaccharide must comply with this limit.

***O*-acetyl groups.** Proceed as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine*. An acceptable value is established for each product and each lot of the meningococcal group C polysaccharide must comply with this limit.

Sialic acid. Proceed as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine*. Not less than 0.800 g of sialic acid per gram of meningococcal group C polysaccharide.

Residual reagents. When relevant, tests must be carried out to determine residues from reagents used during inactivation and purification. An acceptable value for each reagent is established specifically for the product and each lot of the meningococcal group C polysaccharide must comply with this limit. If the methods for removing a residual reagent have been validated, the test on the meningococcal group C polysaccharide can be omitted.

Bacterial endotoxins (5.5.2.2). Not more than 100 EU/µg of meningococcal group C polysaccharide.

CARRIER PROTEIN

The carrier protein, when conjugated to the meningococcal group C polysaccharide, must be capable of inducing an adequate immunogenic activity. Tetanus anatoxin or diphtheria protein CRM 197 are approved for the conjugation. The carrier proteins are produced through culture of the respective microorganisms, where the bacterial purity is checked. The culture can be inactivated and the carrier protein is purified by adequate methods. The carrier protein to be used in the conjugate preparation must meet the following requirements. The microorganisms used for production of the carrier protein must be cultivated in culture media free from substances that may cause toxic or allergenic reactions to humans. If any component of animal origin is used in the preparation or preservation of the seed lot, or in the production process, it must meet the requirements established by the national regulatory authority.

Identification. The carrier protein is identified serologically by an adequate *Immunochemical method* **(5.6)**. The physical-chemical methods that can be used to characterize the protein comprise SDS-PAGE; isoelectric focusing; CLAE; analysis of amino acids; sequence of amino acids; circular dichroism; fluorescence spectroscopy; peptide mapping; and mass spectrometry.

Tetanus anatoxin. The tetanus anatoxin is produced as described in the monograph *Adsorbed tetanus toxoid*. It meets the requirements for purified tetanus anatoxin, except for the sterility test, which is not required. The product presents minimum antigenic purity of 1500 Fl/mg of protein nitrogen.

Diphtheria protein CRM 197. It contains not less than 90% of diphtheria protein CRM 197, determined by an adequate method. Some tests must be carried out, for validation or routinely, to demonstrate absence of toxicity in the product.

The meningococcal group C polysaccharide is chemically modified, being partially depolymerized before or during the procedure. The conjugate is obtained by covalent bond of the activated meningococcal group C oligosaccharide with the carrier protein and must be submitted to the following controls.

Saccharide concentration. The saccharide content is determined by an adequate validated test, such as the assay *Sialic acid*, as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine,* and *Anion exchange liquid chromatography with pulsed amperometric detection* **(5.2.17.3)**.

Protein. The protein content must be determined by a method validated and approved by the national regulatory authority. An acceptable value is established for each product and each batch of bulk conjugate must comply with this limit.

Polysaccharide/protein ratio. Quotient between polysaccharide concentration and protein concentration. For each conjugate, the ratio must comply with the range approved by the national regulatory authority.

Distribution by molecular mass. The percentage of polysaccharide eluted before a given Kd value or within a Kd value range is determined by size-exclusion *High-efficiency liquid chromatography* **(5.2.17.4)**. An acceptable value is established specifically for the product and each batch of bulk polysaccharide conjugate must comply with this limit.

Free polysaccharide. The concentration of free polysaccharide is determined after removing the conjugate, by ion exchange, size-exclusion or hydrophobic chromatographies, ultrafiltration, *Immunochemical assay* **(5.6)** or other validated methods. An acceptable value is established for each product and each batch of bulk conjugate must comply with this limit.

Free protein. The concentration can be determined directly by an adequate method or by derivation, through calculation of results from other tests. The value must be within the limits approved for each product.

Residual reagents. The removal of residual reagents, such as cyanide, is confirmed by adequate tests or by process validation.

Sterility (5.5.3.2.1). Complies with the test. Use for each cultivation medium 10 mL or the equivalent to 100 doses, whichever is lowest.

In the preparation of the finished bulk vaccine product, an adjuvant, a preservative and a stabilizer may be added before the final dilution with adequate diluent. Before packaging, the product samples must be submitted to *Sterility* and *Thiomersal* tests.

The product is packaged in adequate containers and, if this is the case, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

The identity test must be carried out by an approved *Immunochemical method* **(5.6)**, using specific antibodies for the purified polysaccharide.

CHARACTERISTICS

pH (5.2.19). Determine the pH in the vaccine sample or, in the case of the lyophilized component, pH must be determined after reconstitution with the adequate diluent. The limits must be determined by the history in the product registration.

Determination of volume (5.1.2). Complies with the test.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

Thiomersal. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Aluminum. Proceed as described in the monograph *Vaccines for human use*. Not more than 1.25 mg/individual human dose. The manufacturer has the option of using the result obtained in the product before packaging.

Free polysaccharide. The concentration of free polysaccharide is determined after removing the conjugate by ion exchange, size-exclusion or hydrophobic chromatographies, ultrafiltration, *Immunochemical method* **(5.6)** or other validated methods. An acceptable value consistent with adequate immunogenicity, as described in clinical trials, is established for each product and each final batch must comply with this limit.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Bacterial endotoxins (5.5.2.2). Complies with the test. Not more than 25 EU per human dose.

ASSAY

Saccharide concentration. Determine the polysaccharide concentration by the *Sialic acid* assay, as described in the monograph *Meningococcal ACWY (polysaccharidic) vaccine,* or by *Anion exchange liquid chromatography with pulsed amperometric detection* **(5.2.17.3)**. Not less than 80% of the concentration of group C polysaccharide stated on the label.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

POLIOMYELITIS 1, 2 AND 3 (ATTENUATED) VACCINE
Vaccinum poliomyelitidis perorale typus I, II, III

poliomyelitis 1, 2 and 3 (attenuated) vaccine; 09051

The oral vaccine against poliomyelitis consists of mixing attenuated polioviruses types 1, 2 and 3. It is presented as an aqueous homogeneous clear suspension, and may present color due to the presence of pH indicator.

The vaccine production is based on the seed lot system and the strain of each virus serotype cannot contain more than three sub-cultivations from the original lot, and cannot induce neuropathogenesis in monkeys susceptible to the three types of poliovirus. The virus strain must demonstrate adequate immunogenicity and safety for humans.

The replication of each one of the three polioviruses is carried out in culture of susceptible cells and the virus suspension is identified and controlled as for sterility. Stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added after clarification of the virus suspension by an adequate method to remove cell residues. Before the formulation, each purified virus suspension is assessed as for identification, virus concentration, sterility, consistency of the virus characteristic, and neurovirulence in susceptible monkeys. After the mixture, the bulk trivalent vaccine is submitted to controls of virus concentration, sterility, content of stabilizer used, and pH.

The product is packaged in adequate containers, labeled and submitted to the required controls.

Further information related to production criteria and controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Dilute the sample, add an equal volume of mixture of anti-poliovirus 1, 2, 3 sera and incubate at 37°C for one hour. After the incubation, inoculate the mixture in susceptible cells and incubate at 35°C for seven days. As control, use a cell culture inoculated with the vaccine dilution and another not inoculated, which show, respectively, presence and absence of cytopathogenic effect (CPE). The absence of CPE in the cell culture inoculated with the vaccine mixture and anti-poliovirus sera identifies vaccinal viruses.

CHARACTERISTICS

Aspect. Mobile liquid with pinkish to reddish color.

Determination of volume (5.1.2). Complies with the test.

pH (5.2.19). 6.0 to 7.0.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Dilute, in an adequate culture medium, two samples of the vaccine to be analyzed and one sample of reference vaccine. The interval between dilutions is not less than 0.5 $\log_{10}$, and the samples are diluted separately. For determination of each type of poliovirus, add equal volumes of each dilution of vaccine to the adequate mixture of anti-poliovirus serum. Thus, for determining the poliovirus type 1, add dilutions of the sample to the mixture of anti-polio types 2 and 3 sera; for poliovirus type 2, add dilutions to the mixture of anti-polio types 1 and 3 sera; and for poliovirus type 3, add dilutions of vaccine to the mixture of anti-polio types 1 and 2 sera. To determine the total virus, add equal volumes of each dilution of vaccine to the culture medium used in the dilution. Incubate for one to three hours at 35°C to 36°C. After the incubation, inoculate each dilution of vaccine in not less than eight microplate wells with the suspension of Hep2_C cells. Incubate the microplates at 35°C for seven days. Observe the presence or absence of CPE in the cell cultures. Calculate the titer of each serotype by a validated statistical method.

The vaccine potency is the value of the geometric mean of the vials analyzed, expressed in CCID_{50} (cell culture infecting dose 50%) per dose. For the determination to be considered valid, it is necessary that: (a) the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ CCID_{50} for each serotype; (c) the reference vaccine potency varies not more than 0.5 $\log_{10}$ CCID_{50} of the mean titer from each serotype; (d) the CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The potency is not less than 10^6 for the poliovirus type 1, 10^5 for the poliovirus type 2, and $10^{5.78}$ for the poliovirus type 3. The confidence interval of 95% of the assay cannot differ from a factor greater than $10^{0.5}$ of the CCID_{50} estimated, for each type of virus contained in the vaccine. If the sample does not meet the requisites, repeat the test for the type(s) of virus where the potency is below the minimum value specified. The potency is the average of the two determinations carried out.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate two samples of vaccine at the temperature of 37°C for 48 hours and determine the total content of virus (type 1 + type 2 + type 3), using the method described in *Assay*. The vaccine may lose not more than 0.5 $\log_{10}$ CCID_{50} in relation to the total virus titer, determined in the sample stored in adequate temperature conditions. If it does not meet the requisite, repeat the test. The final titer is the average of the two assays carried out.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

POLIOMYELITIS 1, 2 AND 3 (INACTIVATED) VACCINE

Vaccinum poliomyelitidis inactivatum

The inactivated poliomyelitis vaccine is composed of a mixture of inactivated polioviruses types 1, 2 and 3 and presented as a clear liquid, and some color may develop to the presence of pH indicator.

The vaccine production is based on the seed lot system and each serotype resent can contain not more than 10 sub-cultivations from the original lot. The virus replication is carried out in a culture of susceptible cells and the virus suspension is identified and controlled as to sterility. After clarification of the virus suspension by an adequate method for removing cell residues, each virus suspension is concentrated and purified. The suspension of each type of virus is identified, assessed as for sterility, mycoplasmas and virus concentration. The inactivation of each virus suspension is conducted separately, by an appropriate method such as addition of chemical agents in adequate conditions. The most commonly used chemical agent is formaldehyde. Before mixing the three types of inactivated polioviruses and adding preservative and other substances, each virus suspension is assessed as to the inactivation effectiveness. After mixing the three polioviruses and before packaging, controls on absence of infective particles in susceptible cells, sterility and preservative concentration are carried out.

The vaccine is packaged in adequate containers, labeled and submitted to the required controls.

Further information related to production criteria and controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Meets the requisites described in *Assay.*

PHYSICOCHEMICAL TESTS

Protein. Use the Kjeldahl method **(5.3.3.2)** or another adequate and validated method. Not more than10 µg/dose.

Residual formaldehyde. Proceed as described in the monograph *Vaccines for human use*. Not more than 200 ppm. The manufacturer has the option of using the result obtained in the product before packaging.

Bovine albumin. Not more than 50 ng per human dose, determined *by Immunochemical method* **(5.6)**.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Proceed as described in the monograph *Vaccines for human use*.

Bacterial endotoxins (5.5.2.2). Not more than 5 EU per dose.

ASSAY

Use an immunoenzymatic method with proven sensitivity for assessment of antigen D concentration from each of the three poliovirus serotypes present in the vaccine. Assess the reference vaccine in parallel.

The potency is expressed in units of antigen D per dose for the poliovirus types 1, 2 and 3 whose minimum limits must comply with the history of product registration, approved by the national regulatory authority.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

RABIES (INACTIVATED) VACCINE

Vaccinum rabiei inactivatum

rabies (inactivated) vaccine; 09053

The vaccine is an inactivated suspension prepared from the rabies virus replicated in cell culture and can be presented in the lyophilized form or in suspension. After the reconstitution with appropriate diluent, the lyophilized vaccine has the aspect of clear homogeneous suspension, and can present color due to the presence of pH indicator.

The vaccine production is based on the virus seed lot system, which must be duly characterized. The lots are submitted to virus identification, sterility and infective potency controls. Additionally, it is necessary that the virus strain demonstrates adequate immunogenicity for humans. The virus replication is carried out in culture of susceptible cell and controlled as for sterility, virus identification and infective potency. In the production process, an intermediate virus suspension with known concentration is prepared and submitted to centrifugation, purification and virus inactivation by a validated method, where beta-propiolactone at 1:4000 or irradiation by ultraviolet is commonly used. After the inactivation, the product is concentrated and sterility, virus inactivation and immunogenic activity tests are carried out. The final preparation must be made isotonic and may contain preservatives and pH indicator. Before packaging, the product is submitted to sterility, immunogenic activity and preservatives controls.

The vaccine is packaged in adequate containers, and can be lyophilized, labeled and submitted to adequate controls.

IDENTIFICATION

The *Determination of immunogenic activity* can be used.

Phenol. Assay applied when preservative is present. Proceed as described in the monograph *Vaccines for human use*. Not more than 0.15% (1500 ppm). The manufacturer has the option of using the result obtained in the product before packaging.

Thiomersal. Assay applied when preservative is present. Proceed as described in the monograph *Vaccines for human use*. Not more than 0.015% (150 ppm). The manufacturer has the option of using the result obtained in the product before packaging.

Residual humidity. Assay applied to the lyophilized product. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

Pyrogens (5.5.2.1). Complies with the test. Inject in each rabbit one human dose of vaccine diluted (1:10) in sterile and pyrogen-free saline solution.

Verification of virus inactivation.

Use one of the methods described below.

A. Inoculate, intracerebrally, 10 µL of the sample in not less than 20 nursing mice (5 to 10 days) and 30 µL in not less than 20 Swiss albino mice with 10 g to 15 g. Observe the inoculated animals for 21 days. During the observation period, the animals cannot present neurological symptoms or death. If this occurs, carry out the *Direct immunofluorescence* test on the brain of suspected animals. The product meets the requisites if none of the inoculated animals presents classic symptoms of rabies. If evolution of neurological symptoms or death of inoculated animals is verified, the confirmation of rabies infection will depend on the positivity detected in the *Direct immunofluorescence* assay.

Direct immunofluorescence: cut the brain of the mouse with suspected rabies, so that the central sections are faced upward. Prepare paired impressions on glass slide for microscopy, duly identified. In parallel, prepare in slides duly identified impressions for control, using mice knowingly negative and positive for rabies, which will respectively be used as negative and positive controls. Allow the slides to dry for 30 minutes at temperature of 20°C to 25°C and fix the impressions in acetone previously chilled at -20°C, maintaining them incubated for two to four hours at -20°C. Allow the slides to dry at temperature of 20°C to 25°C. Proceed to staining, circling the impressions with a substance that can be used to retain the conjugate during the incubation period. Enamel can be used. Thaw, at the moment of use, two aliquots of conjugate working dilution for identification of the rabies virus (antibodies against the rabies virus usually marked with fluorescein isothiocyanate) and dilute one of the aliquots, in the ratio 1:5, with suspension at 20% of brain from non-infected mice (SCN). Dilute the other aliquot likewise, but in suspension at 20% of brain from mice infected with the rabies virus (SCI), and homogenize. Position the slide so that its identification faces the left side of the operator and place the mixtures on the impressions, using different Pasteur pipettes. Cover the impression on the left with the mixture "conjugate + SCN" and the one on the right with the mixture "conjugate + SCI" and incubate in humid chamber for 30 minutes at 37°C. After incubation, wash with saline phosphate buffer PBS (with pH of 7.6 to 8.0) and leave for 10 minutes immersed in the same diluent. Drain the diluent and wash with distilled water to avoid crystals formation. Allow to dry and assemble with buffered glycerin pH 8.5 to 9.0 to increase the intensity of fluorescence, putting a coverslip. Examine in fluorescence microscope with magnification of 100 times. Examine first the negative control impression treated with the mixture "conjugate + SCN". The SCN is free from rabies virus; therefore, the conjugate remains free to react with the reagent present in the impression, thus there is fluorescence emission. Accordingly, the antigen will be evidenced as inclusions or fine dust, in green color. Then, observe the positive control impression treated with the mixture "conjugate + SCI". The conjugate is adsorbed by the antigen present in the SCI, therefore there is no conjugate left available to react with the rabies antigen present in the impression, and there is no fluorescence emission. The antigen will not be evidenced in this impression. Observe the impressions from the negative control slide in the same order. No fluorescence mus be observed; after checking that the controls are satisfactory, observe the impressions of the material being tested; the presence of rabies virus in the material analyzed, that is, positivity, is verified when fluorescence is observed, on the test slide, only in the impression that received the mixture "conjugate + SCN", which is not verified on the impression where the mixture "conjugate + SCI" was placed; the absence of rabies virus in the material examined, that is, negativity, is verified when no fluorescence is observed.

B. Amplified method for verification of virus inactivation.

Inoculate an amount equivalent to not less than 25 doses of *Rabies (inactivated) vaccine* in five cell cultures, from the same type used in the vaccine production or another cell culture with sensitivity similar to the rabies virus. The ratio used is of 3 cm^2 of culture per milliliter of vaccine. After the virus adsorption, culture medium is added in ratio not greater than 1:3 of the volume of the vaccine used. The cultures are observed for 21 days and the detection of presence of rabies virus can be carried out by inoculation in mice or by immunofluorescence.

By inoculation in mice, on the 14th and 21st days of cultivation: 0.03 mL of a mixture of samples from supernatant of cultures is inoculated, intracerebrally, in 20 Swiss albino mice with 12 g to 15 g. The animals are observed for 14 days and any rabies symptom must be confirmed by immunofluorescence.

By immunofluorescence: the cultures are examined on the 21st day after inoculation and the presence of rabies virus is searched through immunofluorescence test.

The test is considered satisfactory if the presence of rabies virus or onset of cytopathic effects in the cultures is not detected.

DETERMINATION OF IMMUNOGENIC ACTIVITY

Challenge method in mice: prepare, at least, three dilutions of the sample and the reference vaccine in buffered phosphated saline solution with pH 7.6 and inoculate, intraperitoneally, 0.5 mL of each dilution in, at least, 16 Swiss albino mice with 10 g to 15 g. Reserve 30 animals not inoculated for control of the challenge virus titer. Conduct booster immunization by inoculating the same dilutions in each group of mice, seven days after the first immunization. Seven days after the second immunization, carry out the challenge, inoculating in each mouse the volume of 30 µL, with approximately 50 LD_{50} of fixed rabies virus from the strain CVS (*challenge virus standard*), intracerebrally, from each mouse. Prepare two decimal dilutions from the challenge dilution and inoculate 30 µL of these dilutions and the challenge dilution, intracerebrally, in the three groups of 10 mice from non-immunized animals. Observe the animals for 14 days, recording the number of live animals from each mixture. The animals dead before the fifth day after the inoculation must not be considered for calculation of the immunogenic activity. Calculate the effective doses 50% (ED_{50}) of the sample and reference vaccine, as well as the LD_{50} of the challenge virus, by a statistically validated method. The response range produced by the test sample (survival percentage) must be between the highest and the lowest dilution used in the standard regression curve, which must present a linear relation. The immunogenic activity is determined by the formula:

$$IA\ (IU/mL) = \frac{ED_{50}\text{ of the sample} \times IU/mL\text{ of the reference vaccine}}{ED_{50}\text{ of the reference vaccine}}$$

IA = immunogenic activity

Not less than 2.5 IU/individual human dose. When the value of immunogenic activity (IU/mL) is mentioned, the real LD_{50} number obtained in the challenge virus titration, which is equal to the antilogarithm of the difference between the LD_{50} calculated and the dilution of the challenge dose used, must be mentioned. The confidence limits must not be below 25% or above 400% of the activity determined. The challenge suspension titration must present not less than 10 LD_{50}. The statistical analysis must demonstrate that there are no deviations of linearity and parallelism of the dose-response curves.

THERMOSTABILITY

Incubate the sample at temperature between 35°C and 37°C for four weeks and proceed to the *Determination of immunogenic activity*. Not less than 2.5 IU/individual human dose.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

HUMAN ROTAVIRUS (ATTENTUATED) VACCINE

Vaccinum rotaviri vivum perorale

The rotavirus (attenuated) vaccine is obtained from one or more serotypes of a live attenuated strain of human rotavirus, cultivated in an adequate cell substrate. It is presented as an homogeneous clear aqueous or lyophilized suspension to be immediately reconstituted to result in a slightly turbid suspension. The vaccine may present color due to the presence of a pH indicator.

The vaccine production is based on the seed lot system and a cell bank system, and the method must demonstrate production of vaccines with consistency and meet the requisites for immunogenicity, safety and stability. The virus replication is conducted in a culture of susceptible cells, deriving from a primary cell bank. The virus in the final vaccine must not have a higher number of passages than the ones used to prepare the vaccine used on clinical trials. The strain from each of the virus serotypes used for the preparation of the primary seed lot must have historical records as to its origin and subsequent manipulations, and also comply with controls on identity, virus concentration and adventitious agents. Virus intermediate cultures or production inoculates are submitted to assays on identity, sterility, virus concentration and adventitious agents. Monovalent virus suspensions or the mixture of several suspensions obtained for the vaccine production is controlled as for identity, sterility, adventitious agents and virus concentration. The monovalent virus suspension is then purified by an adequate method for removing cell residues and, before the formulation, must be assessed as for virus concentration, sterility and residual cellular DNA.

The bulk finished product is obtained by mixing one or several purified monovalent virus suspensions, and may contain more than one serotype and stabilizer substances that do not change the product efficacy. Before packaging, the bulk vaccine is submitted to sterility test.

The product is packaged in adequate containers, labeled and submitted to the required controls.

Further information related to production criteria and controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

A. Use an immunological assay with specific antibodies for each type of virus that composes the vaccine.

B. Meets the requisites described in *Assay*.

CHARACTERISTICS

Determination of volume (5.1.2). Complies with the test.

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

A. By *Infectivity on cells*

The assay is based on visualization of infected areas from a single layer of MA-104, Vero cells or other susceptible cells cultivated in adequate culture medium and there must be no interference or potentiation between the serotypes present in the vaccine. The rotavirus vaccine assay is conducted in not less than three vials of sample and one vial of the reference vaccine in triplicate to validate it. If the vaccine has more than one serotype of rotavirus, calculate the titer individually by a method that demonstrates specificity. Calculate the vaccine titer by an adequate statistical method and express in $\log_{10}$ $CCID_{50}$ (cell culture infecting dose 50%) per dose.

For the determination to be considered valid, it is necessary that: (a) at the end of the assay the cell culture control presents an unchanged single layer; (b) the variation of potency between the three vaccine vials is not more than 0.5 $\log_{10}$ $CCID_{50}$; (c) the confidence interval ($P = 0.95$) of the estimated virus concentration on the reference vaccine for the three replicates combined is lower than ± 0.3 log 10 $CCID_{50}$; (d) the cytopathic effect (CPE) is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The assay must be repeated if the confidence interval ($P = 0.95$) of the combined concentration of virus in the vaccine is greater than ± 0.3 log 10 $CCID_{50}$ (or an equivalent value expressed in the unit of the method used for the assay). The data generated from valid assays must be combined by adequate statistical methods to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the concentration of combined viruses must be lower than ± 0.3 log 10 $CCID_{50}$.

The minimum titer of each virus serotype is provided by the producer to the national regulatory authority, according to analysis of results from clinical trials.

B. By *Assay based on the comparison of the vaccine capacity to produce viral RNA*

For each lot, use at least three vials of sample and one vial of the reference vaccine in triplicate. Infect cell cultures in microtitration plates with serial dilutions of the sample being tested and the reference vaccine. After incubation to allow the virus replication, the viral RNA in individual wells is cleared from cells and quantified by *Nucleic acid amplification techniques* **(5.5.1.10)**, such as the real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Calculate the concentration of individual virus for each vaccine container against the reference preparation, as well as the corresponding combined concentrations of virus, using an adequate statistical method. The combined estimate of virus concentration for the three vaccine vials cannot be lower than that indicated on the label.

For the determination to be considered valid, it is necessary that (a) the negative control must prove to be undeniably negative; (b) the positive control must provide positive reaction; (c) no changes in non-infected cells must be observed in the cell control; (d) there must be positive detection in the control of cells contaminated with viral RNA; (e) the dose-response curves must result in a significant slope without significant deviations of linearity or parallelism.

The assay must be repeated if the confidence interval ($P = 0.95$) of the combined concentration of virus in the vaccine is greater than ± 0.3 log 10 $CCID_{50}$ (or an equivalent value expressed in the unit of the method used for the assay). The data generated from valid assays must be combined by adequate statistical methods to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the concentration of combined viruses must be lower than ± 0.3 log 10 $CCID_{50}$.

The minimum titer of each virus serotype is provided by the producer to the national regulatory authority, according to analysis of results from clinical trials.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate the vaccine sample at 37°C, for seven days, and analyze according to methodology described for the product potency. The vaccine can lose not more than 0.5 $\log_{10}$ $CCID_{50}$/dose, in relation to the titer of the vaccine stored in adequate temperature conditions. The vaccine being tested cannot present a titer below the one specified for the product potency.

PACKAGING AND STORAGE

Proceed as described in the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

RUBELLA (ATTENUATED) VACCINE
Vaccinum rubellae vivum

rubella (attenuated) vaccine; 09054

The vaccine is composed of live attenuated viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and can develop coloration due to the presence of pH indicator.

The vaccine production is based on the seed lot system, and the strain of virus used or five consecutive lots of the vaccine cannot induce neuropathogenesis in monkeys susceptible to the rubella virus. Additionally, the virus strain must demonstrate adequate immunogenicity and safety for humans. The virus replication is carried out in a culture of susceptible cells and the virus suspension is identified and controlled as to sterility. Stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added after clarification of the virus suspension by an adequate method to remove cell residues. Before packaging and lyophilization, the product is analyzed as for sterility, concentration of viruses and proteins derived from animal serum used in the cultivation.

The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

Further information related to the production criteria and their controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Reconstitute the vaccine with an appropriate diluent and add to an equal volume of serum containing neutralizing antibodies for the rubella virus. Incubate for 90 minutes at temperature of 4°C to 8°C. After incubation, inoculate the mixture of vaccine with serum in a culture of susceptible cells, and store for 12 days at temperature of 32°C to 33°C. As control, a cell culture inoculated with the vaccinal virus and other not inoculated, respectively, must show presence and absence of cytopathogenic effect (CPE). The absence of CPE in the cell culture identifies the vaccinal virus.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 2%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Proceed protected from direct light. Dilute, in intervals of not more than 1.0 $\log_{10}$, two samples of the vaccine to be analyzed and one sample of reference vaccine in adequate culture medium. Inoculate each dilution in not less than 10 microplate wells containing RK-13 cells in suspension and incubate at temperature of 32°C to 33°C for 12 days. Observe the presence or absence of CPE in the cell cultures. Calculate the vaccine titer by a validated statistical method. The vaccine potency is the value of the geometric mean of the vials analyzed, expressed in $CCID_{50}$ (cell culture infecting dose 50%) per dose. For the determination to be considered valid, it is necessary that: (a) the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ $CCID_{50}$; (c) the reference vaccine potency does not vary more than 0.5 $\log_{10}$ $CCID_{50}$ from its mean titer; (d) CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The potency is not less than 10^3 $CCID_{50}$/dose. If the sample does not meet the requisites, repeat the test. The potency is the average of the two determinations carried out. The plaque forming units (PFU) method can also be used and its potency value for product approval must be correlated with the $CCID_{50}$ one.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate the sample at temperature of 37°C for seven days and proceed as established in *Assay.* The vaccine can lose not more than 1.0 $\log_{10}$ $CCID_{50}$ in relation to the titer determined on the sample stored in adequate conditions. Additionally, it cannot have a titer below the one established for approval in *Assay*. If it does not meet the requisite, repeat the test and the final titer is the average of the two assays conducted.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MEASLES (ATTENUATED) VACCINE

Vaccinum morbillorum vivum

measles (attenuated) vaccine; 09056

The vaccine is composed of live attenuated viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and can develop coloration due to the presence of pH indicator.

The vaccine production is based on the seed lot system, and the strain of virus used or five consecutive lots of the vaccine cannot induce neuropathogenesis in monkeys susceptible to the measles virus. Additionally, the virus strain must demonstrate adequate immunogenicity and safety for humans. The virus replication is carried out in culture of susceptible cells and the virus suspension is identified and controlled as for sterility. Stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added after clarification of the virus suspension by an adequate method to remove cell residues. Before packaging and lyophilization, the product is analyzed as for sterility, concentration of viruses and proteins derived from animal serum.

The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

Further information related to production criteria and their controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

Reconstitute the vaccine with an appropriate diluent and add an equal volume of serum containing neutralizing antibodies for the measles virus. Incubate at 36°C for one hour. After incubation, inoculate the mixture in culture of susceptible cells and keep at temperature of 36°C for seven days. Use as control a cell culture inoculated with the vaccinal virus and other not inoculated, which present, at the end of the test, presence and absence of cytopathogenic effect (CPE), respectively. The absence of CPE in the cell culture identifies the vaccinal virus.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Proceed protected from direct light. Dilute two samples of the vaccine to be analyzed and one sample of reference vaccine, in intervals of not more than 1.0 $\log_{10}$, in adequate culture medium. Inoculate each dilution in not less than 10 wells of the microplate containing Vero cells in suspension. Incubate for seven to nine days between 35°C and 37°C. The cell cultures are observed for the presence or absence of CPE, and the vaccine titer is calculated according to the Spearman & Karber estimation method. The vaccine potency is the value of the geometric mean of the vials analyzed, expressed in $CCID_{50}$ (cell culture infecting dose 50%) per dose.

For the determination to be considered valid, it is necessary that: (a) at the end of the assay the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ $CCID_{50}$; (c) the reference vaccine potency varies not more than 0.5 $\log_{10}$ $CCID_{50}$ from its mean titer; (d) CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The vaccine potency must be not less than $10^{3.7}$ $CCID_{50}$/dose for the Biken Cam 70 strain and $10^{3.0}$ $CCID_{50}$/dose for the other strains. If it does not meet the requisites, repeat the determination of potency and the result is the geometric mean of the two assays conducted.

The plaque forming units (PFU) method can also be used and its potency value for product approval must be correlated with the $CCID_{50}$ one.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate a vaccine sample between 35°C and 37°C for seven days, and analyze according to the method described for the product *Assay*. The vaccine can lose not more than 1.0 $\log_{10}$ $CCID_{50}$ in relation to the titer determined on the sample stored in adequate temperature conditions. It cannot present a titer below the one specified for the product potency.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MEASLES, MUMPS, RUBELLA VACCINE
Vaccinum parotiditis et rubellae et morbillorum vivum

measles, mumps, rubella vaccine; 09055

The vaccine is composed of the mixture of live attenuated mumps, rubella and measles viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and can develop coloration due to the presence of pH indicator.

Each virus component present in the vaccine is produced separately, as described in the specific monographs.

The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

Reconstitute the vaccine with an appropriate diluent and add an equal volume of the mixture of sera containing neutralizing antibodies for the mumps, rubella and measles viruses. Keep for 90 minutes at temperature of 4°C to 8°C, inoculated in culture of susceptible cells and store for 10 days. As test control, cultures of cells inoculated with the vaccine not neutralized with the mixture of sera with antibodies for the three types of viruses and another non-inoculated must indicate presence and absence of cytopathogenic effect, respectively. The absence of CPE in the culture of cells inoculated with the mixture of vaccines plus antibodies identifies the product.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 2%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Proceed protected from direct light. Dilute two samples of the vaccine to be analyzed and one sample of reference vaccine, in intervals of not more than 1.0 $\log_{10}$, in adequate culture medium. To titer each type of virus, add to each vaccine dilution an equal volume of specific heterologous antiserum, according to the following scheme:

Virus to be titrated	***Serum***
Mumps	anti-measles
Rubella	anti-mumps
Measles	anti-mumps

Keep the mixtures for 90 minutes at temperature of 4°C to 8°C, for suitable neutralization. Inoculate each dilution in 10 microplate wells containing the suspension of susceptible cells. For titration of mumps and measles viruses, inoculation is carried out in Vero cells, whereas for rubella, in RK-13 cells. Incubate the microplates containing Vero cells at 36°C for 10 days and the microplates with RK-13 cells at temperature of 32°C to 33°C for 12 days. Observe the cell cultures for the presence or absence of CPE and calculate the titers of each virus present in the vaccine, according to a validated statistical method. The potency of each virus is the value of the geometric mean of the vials analyzed, expressed in $CCID_{50}$ (cell culture infecting dose 50%) per dose. For the determination to be considered valid, it is necessary that: (a) at the end of the assay the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ $CCID_{50}$ for each type of virus; (c) the variation of the reference vaccine titer is not more than 0.5 $\log_{10}$ $CCID_{50}$ of its mean titer for each type of virus; (d) CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The vaccine potency is not less than $10^{3.7}$ $CCID_{50}$/dose for mumps virus and 10^3 $CCID_{50}$/dose for measles and rubella viruses. If the product does not meet the potency requisites, the assay is repeated for the type(s) of virus where the limit titer for approval was not obtained. The vaccine potency is the geometric mean of the two assays carried out.

The plaque forming units (PFU) method can also be used and the potency value for product approval must be correlated with the $CCID_{50}$ one.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate a vaccine sample at 37°C for seven days and analyze according to methodology described for the product potency. The vaccine can lose not more than 1.0 $\log_{10}$ $CCID_{50}$/dose in relation to the titer determined on the sample stored in adequate temperature conditions for each virus type. Additionally, it cannot have a titer below the one established for approval in *Assay*. If it does not meet the requisites, repeat the test and the final titer is the average of the two tests carried out.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MEASLES, MUMPS, RUBELLA AND VARICELLA (ATTENUATED) VACCINE

Vaccinum parotiditis et rubellae et morbillorum et varicellae vivum

The vaccine is composed of a mixture of the live attenuated viruses of mumps, rubella, measles and varicella. The vaccine is presented in lyophilized form and, after the reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and coloration can be developed due to the presence of pH indicator.

The components are produced as described in the specific monographs.

The virus suspensions of each component are mixed and the bulk product is submitted to sterility control.

The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

Reconstitute the vaccine with appropriate diluent and mix with specific antibodies for the mumps, rubella, measles and varicella viruses. When the vaccine is mixed with quantities of antibodies sufficient to neutralize three components, the fourth component must present CPE in the culture of susceptible cells.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. The maximum limit must ensure that the product maintains its stability, according to the registration submitted to the national regulatory authority.

BIOLOGICAL SAFETY TESTS

Sterility. Proceed as described in the monograph *Vaccines for human use*.

ASSAY

The cell lineages and specific antisera must ensure that the potency of each virus is determined without interference from the other three components. Dilute not less than three vials of the vaccine to be analyzed and one sample of reference vaccine in duplicate, in intervals of not more than 1.0 $\log_{10,}$ in adequate culture medium.

Proceed as described in the monographs *Measles, mumps, rubella (attenuated) vaccine* and *Varicella (attenuated) vaccine*. For the determination to be considered valid, it is necessary that: (a) the confidence interval ($P = 0.95$) of the estimated concentration of the reference vaccine for the combined duplicate is lower than ± 0.3 $\log_{10}$ CCID50; (b) the reference vaccine potency varies not more than 0.5 $\log_{10}$ CCID50 for measles, mumps and rubella viruses and 0.5 $\log_{10}$ PFU for the varicella virus from their established titer; (c) the CCID50 number is decreasing in relation to increasing dilutions.

The assay must be repeated if the confidence interval (P = 0.95) of the combined concentration of the viral vaccine concentration is higher than 0.3 log10 CCID50 (measles, mumps, rubella) and 0.3 log10 PFU (varicella); the data obtained from valid assays can only be combined by adequate statistical methods to calculate the viral concentration of the sample. The confidence interval (P = 0.95) of the combined concentration must be not more than 0.3 log10 CCID50 (measles, mumps, rubella) and 0,3 log10 PFU (varicella). Other assay methods can be used, provided they are justified and approved by national regulatory authorities. However, if the vaccine is assessed by the method described above, it must meet the requirements already established.

The vaccine potency is not less than $10^{3.7}$ CCID50/dose for mumps virus and $10^{3.0}$ CCID50/dose for measles and rubella viruses. The minimum concentration of the varicella virus must be approved by the regulatory authorities, according to studies on efficacy and safety described in the vaccine registration.

THERMOSTABILITY

The test is conducted for the components: measles, mumps and rubella in parallel to *Assay*. Incubate a vaccine sample at 37°C for seven days, and analyze according to method described for the product potency. The vaccine can lose not more than 1 $\log_{10}$ $CCID_{50}$/dose in relation to the titer determined on the sample stored in adequate temperature conditions for each virus type. Additionally, it cannot have a titer below the one established for approval of determination of potency.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

MEASLES, RUBELLA VACCINE
Vaccinum rubellae et morbillorum vivum

measles, rubella vaccine; 09057

The vaccine is composed of the mixture of live attenuated rubella and measles viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear homogeneous suspension, and can develop coloration due to the presence of pH indicator.

Each virus component present in the vaccine is produced separately, as described in the specific monographs. The product is packaged in adequate containers, lyophilized, labeled and submitted to the required controls.

IDENTIFICATION

Reconstitute the vaccine with an appropriate diluent and add an equal volume of the mixture of sera with neutralizing antibodies for rubella and measles viruses. Keep for 90 minutes at temperature of 4°C to 8°C. Inoculate in culture of susceptible cells and store for 10 days. As test control, a cell culture, inoculated with the vaccine not neutralized with the mixture of sera containing antibodies for the two types of viruses and another non-inoculated, must indicate presence and absence of cytopathogenic effect (CPE), respectively. The absence of CPE in the culture of cells inoculated with the mixture of vaccines plus antibodies identifies the product.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. Not more than 3%.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Proceed protected from direct light. Dilute two samples of the vaccine to be analyzed and one sample of reference vaccine, in intervals of not more than 1.0 $\log_{10}$, in adequate culture medium.

Inoculate each dilution in 10 microplate wells containing the suspension of susceptible cells. For titrating the measles virus, inoculation is conducted in Vero cells, whereas for the rubella virus, in RK-13 cells. Incubate the microplates containing Vero cells at 36°C for 10 days and the microplates with RK-13 cells at temperature of 32°C to 33°C for 12 days. Observe the cell cultures for the presence or absence of CPE and calculate the titers of each virus present in the vaccine, according to a validated statistical method.

The potency of each virus is the value of the geometric mean of the vials analyzed, expressed in $CCID_{50}$ (cell culture infecting dose 50%) per dose. For the determination to be considered valid, it is necessary that: (a) at the end of the assay, the cell culture control presents an unchanged single layer; (b) the variation of potency between the two vaccine samples is not more than 0.5 $\log_{10}$ $CCID_{50}$ for each type of virus; (c) the variation of the reference vaccine titer is not more than 0.5 $\log_{10}$ $CCID_{50}$ from its mean titer for each type of virus; (d) CPE is decreasing in relation to increasing dilutions; (e) the dilutions used in the assay are between 10% and 90% of the inoculated cell cultures.

The vaccine potency is, not less than $10^{3.7}$ $CCID_{50}$/dose for the Biken Cam 70 strain and 10^3 $CCID_{50}$/dose for the other strains of measles and rubella viruses. If the product does not meet the potency requisites, the assay is repeated for the type(s) of virus where the limit titer for approval was not obtained. The vaccine potency is the geometric mean of the two assays carried out.

The plaque forming units (PFU) method can also be used, and the potency value for product approval must be correlated with the $CCID_{50}$ one.

THERMOSTABILITY

The test is carried out in parallel to *Assay*. Incubate a vaccine sample at 37°C for seven days and analyze according to methodology described for the product potency. The vaccine can lose not more than 1.0 $\log_{10}$ $CCID_{50}$/dose in relation to the titer determined on the sample stored in adequate temperature conditions for each virus type. Additionally, it cannot have a titer below the one established for approval in *Assay*. If it does not meet the requisites, repeat the test. The final titer is the average of the two tests carried out.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

VARICELLA (ATTENUATED) VACCINE

Vaccinum varicellae vivum

The vaccine is composed of live attenuated varicella viruses and presented in lyophilized form. After reconstitution with appropriate diluent, it has the aspect of clear, homogeneous suspension, and can develop coloration due to the presence of pH indicator.

The vaccine production is based on the seed lot system, and the virus strain used in vaccine production must demonstrate adequate immunogenicity and safety for humans, and cannot induce neuropathogenesis in monkeys susceptible to the varicella virus.

Virus replication is carried out in culture of susceptible human diploid cell and the virus suspension is submitted to tests on identification, sterility, virus concentration and adventitious agents. Stabilizer substances, that demonstrably do not change the product efficacy or safety, can be added after clarification of the virus suspension by an adequate method to remove cell residues. The clarified product is submitted to identification and virus concentration controls.

Before packaging and lyophilization, the product is analyzed as for sterility. The product is then packaged in adequate containers, lyophilized, labeled and submitted to required the controls.

Further information related to production criteria and their controls is indicated in the monograph *Vaccines for human use*.

IDENTIFICATION

The vaccine, when neutralized with serum containing specific antibody for the varicella virus, inhibits the formation of plaque forming units (PFU) in susceptible cells as described in *Assay*.

PHYSICOCHEMICAL TESTS

Residual humidity. Proceed as described in the monograph *Vaccines for human use*. The maximum limit must ensure that the product maintains its stability, according to the registration submitted to the national regulatory authority.

BIOLOGICAL SAFETY TESTS

Sterility (5.5.3.2.1). Complies with the test.

ASSAY

Not less than three vials of lyophilized vaccine and one of reference vaccine are submitted to the method of plaque forming units (PFU). The virus concentration of the reference vaccine is established based on historical data from each laboratory and must be monitored by control chart. Dilute the vaccine samples and inoculate in plates or vials containing a single layer of culture of susceptible human diploid cell.

Calculate the individual virus concentration for each vaccine vial and for each replicate of the reference vaccine by validated statistical methods, which is expressed in log10 PFU/dose. For the determination to be considered valid, it is necessary that (a) the confidence interval (P = 0.95) of the estimated concentration of the reference vaccine for the three replicates combined is lower than ± 0.3 log10 PFU; (b) the reference vaccine potency varies not more than 0.5 log10 PFU from its established titer.

The assay must be repeated if the confidence interval (P = 0.95) of the vaccine combined virus concentration is greater than ± 0.3 log10 PFU; data obtained from valid assays can only be combined by adequate statistical methods to calculate the sample viral concentration. The confidence interval (P = 0.95) of the combined virus concentration is not more than ± 0.3 log10 PFU.

Other assay methods can be used, provided they are justified and approved by national regulatory authorities. However, if the vaccine is assessed by the method described above, it must meet the requirements already established.

The minimum potency must be approved by the regulatory authorities, according to studies on efficacy and safety described in the vaccine registration.

PACKAGING AND STORAGE

Comply with the monograph *Vaccines for human use*.

LABELLING

Comply with current legislation.

VACCINES FOR HUMAN USE
Vaccina ad usum humanum

The vaccines for human use are, essentially, medications of prophylactic nature, capable of inducing specific immunity against an infectious agent. Their efficacy and safety must be proven through studies approved by the national quality control authority.

The vaccines can be composed of inactivated microorganisms, attenuated microorganisms, substances produced by microorganisms and antigen fractions. The methods employed for preparation of vaccines depend on each type of product and must comply with standards on good manufacturing practices for pharmaceutical products.

During the vaccine production processes, some substances, such as stabilizers, adjuvants and preservatives, can be added. At the end product, very low concentrations of antibiotics are permitted, with the exception of streptomycin, penicillin and their derivatives. If serum of animal origin is used in the production process, the end product cannot contain more than 50 ng/dose of proteins derived from the serum. Human albumin may be used, provided that the absence of antibodies to hepatitis B, hepatitis C, and HIV 1 and 2 is proven.

BACTERIAL VACCINES

Bacterial vaccines are produced in liquid or solid media, using adequate strains of inactivated bacteria, attenuated (live) bacteria, or their antigenic components. They are presented as a colorless liquid or with different levels of opacity or lyophilized.

For the preparation of these vaccines, both total microorganisms cultivated in adequate culture media and fractions of these microbial agents can be used. The inactivated vaccines must be prepared by physical or chemical methods that do not destroy their antigenic capacity, whereas vaccines from live bacteria are produced with attenuated strains, capable of inducing immunity against a microorganism from the same species or from antigenically related species.

BACTERIAL TOXOIDS

Bacterial toxoids are toxins detoxified by physicochemical treatment, which, despite losing their toxic capacity, maintain the immunogenic activity. The production is based on the seed lot system of strains from specific microorganisms, cultivated in culture media free from substances that may cause toxic, allergic effects and other undesirable reactions to humans.

The toxoids can be presented in liquid or lyophilized form and, in both cases, they can be purified or adsorbed. The ones adsorbed are presented in the form of opalescent suspension in white or slightly brownish color and can form sediment on the bottom of the packaging container.

VIRAL VACCINES

Viral vaccines consist of suspension of attenuated, inactivated or fractions of viruses, and can be presented in lyophilized or suspension form. Very low concentrations of antibiotics may be present, except for streptomycin, penicillin and their derivatives. The product can contain not more than 50 ng/dose of proteins derived from serum of animal origin. Human albumin may be used, provided that the absence of antibodies to hepatitis B, hepatitis C, and HIV 1 and 2 is proven.

The vaccine production is based on the seed lot system, and the virus strain used must demonstrate adequate immunogenicity and be safe for humans. The replication of the vaccinal virus strain is obtained in an adequate host system (animals, avian embryos or cell culture) and the production methodologies are indicated in the monographs for each product.

In the case of culture of mammal cells for replication of the vaccinal virus, separate for control 5% or 500 mL, whichever is the highest volume. At the end of the vaccine production, these cell cultures cannot present cytopathogenic effect (CPE). Additionally, aliquots of the growth medium are inoculated in appropriate culture media to prove absence of contaminant microorganisms (fungi, bacteria and mycoplasmas). The cells must also demonstrate absence of other contaminant agents, especially viruses from animal species from which the cell culture was derived, through hemadsorption test with red blood cells from guinea pigs and inoculation in cell cultures, laboratory animals and embryonated eggs.

If the cell culture used derives from primary lineage of avian embryo, in addition to controls mentioned in the previous paragraph, egg farms must demonstrate adequate production conditions environments free from specific pathogens. Regularly, the avian species are monitored for infections caused by retrovirus, Newcastle virus, parainfluenza virus, smallpox virus, encephalomyelitis virus, laryngotracheitis virus, reticuloendotheliosis virus, Marek virus, adenovirus, influenza virus, mycobacteria*, Haemophilus paragallinarum, Salmonella gallinarum, Salmonella pullorum, Mycoplasma gallisepticum, Mycoplasma synoviae* , among other pathogenic agents from avian species.

If the cell culture used derives from primary lineage of rabbit kidney (*Oryctolagus cuniculus*), in addition to the controls mentioned in the third paragraph, the rabbits must be raised in adequate microbiological control conditions and monitored regularly as for infections caused by fungi, bacteria and viruses, such as: coccidiosis, myxomatosis, smallpox, fibromatosis, herpes virus, tuberculosis, *Nosema cuniculi*, toxoplasmosis, among other infections caused by microorganisms that naturally occur in rabbits. When using culture of primary lineage cells from monkey kidney, the animals must be healthy and never been used for other purposes. Before having their kidneys removed, the animals must have been stored in quarantine for a period of not less than six weeks and prove to be free from antibodies to virus B (*herpes virus*) and to immunodeficiency virus.

If human diploid cells or continuous lineage cells are used, they must derive from a cell bank certified by national control authority and demonstrate absence of contaminant microorganisms, as described in the third paragraph. They cannot be tumorigenic and are identified as to the origin species. The number of passages of human diploid cells cannot exceed two thirds of their maximum number of passage and their karyotype must be normal. When the vaccine is produced in continuous lineage cells, the virus pool must be purified by a process proving that, in the end product, the residual DNA is below 100 pg per dose.

The serum and trypsin used in the preparation of cell culture must be free from contaminant microorganisms (bacteria, fungi, mycoplasmas and viruses). Additionally, the serum must derive from cattle with certificate of absence of bovine spongiform encephalopathy.

COMBINED VACCINES

Combined vaccines are composed of a mixture of two or more different antigens and can be presented in lyophilized or suspension form. These immunobiological agents can contain, in their formulation, attenuated microorganisms, inactivated microorganisms, substances produced by microorganisms, and antigen fractions. The production and quality control process must comply with the specific monograph for each product present in this vaccine.

IDENTIFICATION

Proceed as described in the specific monograph.

CHARACTERISTICS

Proceed as described in the specific monograph.

PHYSICOCHEMICAL TESTS

Aluminum

A. Proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**.

Acetate buffer: dissolve 27.5 g of ammonium acetate in 50 mL of purified water and add 0.5 mL of hydrochloric acid at 25% (w/v). Complete the volume to 100 mL with purified water.

Carbonate buffer: dissolve 20 g of ammonium carbonate in 20 mL of diluted ammonia solution (dilute 17.5 mL of ammonium hydroxide at 10% (w/v) with 32.5 mL of purified water) and complete the volume to 100 mL with purified water.

Transfer to a Kjeldahl flask 1 mL of the sample and add 2 mL of nitric acid. Digest the mixture until the solution is clear. Transfer to a 25 mL volumetric flask and complete the volume with *Acetate buffer*. Transfer 2 mL of this solution to a 50 mL volumetric flask and add 2 mL of fresh-prepared solution of thioglycolic acid at 1% (v/v). Allow to stand for two minutes, add 15 mL of the aluminon reagent and heat in water bath (100°C) for 15 minutes. Cool, add 10 mL of *Carbonate buffer* and complete the volume with purified water. Prepare blank containing water in place of sample. The sample and the standards readings are conducted in spectrophotometer at the wavelength of 530 nm, using the blank for zero adjustment. Calculate the concentration of aluminum (III) in the sample by graphic interpolation or linear regression. The result must be expressed in mg of aluminum (III) per dose.

B. Proceed as described in *Atomic absorption spectrophotometry* **(5.2.13.1)**. Transfer to a Kjeldahl flask 2 mL of the sample and add 4 mL of nitric acid. Digest the mixture until the solution is clear. Transfer to a 25 mL volumetric flask and complete the volume with purified water. In parallel, prepare a blank with purified water in place of sample and calibration curve of aluminum with the concentrations of 20, 40, 60 and 80 ppm. Add to the sample, to the solutions for the calibration curve and to the blank a determined amount of ionization suppressor, so that it contains, at the end, concentration of 2000 ppm of potassium. Determine the concentration of aluminum (III) in the sample, in atomic absorption spectrophotometer at the wavelength of 309.3 nm, slit opening 0.2 nm, lamp current for aluminum of 10 mA, and nitrous oxide/acetylene flame.

Phenol. Dilute the sample so that the phenol concentration is between 5 ppm and 30 ppm. Add 5 mL of borate buffer pH 9.0, 5 mL of the 4-aminoantipyrine solution at 0.1% (w/v) and 5 mL of potassium ferricyanide aqueous solution at 5% (w/v). In parallel, prepare the blank and phenol calibration curve with concentrations ranging from 5 ppm to 30 ppm. Read the absorbances from the sample and the standards at the wavelength of 546 nm, 10 minutes after the reaction ends, using the blank to zero the device. Use the reading of standards to build the calibration curve. Determine the phenol concentration in the sample by graphic interpolation or linear regression. The manufacturer has the option of using the result obtained in the product before packaging.

Residual formaldehyde. Add to 1 mL of the sample, slowly and with agitation, 3 mL of trichloroacetic acid at 2.5% (v/v). Allow to stand for five minutes, centrifuge at 2000 g for 10 minutes, and transfer the supernatant to a test tube. In parallel, prepare the formaldehyde calibration curve with the concentrations of 2.5, 5, 7.5 and 10 mL/mL, being the volume of 4 mL/tube. Prepare blank with purified water in place of sample. Add 4 mL of Hantzach reagent to each one of the six test tubes prepared in advance, leave in water bath at 58°C for five minutes, and cool. Immediately read the absorbances from the sample and the standards, at the wavelength of 412 nm, using the blank to zero the device. The standard readings are used to plot the calibration curve. The concentration of residual formaldehyde in the sample is determined by graphic interpolation or linear regression.

Protein nitrogen (5.3.3.2). Complies with the test.

Thiomersal

Use one of the methods described below.

A. Proceed as described in *Visible absorption spectrophotometry* **(5.2.14)**. After mixing the sample rigorously , transfer 1 mL, in duplicate, to beakers and add 3 mL of purified water (dilution 1:4), then take 1 mL of this solution and transfer to a digestion tube. Add 1 mL of purified water and 2 mL of a mixture of equal volume of sulfuric acid R and nitric acid R. Take the mixture to ebullition for 10 minutes. Cool. Add 10 mL of purified water and 2 mL of hydroxylamine hydrochloride at 50% (w/v). Take to ebullition again for one minute, cool and transfer the liquid to a separation filter, filtering through cotton. Wash the tube with 70 mL of purified water and transfer likewise to the separation funnel. Add 10 mL of the dithizone solution (1:7), shake vigorously for one minute. Allow to stand for one minute, filter in cotton and collect the organic (chloroform) phase in Erlenmeyer flask. Proceed immediately to reading the filtrate in spectrophotometer at 490 nm. Preparation of standards and calibration curve: Prepare a stock solution of thiomersal (1200 µg/mL in thiomersal or 600 µg/mL in mercury). From this solution, prepare the standard solutions in 100 mL volumetric flasks. Establish the calibration curve with concentrations of 6 µg Hg/mL to 24 µg Hg/mL. After preparing the standard solutions, proceed as described for the sample. The blank is prepared using 2 mL of purified water in place of sample. Use the standard reading to plot a calibration curve and determine the thiomersal concentration in the sample by graphic interpolation or linear regression.

B. Proceed as described in *Atomic absorption spectrophotometry* **(5.2.13.1)**. Transfer, quantitatively, 1 mL of the sample to a 50 mL volumetric flask, add 0.5 mL of nitric acid and complete the volume with purified water. Prepare blank with purified water. From the stock solution of 1000 ppm of Hg, prepare an intermediate standard of 1 ppm of Hg and take different aliquots from it, according to the working interval, transferring to the reaction cells containing potassium permanganate solution. Determine the absorbance at 253.6 nm by cold vapor, in atomic absorption spectrophotometer, using nitrogen as carrier gas.

Residual humidity. Transfer 80 mg of the sample to a weighing bottle previously dried and tared. Store the sample for three hours in an anhydrous phosphorus pentoxide atmosphere, under pressure not greater than 5 mm of mercury, at temperature of 60°C. The weighing bottle is cooled for 20 minutes in a dryer with silica gel and immediately weighed. The heating and cooling phase is repeated until obtaining constant weight. The value of the residual humidity is the average percentage of weight loss from not less than three assessments of the sample. The volumetric method for *Determination of water* **(5.2.20.1)** can also be used.

BIOLOGICAL SAFETY TESTS

Bacterial endotoxins. Proceed as described in the specific monograph.

Sterility (5.5.3.2.1). Complies with the test.

Pyrogens. Proceed as described in the specific monograph.

ASSAY

Proceed as described in the specific monograph.

THERMOSTABILITY

Proceed as described in the specific monograph.

PACKAGING AND STORAGE

The temperature and the expiry date are indicated by the vaccine manufacturer, based on experimental evidence approved by the national regulatory authority.

LABELLING

Comply with current legislation.