MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION

GENERAL PHARMACOPOEIA MONOGRAPH

Agarose gel immunoelectrophoresis **GPM**.1.8.2.0002.15
Replaces the Method described in the Pharmacopoeia Monograph 42-3874-99

The present General Pharmacopoeia Monograph applies to the investigation of the antigenic composition of biological materials, as well as to the determination of the purity, qualitative and quantitative composition of immunobiological medicinal products, by means of agarose gel immunoelectrophoresis (AGI), which combines the zonal electrophoresis and immunodiffusion methods.

During immunoelectrophoresis conducted in gels or cellulose acetate films by means of single or double immunodiffusion, a reaction occurs between soluble proteins and precipitating antibodies developing against these proteins. Quantification of proteins may be performed by electrophoresis in an antibody-containing environment (electroimmunoassay, electroimmunodiffusion, rocket immunoelectrophoresis). The antigenic nature of protein components may be investigated by comparing them with known markers.

The efficacy of immunochemical tests depends on the specificity of antisera used, as well as on their titre and affinity to the respective antigen.

In most cases, immunoelectrophoresis is a combination of agarose (or agar) gel electrophoresis with subsequent double immunodiffusion performed in the same medium. During two-dimensional double immunodiffusion, the antigen and antibody placed in round-shaped or rectangular holes in the gel migrate towards each other, which produces precipitation lines (arcs) when they meet. The position of precipitation bands depends on the diffusion coefficient of the antigen and its

concentration expressed in relation to the antibody level (the diffusion rate of the antibody may be regarded as a constant). The certain ratio of the antigen and antibody concentrations, which is optimal for the formation of a precipitate, is called the equivalence zone. Clear-cut precipitation lines thus form.

The concentration (titre) of an antibody in immune serum may considerably vary as well. There is a possibility that the equivalence zones of different components of the mixture will not overlap. To obtain the equivalent antigen to antibody ratio, it is recommended that immunoelectrophoresis of multi-component mixtures should be conducted for several antigen to antibody relative concentrations, because some components may remain undetected if only one concentration is used.

The agarose gel immunoelectrophoresis method

An alcohol-processed glass plate with dimensions 90 x 120 mm is placed strictly horizontally on an object table. Agarose gel cooled beforehand to (45 ± 5) ^{0}C is applied on the glass plate (volume, 18.0 to 20.0 mL). As the gel sets at room temperature, an agarose layer 2.0 to 2.5 mm thick forms on the plate in 20 to 30 minutes.

After the gel has fastened, 6 to 7 wells measuring 1 to 2 mm in diameter are cut in it using a special template. The wells are filled with the tested sample, the volume of which should not exceed the well volume (2 wells for each tested sample). A control sample, normal human serum («a standard sample of the test system for the determination of the fractional (antigenic) composition of human serum preparations by immunoelectrophoresis» or a different control sample, as specified in the Pharmacopoeia Monograph or in the Normative Document) stained with Pyronin B (or a with different dye specified in the Pharmacopoeia Monograph or in the Normative Document), is introduced into the upper and lower wells of the gel-containing glass plate.

A 500 mL volume of the Veronal – Medinal or borate buffer solution is transferred into a 1000 mL volumetric cylinder, then the volume of the solution is brought to the mark with purified water, and mixing follows. The resulting solution

(or a different buffer solution specified in the Pharmacopoeia Monograph or in the Normative Document) is poured into the electrode sections of the chamber of the electrophoresis device.

The plate with the prepared gel is placed inside the electrophoresis device and joined with the buffer solution in the electrode chambers of the electrophoresis device using a few layers of filter paper. If the design of the electrophoresis device necessitates joining the agar on the plate with the electrode buffer by means of agarose columns, the plate is placed in the equilibrated device and flushed with the melted agar until it connects with the agarose columns and a gel layer with a thickness of 1 to 2 mm forms.

The electrophoresis device is closed with the cover, and the power supply is turned on (voltage 70 – 200 V, current 10 – 40 mA). Electrophoresis is performed over a period of 1.5 to 3 hours, until the Pyronin B spot corresponding to the migration of albumin is at a distance of 20 to 25 mm from the well.

After the electrophoresis procedure, the specially designed template is used cut longitudinal “grooves” between the wells in the agarose gel (parallel to the direction of the migration). A 0.25 mL portion of the precipitating antiserum is introduced into each groove: an antiserum to human serum proteins (for fractional composition tests) or a polyvalent serum against human, bovine, horse, and porcine serum proteins (for identity tests).

The agarose gel plate is placed inside a moist chamber and exposed to a temperature of (5 ± 3) ^{0}C for 24 to 48 hours.

To wash off proteins that did not engage in the precipitation reaction, the agarose gel plate is placed in dishes, flooded with 0.9 % sodium chloride solution, and left to stand for 16 to 18 hours. The solution is changed 3 or 4 times. After that, the plate is taken out of the 0.9 % sodium chloride solution, covered with filter paper soaked in 0.9 % sodium chloride solution, and dried in the air until the agarose gel turns into a thin film. Afterwards, the plate with the dried gel is covered with filter paper soaked in 0.9 % sodium chloride solution, avoiding leaving any air bubbles, and dried at room temperature or in an exsiccator at a

temperature not exceeding 40 °C. After the plate has dried out, the filter paper is soaked in water and carefully removed.

Proteins are dyed with the Amino Black 10B stain or a different appropriate stain (as described in the Pharmacopoeia Monograph or in the Normative Document), by placing the plate in a dish containing the staining solution for 30 to 40 minutes. After that, the plate is washed in the agar wash solution (2 % acetic acid solution or a different solution, as described in the Normative Document) over 15 to 40 minutes, until the coloured background is completely absent, and dried again at room temperature or in an exsiccator at a temperature not exceeding 40 °C.

Lipoproteins are stained with the Sudan Black B dye. To allow staining, the completely dried plates are placed for 3 hours in the staining solution, and then discoloured with 50 % ethanol until the background becomes absolutely clear. Lipoproteins are stained dark blue. Staining should be done on an absolutely dry product, because Sudan Black B is water-insoluble and stains moist gel.

Interpretation of results obtained in fractional composition tests performed for human immunoglobulin preparations should be carried out by visual examination, comparing the electrophoregram of the tested sample with the electrophoregram of the control sample, normal human serum («a standard sample of the test system for the determination of the fractional (antigenic) composition of human serum preparations by immunoelectrophoresis» or a different control sample, as specified in the Normative Document), which should display the required number of precipitation lines with the serum against human serum proteins (not less than 15 precipitation lines with the «standard sample of the test system for the determination of the fractional (antigenic) composition of human serum preparations by immunoelectrophoresis»). The main component of a human immunoglobulin preparation should correspond to the immunoglobulin G (Ig G) found in normal human serum.

Interpretation of results obtained in identification tests performed for human blood preparations should be carried out by visual examination, analyzing

precipitation lines formed with the serum against human, bovine, horse, and porcine serum proteins. The test should produce precipitation lines only with the serum against human serum proteins.

Notes

1. Preparation of the 0.05 M Veronal – Medinal buffer solution (pH 8.6 ± 0.1). Transfer 1.38 g of Veronal and 8.76 g of Medinal into a 1000 mL volumetric flask, bring the volume of the solution to the mark with purified water, and stir until complete dissolution of the substance.

2. Preparation of the 0.05 M borate buffer solution (pH 8.6 ± 0.1). Transfer 6.7 g of boric acid and 13.4 g of sodium tetraborate decahydrate into a 1000 mL volumetric flask, bring the volume of the solution to the mark with purified water, and stir.

3. Preparation of the 1.25 % agarose gel. Preparation of the agarose gel is performed using one of the following methods:

a) To prepare 1.25 % agarose gel, use a 0.05 M Veronal – Medinal buffer solution (pH 8.6 ± 0.1) with a double concentration of the salts (2.76 g of Veronal and 17.52 g of Medinal, respectively, in 1000 mL of purified water).

b) To prepare 1.25 % agarose gel, use a borate buffer solution (pH 8.6 ± 0.1) with a double concentration of the salts (13.4 g of boric acid and 26.8 g of sodium tetraborate decahydrate, respectively, in 1000 mL of purified water).

Transfer 12.5 g of agar into a 1000 mL chemical glass, add 500 mL of purified water, and leave the gel for one hour at (20±1) ^{0}C to swell. The glass with its contents is placed in a boiling water bath and left to stand until the agar has completely melted. The volume of the gel solution is brought to 500 mL with water, and then an equal volume of the Veronal – Medinal or borate buffer solution (or a different buffer solution specified in the Pharmacopoeia Monograph or in the Normative Document) is added. The agar solution is filtered through 2 or 3 layers of gauze, and thiomersal is added to a concentration of 100 μg/mL; this solution is then poured into vials, 40 to 50 mL in each (the amount required for two plates). The melted agar should be transparent.

4. Preparation of the Amido Black 10B staining solution. The staining solution is prepared using one of the following methods:

a) Transfer 1.0 g of Amido Black 10B, 450 mL of 0.1 M acetic acid solution, and 550 mL of 0.1 M sodium acetate solution into a 1000 mL volumetric flask, then stir.

b) Transfer 1.0 g of Amido Black 10B and 100 mL of glacial acetic acid into a 1000 mL volumetric flask, bring the volume of the solution to the mark with purified water, and stir. Filter through a paper filter in 12 hours.

5. Preparation of the Sudan Black B staining solution. Transfer 1.2 g of Sudan Black B, 20 mL of 1 M sodium hydroxide solution, and 380 mL of purified water into a 1000 mL volumetric flask, bring the volume of the solution to the mark with absolute ethanol, and stir. Boil the mixture over a short time, and cool down to room temperature. Once cooled, transfer the stain into a dark glass receptacle equipped with a ground stopper. This reagent may be stored at room temperature for 3 months.

6. Preparation of the 2 % acetic acid solution for agar washing. Transfer 20.0 mL of glacial acetic acid into a 1000 mL volumetric flask, bring the volume of the solution to the mark with purified water, and stir. This reagent may be stored at room temperature for 3 months.