**MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION**

# GENERAL PHARMACOPOEIA MONOGRAPH

**Determination of protein** **GPM**.1.2.3.0012.15

Replaces the State Pharmacopoeia of the Russian Federation XII, Part 1 Monograph, GPM 42-0053-07

Determination of protein is carried out in drug products obtained from natural sources or in biotechnological methods.

Modified protein determination methods specified in the Pharmacopoeia Monographs may be used for such drug products; the recommended protein concentration ranges and / or volumes of tested solutions and reagents, as well as some other conditions, may be modified in these methods depending on individual characteristics of the component being determined.

The colourimetric and some of the spectrophotometric methods require the use of a standard sample. The following can be used as a standard protein sample: a standard sample of the protein present in the drug product, or a bovine serum albumin, or a human serum albumin dried before the test so that a constant weight is achieved (the standard sample and the exsiccation conditions should be specified in the Pharmacopoeia Monograph).

Spectrophotometric, colourimetric, and spectrofluorimetric methods are used for the quantification of protein.

**Method 1 (By spectrophotometry)**

This method is based on the ability of aromatic amino acids (tyrosine, tryptophane, and, to a lesser extent, phenylalanine) present in the protein molecule sequence to absorb ultraviolet light at wavelengths around 280 nm.

Different solutions should be used to dissolve protein molecules: water, 0.9 % sodium chloride solution, various buffer solutions, etc.

When a buffer solution is used to dissolve the molecules of a protein, a high optical density value relative to water indicates the presence of an interfering substance in the protein molecule. A buffer solution should be used instead of water as the reference solution in order to offset the effects of the interfering substance on test results. If the interfering substances have high optical density, test results obtained may be doubtful.

At low concentrations, protein is adsorbed on the dish walls, which may produce too low protein content results for the tested solution; in this case, the tested solution of the drug product should be concentrated beforehand or non-ionic detergents should be added when preparing the tested solution.

*Tested solution.* A solution of the tested substance should be prepared using the buffer solution specified in the Pharmacopoeia Monograph, so that the concentration of protein is between 0.2 mg/mL and 2 mg/mL.

*Standard solution*. A solution of the respective standard sample should be prepared in the same buffer solution and with the same protein concentration as that of the tested solution.

*Procedure description*. The tested solution, standard solution, and reference solution are left to stand at the same temperature. The temperature and incubation time should be specified in the Pharmacopoeia Monograph. The optical densities of the tested and the standard solutions are determined in quartz cuvettes at wavelength 280 nm, using the same buffer solution as the reference solution.

To obtain reliable and accurate results, the optical density values of the solutions should meet the linearity requirements over the range of determined protein concentrations.

For highly purified proteins, the protein concentration of the solution should be calculated using the specific absorbance index.

*Light scattering.* The accuracy of protein content analysis in the ultraviolet range decreases if the protein is present in the solution as particles with dimensions comparable to the wavelength of the measured light (250 to 300 nm). Scattering of a beam of light results in a higher absorbance of the tested solution. When

calculating the optical density of the tested solution at wavelength 280 nm that is due to light scattering, determinations should be performed at wavelengths 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, and 350 nm. A diagram is charted for the dependence of the optical density decimal logarithm (lg) and the respective wavelength logarithm. The curve is extrapolated using the linear regression method to determine the optical density logarithm at wavelength 280 nm. The antilogarithm of this value corresponds to the scattering-caused optical density of the solution. To calculate the true protein content of the tested solution, the optical density of the solution obtained at 280 nm should be adjusted by subtracting the optical density caused by scattered light.

The effect of scattered light in opalescent solutions may be decreased by filtration through a 0.2 μm non-protein-adsorbing filter or by centrifugation. The filtration and centrifugation conditions are specified in the Pharmacopoeia Monograph.

*Calculations.* The protein content in the tested solution (C) expressed in mg/mL is calculated according to the following equation:

$$C = C_0 \cdot A/A_0, \quad \text{where}$$

$C_0$ is the protein concentration in the standard sample solution, expressed in mg/mL;

A and $A_0$ are the adjusted optical density values of the tested solution and the standard sample solution, respectively.

**Method 2 (The Lowry method, by colourimetry)**

This method is based on the reaction of proteins with copper (II) salts in an alkaline solution and reduction of the phosphorus-molybdenum-tungsten reagent (or the Folin reagent) resulting in the production of coloured products whose colour intensity should be determined by their optical density at wavelength 750 nm. The Folin reagent interacts with the residues of the protein's amino acids (predominantly tyrosine, as well as tryptophane and phenylalanine, and, to a lesser extent, cysteine). The colouration achieves its maximum in 20 to 30 minutes at

room temperature, and after that the colour intensity decreases. The colouration degree depends on the nature of the protein. Since different types of protein may produce colour reactions of various intensity, the tested protein should correspond to the standard sample.

The determination can be interfered by some salts, thiol compounds, carbohydrates, lipids, non-ionic detergents, organic solvents, complexones, and some other compounds. The majority of interfering substances produce a slight colouration, but the use of some detergents leads to a significantly more intensive colour. A high concentration of a salt may cause sedimentation. To offset the effects of substances interfering with the determination, the solution is additionally diluted to produce a concentration of the tested protein at a level sufficient for accurate measurements or the proteins are precipitated using sodium deoxycholate and trichloroacetic acid solutions.

*Standard solutions*. The respective protein standard sample is dissolved in the buffer solution specified in the Pharmacopoeia Monograph. Parts of the resulting solution are diluted with the same buffer solution to obtain not less than five standard solutions with protein concentrations evenly distributed over the range from 5 μg/mL to 100 μg/mL.

*Tested solution*. A solution of the tested drug product should be prepared in the buffer solution specified in the Pharmacopoeia Monograph and with a protein concentration lying within the concentration range of the calibration graph.

*Control solution.* Utilize the buffer solution used for the preparation of the standard and tested solutions.

***Method A (without preliminary protein sedimentation).*** A 5.0 mL portion of Reagent B is added to 1.0 mL of each of the standard solutions, tested solution, and to 1.0 mL of the control solution. The contents of the test tubes are mixed and left to stand at room temperature for 10 to 30 minutes. After that, 0.5 mL of Folin's reagent diluted with the equal volume of water before use is added into each test tube, the mixtures are promptly and thoroughly mixed and left to stand at room temperature for 30 minutes. The optical densities of the tested and the standard

solutions are measured on a spectrophotometer at wavelength 750 nm (or the wavelength specified in the Pharmacopoeia Monograph), using the control solution as a reference. The colour remains stable for 2 hours. The commercial Folin – Ciocalteu reagent may be used.

The protein concentration to optical density relationship is non-linear; nevertheless, if the concentration range used to chart the calibration graph is narrow, it approximates a linear pattern. The relationships between the protein concentration and the optical density of the standard solution are charted, and linear regression is used to plot a calibration curve. The protein concentration of the tested solution is determined using the calibration curve and the optical density of the tested solution.

A calibration graph should be plotted each time when preparing new reagents or using a different spectrophotometer, in any case at least once in 3 months.

**Notes:**

*1. Preparation of Reagent A.* Take 2 g of sodium carbonate into a 100 mL volumetric flask, dissolve in 0.1 M sodium hydroxide solution, and bring the volume of the solution to the mark using the same solution, then stir.

The shelf-life of this solution is 1 month.

*2. Preparation of Reagent B.* Take 0.5 g of copper sulfate and 1 g of potassium – sodium tartrate into a 100 mL volumetric flask, dissolve in water, and bring the volume of the solution to the mark.

The shelf-life of this solution is 2 months.

*3. Preparation of Solution C.* Mix 50.0 mL of Reagent A and 1.0 mL of Reagent B before the analysis.

***Method B (with sodium dodecyl sulfate)***. The test is carried out as described for Method A, except that 1 mL of the alkaline copper reagent and 0.5 mL of the diluted Folin's reagent should be added to each of the solutions instead of 5 mL of Reagent C.

**Notes:**

*1. Preparation of the copper sulfate reagent.* Dissolve 0.2 g of copper sulfate and 0.4 g of sodium tartrate in a 100 mL volumetric flask, bring the volume of the solution to the mark using water, and mix. Transfer 10 g of sodium carbonate into

a 100 mL volumetric flask and dissolve in water, then stir. Slowly pour the sodium carbonate solution into the copper sulfate solution while mixing. Use this solution within 24 hours.

*2. Preparation of the alkaline copper reagent.* Mix 1 volume of the resulting copper sulfate reagent solution with 2 volumes of 5 % sodium dodecyl sulfate solution (50 g/L) and 1 volume of 3.2 % sodium hydroxide solution (32 g/L).

The shelf-life of this solution is 2 weeks at room temperature.

*3. Preparation of the diluted Folin's reagent.* Mix 5 mL of Folin's reagent with 55 mL of water.

This solution should be stored in dark glass bottles at room temperature.

***Method C (with preliminary protein sedimentation).***

*Procedure description*. Add 0.1 mL of sodium deoxycholate solution to 1.0 mL of the tested solution. Vortex this solution, and leave to stand at room temperature for 10 minutes. Add 0.1 mL of 72 % trichloroacetic acid solution, and vortex. Separate the sediment by 30-minute centrifugation at 3000 g. Carefully remove the supernatant, and dissolve the remaining sediment in 1 mL of the alkaline copper reagent. Then proceed as described above for Method B.

When plotting a calibration graph, protein standard solutions should be processed in the same manner.

As the reference solution, use a sample containing 0.1 mL of 0.1 M sodium hydroxide solution, 0.9 mL of water, 5.0 mL of reagent B, and 0.5 mL of Folin's reagent pre-diluted 50 : 50.

**Notes**:

*1. Preparation of 0.15 % sodium deoxycholate solution.* Transfer 0.15 g of sodium deoxycholate into a 100 mL volumetric flask, and dissolve in water. Bring the volume of the solution to the mark with water, and stir thoroughly.

*2. Preparation of 72 % trichloroacetic acid solution.* Transfer 360 g of trichloroacetic acid into a 500 mL volumetric flask, and dissolve in water. Bring the volume of the solution to the mark with water, and stir thoroughly.

The shelf-life of 72 % trichloroacetic acid solution is 1 month.

**Method 3 (The Bradford method, by colourimetry)**

This method is based on the shift in the absorbance maximum of the optical density of the Acid Blue 90 dye (Coumassie Brilliant Blue R-250) from 470 nm to 595 nm observed as a result of the binding between the protein and the dye. The

dye most intensively binds arginine and lysine residues of the protein, which may produce inaccuracies during the quantification of different types of protein. The protein used as a standard sample should be the same as the tested protein.

There is a relatively small influence of interfering substances that can be avoided without the use of detergents and ampholytes in the tested sample. Strongly alkaline samples may interact with an acidic reagent.

*Standard solutions*. Dissolve the respective protein standard sample in the buffer solution specified in the Pharmacopoeia Monograph. Dilute portions of the obtained solutions with the same buffer solution to obtain not less than five standard solutions with protein concentrations evenly distributed over the range from 0.1 mg/mL to 1 mg/mL.

*Tested solution*. A solution of the tested drug product should be prepared in the buffer solution specified in the Pharmacopoeia Monograph and with a protein concentration lying within the concentration range of the calibration graph.

*Control solution.* Utilize the buffer solution used for the preparation of the standard solutions and the tested solution.

*Procedure description*. Add 5 mL of the Bradford reagent to a 0.1 mL portion of each standard solution, the tested solution, and the control solution. Mix thoroughly turning the receptacle upside down. Avoid foaming, which will result in poor reproducibility. Leave to stand at room temperature for 10 minutes, and determine the optical densities of the standard solutions and the tested solution on a spectrophotometer at wavelength 595 nm, using the control solution as a reference solution containing the solvent and the Bradford reagent. Quartz cuvettes for spectrophotometry should not be used for this determination, because the dye binds to these materials. The colour remains stable for 1 hour.

The protein concentration to optical density relationship is non-linear; nevertheless, if the concentration range used to chart the calibration graph is narrow, it approximates a linear pattern. The relationships between the protein concentration and the optical density of the standard solution are charted, and linear regression is used to plot a calibration curve. The protein concentration of

the tested solution is determined using the calibration curve and the optical density of the tested solution.

The Normative Documents permit changes in the volumes of the tested solution and the Bradford solution, provided that the protein concentration to optical density relationship in the calibration graph is linear.

A calibration graph should be plotted each time when preparing new reagents or using a different spectrophotometer, in any case at least once in 3 months.

**Note**:

*Preparation of the Bradford reagent*. Transfer 0.05 g of the Acid Blue 90 dye (Coumassie Brilliant Blue R-250) into a 500 mL volumetric flask, dissolve in 25 mL of 96 % alcohol, add 50 mL of concentrated phosphoric acid, bring the volume of the solution to the mark with the water, and stir thoroughly. Filter, and store in dark glass bottles at room temperature. If a sediment dyes forms during storage, the reagent should be filtered before use.

The shelf-life is 2 weeks.

**Method 4 (the bicinchoninic acid method, by colourimetry)**

This method is based on the reduction of the bivalent copper ion into the monovalent one upon interaction with residues of cysteine, cystine, tryptophane, tyrosine, the peptide bond of the protein, and the formation of the coloured complex of copper and bicinchoninic acid (2,2'-biquinoline-4,4'-dicarboxylic acid – BCA). The determination can be interfered by reducing substances, such as sugars, ascorbic acid, thiol compounds, and ethylenediaminetetraacetate. The effects of interfering substances can be minimized by a dilution producing a protein concentration at a level sufficient for accurate measurements. The protein sedimentation technique explained in the Lowry method protein determination description may be used as an alternative. Since the colour intensity of the forming complex depends on the nature of the protein, the standard sample protein should be the same as in the tested sample.

*Standard solutions*. The respective protein standard sample is dissolved in the buffer solution specified in the Pharmacopoeia Monograph. Parts of the

resulting solution are diluted with the same buffer solution to obtain not less than five standard solutions with protein concentrations evenly distributed over the range from 10 μg/mL to 1200 μg/mL.

*Tested solution*. A solution of the tested drug product should be prepared in the buffer solution specified in the Pharmacopoeia Monograph and with a protein concentration lying within the concentration range of the calibration graph.

*Control solution.* Utilize the buffer solution used for the preparation of the standard and tested solutions.

*Procedure description*. Add 2.0 mL of the copper bicinchoninic acid reagent to 0.1 mL portions of each standard solution, the tested solution, and the control solution. Leave the solutions to stand at 37 ºC for 30 minutes, record the time, and cool the mixture down to room temperature. In 60 minutes after the end of incubation at 37 ºC, determine the optical densities of the standard solutions and the tested solution with a spectrophotometer at wavelength 562 nm, using quartz cuvettes and the control solution as a reference.

The protein concentration to optical density relationship is non-linear; nevertheless, if the concentration range used to chart the calibration graph is narrow, it approximates a linear pattern. The relationships between the protein concentration and the optical density of the standard solution are charted, and linear regression is used to plot a calibration curve. The protein concentration of the tested solution is determined using the calibration curve and the optical density of the tested solution.

**Notes:**

1. *Preparation of the bicinchoninic acid reagent*. Transfer 10 g of disodium salt of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium hydrocarbonate into a 1 L volumetric flask, and dissolve in water. If necessary, adjust the pH value of the obtained solution to 11.25 with 10 % sodium hydroxide solution or 5 % sodium hydrocarbonate solution. Bring the volume of the solution to the mark with water and stir.

2. *Preparation of the copper bicinchoninic acid reagent.* Mix 1.0 mL of 4 % copper sulfate solution (40 g/L) and 50.0 mL of the bicinchoninic acid reagent.

**Method 5 (Colourimetric method with the Biuret reagent)**

This method is based on the interaction between bivalent copper ions and the peptide bonds in the protein molecule in an alkaline environment leading to the formation of a coloured complex whose optical density is determined at wavelength 540 nm. This method demonstrates the minimal differences between equal quantities of immunoglobulin and albumin proteins.

This method is not recommended for solutions containing ammonium salts, as well as for turbid or precipitating solutions. To eliminate the effects of interfering substances, protein should be precipitated from the tested sample solution in the following manner: add 0.1 volume of 50 % trichloroacetic acid solution to 1 volume of the tested sample solution, vortex, and leave to stand at room temperature for 20 minutes, then centrifuge at 3000 g for 30 minutes. Remove the supernatant and dissolve the sediment in a small volume of 0.5 M sodium hydroxide solution. Use the obtained solution to prepare the tested solution.

When plotting a calibration graph, the protein standard solutions should be treated in the same fashion.

*Standard solutions.* Unless otherwise specified in the Pharmacopoeia Monograph, dissolve the respective protein standard sample in 0.9 % sodium chloride solution. Dilute portions of the obtained solutions with 0.9 % sodium chloride solution to obtain not less than three standard solutions with concentrations ranging from 2 mg/mL to 10 mg/mL.

*Tested solution*. A solution of the tested drug product should be prepared with 0.9 % sodium chloride solution and have a protein concentration lying within the concentration range of the standard solutions.

*Control solution.* Use 0.9 % sodium chloride solution.

*Procedure description.* Add 4.0 mL of the Biuret reagent to a 1.0 mL portion of the tested solution, each of the standard solutions, and the control solution. Mix and leave to stand at room temperature for 30 minutes. Determine the optical densities of the tested solution and the standard solutions at wavelength 540 nm,

using the control solution as a reference solution (or as specified in the Pharmacopoeia Monograph, in the range of 540 to 650 nm).

The protein concentration to optical density relationship is non-linear; nevertheless, if the concentration range used to chart the calibration graph is narrow, it approximates a linear pattern. The relationships between the protein concentration and the optical density of the standard solution are charted, and linear regression is used to plot a calibration curve. The protein concentration of the tested solution is determined using the calibration curve and the optical density of the tested solution.

A calibration graph should be plotted each time when preparing new reagents or using a different spectrophotometer, in any case at least once in 3 months.

**Note**:

*Preparation of 50 % trichloroacetic acid solution.* Transfer 250 g of trichloroacetic acid into a 500 mL volumetric flask, and dissolve in water. Bring the volume of the solution to the mark with water, and stir thoroughly.

The shelf-life of 50 % trichloroacetic acid solution is 1 month.

**Method 6 (Fluorimetric method with o-phthalaldehyde)**

This method is based on derivation of protein with o-phthalaldehyde, which reacts with the primary amino groups of the protein (the N-terminal amino acid and the ε-amino group of the lysine residues), after which the fluorescence of the obtained complex is measured. Quantification sensitivity may be augmented by protein hydrolysis performed before o-phthalaldehyde is added. Hydrolysis makes the α-amino group, which is part of the amino acid sequence, accessible for interaction with the phthalaldehyde reagent. This method is highly sensitive, and requires only a small amount of protein.

The determination is interfered by buffer solutions containing primary amines, such as tris(hydroxymethyl)aminomethane, and amino acids, which interact with *o*-phthalaldehyde. High ammonia concentrations also interact with *o*-

phthalaldehyde. Fluorescence obtained as a result of interaction between amines and *o*-phthalaldehyde may be unstable. Use of automated techniques for standardization of this method allows to boost the accuracy and reproducibility of the determination.

*Standard solutions.* Dissolve the respective protein standard sample in 0.9 % sodium chloride solution. Dilute portions of the obtained solution with 0.9 % sodium chloride solution to obtain not less than five standard solutions with concentrations ranging from 10 μg/mL to 200 μg/mL. Bring the pH value of this solution to 8.0 – 10.5 before adding the phthalaldehyde reagent.

*Tested solution*. A solution of the tested drug product should be prepared with 0.9 % sodium chloride solution and have a protein concentration lying within the concentration range of the standard solutions. Bring the pH value of this solution to 8.0 – 10.5 before adding the phthalaldehyde reagent.

*Control solution.* Use 0.9 % sodium chloride solution.

*Procedure description.* Mix 10 μL of the tested solution, each of the standard solutions, and the control solution with 0.1 mL of the phthalaldehyde reagent, and leave to stand at room temperature for 15 minutes. Add 3 mL of 0.5 M sodium hydroxide solution, and stir. Determine the fluorescence intensity of the tested solution and each of the standard solutions at excitation wavelength 340 nm and an emission wavelength between 440 nm and 455 nm, using the control solution as the reference solution. Determine the fluorescence intensity of the specified solutions only once, because emission decreases fluorescence intensity.

The protein concentration to fluorescence intensity relationship is linear. The relationship between the protein concentration and the fluorescence intensities of the standard solutions is charted on a graph, and linear regression is used to plot a calibration curve. The protein concentration of the tested solution is determined using the calibration curve and the fluorescence intensity of the tested solution.

A calibration graph should be plotted each time when preparing new reagents or using a different spectrophotometer, in any case at least once in 3 months.

**Notes:**

*Preparation of the borate buffer solution.* Transfer 61.83 g of boric acid into a 1000 mL volumetric flask and dissolve in water, then bring the pH value of this solution to 10.4 with potassium hydroxide solution. Bring the volume of the solution to the mark with water, and stir thoroughly.

*Preparation of the phthalaldehyde stock solution*. Dissolve 1.20 g of phthalaldehyde in 1.5 mL of methanol, add 100 mL of the borate buffer solution, and stir. Add 0.6 mL of 30 % of the macrogol 23 lauryl ether (300 g/L) solution, and stir.

Store at room temperature, and use this solution within 3 weeks.

*Preparation of the phthalaldehyde reagent.* Add 15 µL of 2-mercaptoethanol to 5 mL of the phthalaldehyde stock solution. This solution should be prepared not less than 30 minutes before use.

This solution should be used within 24 hours.

**Method 7 (Determination of protein by nitrogen content)**

Determination of protein by nitrogen content is based on the fact that the content of nitrogen in most proteins is practically the same and can be considered 16 %. The determined nitrogen quantity in the taken sample is used to calculate the protein content in the drug product utilizing the nitrogen to protein conversion factor (which is 6.25).

Other nitrogen-containing substances present in the tested sample will have an effect on test results.

Determination of protein by nitrogen content is based on disintegration of the tested sample observed during the test, but it is not limited by protein content in an aqueous environment. When a nitrogen-containing organic compound is heated with concentrated sulfuric acid, nitrogen is converted into ammonium sulfate, which can be assayed.

***Method A (The Kjeldahl method)***

The test is carried out in accordance with the requirements established by the "Determination of nitrogen in organic compounds" General Pharmacopoeia Monograph (Method 2 – "The micro-Kjeldahl method") using an accurately measured weight of the drug product containing 10 to 20 mg of protein. After the nitrogen-containing organic compound is mineralized with concentrated sulfuric

acid, the concentrated nitrogen is converted into ammonium sulfate, which can be assayed.

***Method B***

Most nitrogen determination instruments employ pyrolysis, i. e. incineration of the sample in oxygen at approximately 1000 °C, leading to production of nitric monooxide (NO) and other nitric oxides ($NO_x$) from the nitrogen present in the tested substance. Some instruments convert nitric oxides into nitrogen that can be quantified with a thermal conductivity detector. In other instruments, nitric monooxide (NO) is mixed with ozone ($O_3$) to obtain excited nitric dioxide ($NO_2^*$), which emits light upon disintegration that can be quantified with a chemiluminescence detector. To optimize the tested substance weight and pyrolysis parameters and to ensure stability of parameters during the analysis, a standard sample of appropriate purity and corresponding to the composition of the tested protein should be used.

*Calculations.* The protein concentration is determined by dividing the nitrogen content of the tested sample by the known nitrogen content of the tested protein. The known nitrogen content of a protein can be determined on the basis of the chemical composition of the protein or by comparing with an appropriate standard sample.