MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION

GENERAL PHARMACOPOEIA MONOGRAPH

Determination of molecular parameters of immunoglobulins by HPLC	**GPM**.1.8.2.0006.15
in the Pharmacopoeia Monograph 42-3874-99	Replaces the method described

The present General Pharmacopoeia Monograph applies to the size-exclusion high-performance liquid chromatography (HPLC) method designed for the determination of molecular parameters of immunoglobulins. The mechanism of a size-exclusion high-performance liquid chromatography procedure is chromatographic distribution of immunoglobulin molecules by size and molecular weight. The main aspects of size-exclusion HPLC are described in the General Pharmacopoeia Monograph “HPLC”.

The size-exclusion high-performance liquid chromatography method is introduced to replace the gel filtration method.

The size-exclusion high-performance liquid chromatography method

The process of size-exclusion high-performance liquid chromatography occurs in an aqueous medium and is called gel filtration, it leads to distribution of immunoglobulin molecules according to their size over a broad range of molecular weights, from 10,000 to 600,000 kDa. A specific feature of the size-exclusion HPLC methods is the use of high pressure (up to 400 bars) and fine-grained sorbents (3 to 5 μm in size). This permits quick and complete separation of complex mixtures of substances.

The principal basis of the method is the volume of the size-exclusion column, which can be expressed as the sum of three summands:

$$Vc = Vo + Vi + Vd,$$

where: Vo is the free volume of the mobile phase;

Vi is the pore volume filled with the mobile phase (stationary phase volume);

Vd is the volume of the sorbent matrix (excluding the pores).

The total volume of the column's mobile phase (Vt) is the sum of the free volume of the mobile phase (Vo) and the volume of the stationary phase Vi.

The retention of molecules in the size-exclusion column is determined by the probability of their diffusion into the pores, and largely depends on the size ratio of the molecules and the pores. The coefficient of distribution Kd is the ratio of the substance's concentration in the stationary phase (C_1) and that in the mobile phase (Co): $Kd = C_1/Co$. The retention time of immunoglobulin molecules depends on how strong the interaction between the mobile and stationary phases is.

The immunoglobulins analysis should be conducted in accordance with the procedures described in the Pharmacopoeia Monographs or Normative Documents; the following indispensable parameters should be specified: sample preparation for the tested sample; preparation of standard samples; preparation of the mobile phase. The following should be specified when describing the required chromatographic conditions:

- characteristics of the chromatographic column (its make (type) and dimensions);
- the molecular weight separation range;
- the composition and name of the mobile phase;
- the name of the carrier (sorbent);
- characteristics of the detector;
- the wavelength;
- the injection volume;
- the column temperature;

- the mobile phase flow rate;
- the sequence (order) of sample injections;
- the chromatography time.

As immunoglobulin fractions are separated on the chromatographic column, the protein molecules of an immunoglobulin with a molecular weight exceeding 40,000 kDa pass between the granules of the carrier (sorbent) without moving into or being retained in the porous granules. Smaller molecules, partially, get inside and become retained in the granules, and the mobile phase washes them out more slowly. Very small molecules, with a molecular weight less than 50,000 kDa, get inside the carrier granules, and are slowly washed out by the mobile phase. The retention (elution) times of immunoglobulin components (fractions) are distributed according to the molecular weight decrease, which should be mentioned in the Pharmacopoeia Monographs or Normative Documents: polymers (aggregates), dimers, Ig G monomers (main fraction of serum immunoglobulin), and finally Ig G fragments.

Quantitative analysis of immunoglobulins consists of the following stages: 1) chromatographic separation; 2) determination of the peak area and / or height; 3) calculation of the quantities of immunoglobulin fractions based on chromatographic data; 4) interpretation of obtained results (statistical analyses).

The content of immunoglobulin components (fractions) is calculated automatically using the software of the analytical device, by the peak areas, and reported as a percentage. The percentages of the determined immunoglobulin components should meet the requirements included in the Pharmacopoeia Monograph or the Normative Document.

The chromatography system is tested for suitability using standard samples. A chromatography system is considered suitable if the conditions specified in the Pharmacopoeia Monograph or the Normative Document are fulfilled: the peak resolution factor (R), chromatographic column performance calculated for the peak (the number of theoretical plates should be specified), and relative standard deviation of the peak areas.

Notes

1. Tested solution**.** Immunoglobulin samples are diluted before the analysis as specified in the Pharmacopoeia Monograph or the Normative Document.

2. Mobile phase. The mobile phase is prepared in accordance with the procedure described in the Pharmacopoeia Monograph or the Normative Document.

3. Standard samples:

– chromatography system suitability test solution;

– markers for size-exclusion high-performance liquid chromatography with a molecular weight in the range of 10,000 to 600,000 Da. The markers are prepared in accordance with the Instructions for Use or according to the method described in the Pharmacopoeia Monograph or the Normative Document.