**MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION**

# GENERAL PHARMACOPOEIA MONOGRAPH

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| **High-performance liquid chromatography** | **GPM.1.2.1.2.0005.15** supersedes St. Pharm. XII monograph |

High Performance Liquid Chromatography (HPLC) is a method of gel column chromatography where the mobile phase is a fluid moving through a chromatographic column packed with a stationary phase (a sorbent). Columns for high performance liquid chromatography are characterized by high hydraulic resistance at the inlet.

Depending on the mechanism of separation of substances, there are following types of high performance liquid chromatography: adsorption, partition, ion exchange, size exclusion, chiral chromatography etc., in accordance with the nature of main intermolecular interactions. Adsorption chromatography separates substances based on their differing abilities to adsorb and desorb from the sorbent with a developed surface, e.g., silica gel. Partition high performance liquid chromatography separates substances due to the differences in partition coefficients of separated substances between the stationary (usually chemically grafted to the surface of a stationary carrier) and mobile phases.

Depending on the type of mobile phase and stationary phase, there is normal-phase and reversed-phase chromatography. The stationary phase in normal-phase high performance liquid chromatography is polar (often silica or silica grafted with $NH_2$- or CN-groups, etc.), while the mobile phase is non-polar (hexane, hexane or more polar organic solvents, such as chloroform, alcohols etc.).

The retention of substances increases with increasing polarity. In normal-phase chromatography, the mobile phase eluting ability increases with its polarity.

In reversed phase chromatography, the stationary phase is non-polar (hydrophobic silica grafted with C4, C8, C18 groups etc.); the mobile phase is polar (a mixture of water and polar solvents: acetonitrile, methanol, tetrahydrofuran, etc.). The retention of substances increases with increasing hydrophobicity (non-polarity). The greater is the content of an organic solvent, the higher is the eluting capacity of a mobile phase.

In ion exchange chromatography, molecules of a mixture of substances, dissociated to cations and anions in a solution, are separated as it moves through a sorbent (cation exchanger or anion exchanger) due to various interaction forces of determinable ions with ionic groups of the sorbent.

In size exclusion (molecular sieve, gel permeation, gel filtration) chromatography, substance molecules are separated by size due to their different abilities to penetrate into the pores of a stationary phase, with the largest molecule that can penetrate into a minimum number of pores of a stationary phase being eluted the first from the column, while substances with small size molecules are eluted the last.

Chiral chromatography separates optically active compounds into individual enantiomers. The separation can be carried out on chiral stationary phases or achiral stationary phases using chiral mobile phases.

There are other types of high performance liquid chromatography.

Separation often occurs not via one but via several mechanisms simultaneously, depending on the type of mobile and stationary phases, as well as the nature of the analyzed compound.

## Scope

High performance liquid chromatography has been successfully used for both qualitative and quantitative analysis of drugs in tests "Identification", "Related substances", "Dissolution", "Uniformity of Dosage units", "Assay". It

should be noted that chromatography allows combining several tests in one sample, including "Identification" and "Assay."

## Equipment

Appropriate equipment should be used for the analysis, i.e. liquid chromatographs.

A liquid chromatograph usually consists of the following main components:

- A mobile phase preparation unit, including a mobile phase container (or containers with separate solvents from the mobile phase composition) and a mobile phase degassing system;
- A pumping system;
- A mobile phase mixer (if needed);
- A sample injection system (injector), may be manual or automatic (autosampler);
- A chromatographic column (can be installed in a thermostat);
- A detector (one or several, with different detection methods);
- A chromatography control, data acquisition and data processing system.

In addition, a chromatograph may include: a sample preparation system and a pre-column reactor, a column switching system, a post-column reactor and other equipment.

### *Pumping system*

Pumps feed the mobile phase into the column at a predetermined flow rate. The mobile phase composition and flow rate may be constant or changing during the analysis. In case of a constant mobile phase composition, the process is called isocratic. If the mobile phase composition changes, the process is called gradient. A modern pumping liquid chromatograph system consists of one or more pumps controlled by a computer. This allows changing the mobile phase composition using a particular program in a gradient elution. Pumps for analytical high performance liquid chromatography allow maintaining the mobile phase flow rate into the column within the range of 0.1 to 10 ml/min at a column inlet pressure up

to 40 MPa. Pressure pulsations are minimized by a special damper system included in the pump design. Working pump parts are made of corrosion-resistant materials, which allows to use aggressive components in the mobile phase composition.

### *Mixers*

If the required mixture was not prepared in advance, a common mobile phase is prepared from individual solvents fed by pumps in the mixer. The mobile phase components are mixed in a mixer both at low pressure (before the pumping stage) and high pressure (after the pumping stage). The mixer can also be used to prepare the mobile phase in isocratic elution.

The mixer volume can affect the retention time of the components in the gradient elution.

### *Injectors*

Injectors can be multipurpose, with varying volume of an injected sample or discrete, for injecting only a specific sample volume. Both types of injectors can be automatic ("autoinjectors" or "autosamplers"). A sample (solution) injector is located directly before the chromatographic column. The injector construction allows changing the direction of the mobile phase flow and performing a pre-injection of a certain sample amount (usually from 10 to 100 μl) into a sample loop or a special sampling device with variable volume. The sample loop volume is indicated on its label. The discrete injector design typically allows replacement of a sample loop. Modern automatic injectors can have a number of additional features, for example, serve as a sample preparation station: to perform mixing and dilution of the samples, to carry out a precolumn derivatization reaction.

### *Chromatographic column*

Chromatography columns are typically stainless steel, glass or plastic tubes filled with a sorbent and closed on both sides with filters with a pore diameter of 2 to 5 microns. An analytical column length may range from 5 to 60 cm or more, with the inner diameter of 2 to 10 mm. Columns with internal diameters of less than 2 mm are used in the microcolumn chromatography. There are also capillary

columns with inner diameters of about 0.3 to 0.7 mm. Preparative chromatography columns may have inner diameters of 50 mm or greater.

Short columns (precolumns) may be installed before an analytical column to perform various auxiliary functions, the main of which is the protection of the analytical column. Typically, the analysis is conducted at ambient temperature; however, in order to increase the separation efficiency and reduce the analysis duration, thermostatting of columns up to 80–100°C can be used. The possibility to use elevated temperatures in the separation is limited by the stationary phase stability because its destruction is possible at high temperatures.

***Stationary phase (sorbent)***

The following are typically used as sorbents:

– Silica gel, aluminum oxide are used in normal phase chromatography. The retention mechanism in this case is usually adsorption.

– Silica gel, resins or polymers grafted with acidic or basic groups. The scope is ion exchange and ion chromatography.

– Silica gel or polymers with a specified pore size distribution (size exclusion chromatography).

– Chemically modified sorbents (grafted phase sorbents), often prepared on the basis of silica gel. The retention mechanism is adsorption or distribution between the mobile and stationary phases. The scope depends on the type of the grafted functional groups. Some types of sorbents can be used both in reverse-phase and normal-phase chromatography;

– Chemically modified chiral adsorbents, for example, amylose and cellulose derivatives, proteins and peptides, cyclodextrins, chitosans used for the separation of enantiomers (chiral chromatography).

Sorbents with grafted phases may have varying degrees of chemical modification. The following are most commonly used as grafted phases:

- Octadecyl group [Si-$(CH_2)_{17}$-$CH_3$] (sorbent octadecylsilane (ODS) or $C_{18}$);

– Octyl groups [Si-$(CH_2)_7$-$CH_3$] (octylsilane sorbent or $C_8$);

– Phenyl groups [Si-$(CH_2)_n$-$(C_6H_5)$] (phenylsilane sorbent);
– Cyanopropyl groups [Si-$(CH_2)_3$-CN] (CN sorbent);
– Aminopropyl groups [Si-$(CH_2)_3$-$NH_2$] ($NH_2$ sorbent);
– Diol groups [Si-$(CH_2)_3$-OCH(OH)-$CH_2$-OH] (diol sorbent).

The analysis is most frequently performed on grafted non-polar phases in a reversed-phase mode using $C_{18}$ sorbent.

Sorbents with grafted phases obtained based on silica gel are chemically stable at pH values from 2.0 to 7.0, if not otherwise specified by the manufacturer. Sorbent particles can be spherical or irregular in shape and have varied porosity. The particle size of the sorbent is typically 3 to 10 microns in analytical high performance liquid chromatography and 50 microns and greater in preparative high performance liquid chromatography. There are also monolithic columns, where the sorbent is a monolith with through pores, filling the entire volume of the column.

A high separation efficiency is ensured by a large surface area of the sorbent particles (which is a consequence of their microscopic size and the presence of pores), as well as the uniformity of the sorbent composition and its uniform and dense packing.

### *Detectors*

High performance liquid chromatography uses different detection methods. In general, the mobile phase with dissolved components enters the detector cell after the chromatographic column, where its properties (absorption in the ultraviolet or visible light, fluorescence, refractive index, conductivity, etc.) are continuously measured. The obtained chromatogram is a plot of the relationship between a physical or physico-chemical parameter of the mobile phase and time.

The most common detectors in high performance liquid chromatography are **spectrophotometric**. During elution of substances in specially designed microcuvettes, optical density of the eluate is measured at a preselected wavelength. A wide detector linearity area allows analyzing both impurities, and main components of the mixture on the same chromatogram. A spectrophotometric detector allows performing detection at any wavelength within its operating range

(typically 190-600 nm). Multi-wave-length detectors are also used, allowing to carry out detection at multiple wavelengths simultaneously, as well as diode array detectors allowing to record absorbance simultaneously throughout the entire operating range of wavelengths (typically 190-950 nm). This allows recording the absorption spectra of the components passing through the detector cell.

**A fluorimetric detector** is used to determine fluorescent compounds and non-fluorescent compounds as their fluorescent derivatives. The operating principle of a **fluorimetric** detector is based on measuring the fluorescence emission of the absorbed light. The absorption is typically carried out in the ultraviolet area of the spectrum, the fluorescence emission wavelengths being greater than the absorbed light wavelengths. Fluorimetric detectors have a very high sensitivity and selectivity. The sensitivity of fluorescent detectors is about 1000 times higher than that of spectrophotometric detectors. Modern fluorescent detectors allow not only obtaining chromatograms, but also recording excitation and fluorescence spectra of analytes.

In order to determine the compounds with weak absorption in the ultraviolet and visible area of the spectrum (eg, carbohydrates), **refractometric detectors** (refractometer) are used. Disadvantages of these detectors include their low (compared to spectrophotometric detectors) sensitivity and considerable dependence of the signal intensity on temperature (a detector should be thermostated), as well as their inability to use a gradient elution mode.

The principle of operation of a **laser evaporative light scattering detector** is based on the difference in vapor pressures of chromatographic solvents included in the composition of the mobile phase and analyte. A mobile phase is introduced into an atomizer at the column outlet, is mixed with nitrogen or $CO_2$ and, in the form of a fine mist, enters the heated vaporization tube with a temperature of 30 to 160 °C, where the mobile phase is evaporated. Aerosol particles of non-volatile analytes scatter the light flux in the dispersion cell. Based on the degree of the luminous flux dispersion, the analyte amount can be determined. This type of detectors is more sensitive than the refractometric one, its signal does not depend

on the sample optical properties, a type of functional groups in the analyte or the mobile phase composition and may be used in a gradient elution mode.

Electrochemical detectors (conductometric, amperometric, coulometric, etc.). **An amperometric** detector is used to determine electroactive compounds that can be oxidized or reduced at a solid electrode surface. The analytical signal is the value of oxidation or reduction current. There are at least two electrodes in the detector cell, a working electrode and a reference electrode (silver chloride or steel). A working potential whose value depends on the nature of the analyzed compounds is applied to the electrodes. Measurements can be performed both at a constant potential, and in the pulsed mode, when the potential modification profile of the working electrode is set for one cycle of signal recording. An amperometric detector uses working electrodes made of carbon material (frequently glass-carbon or graphite), or metal: platinum, gold, copper, nickel.

**A conductometric** detector is used for detection of anions and cations in ion chromatography. Its operation is based on measuring the electrical conductivity of the mobile phase during the substance elution.

Extremely informative is a **mass-spectrometric detector**, which has a high sensitivity and selectivity. The latest models of mass spectrometers for liquid chromatography operate in the m/z mass range of 0 to 4000 amu.

High performance liquid chromatography also uses FTIR detectors, radioactivity detectors and several others.

### *Data acquisition and processing system*

A modern data processing system is a PC coupled with a chromatograph, with software that allows recording and processing a chromatogram, as well as controlling the chromatograph operation and following the basic parameters of the chromatographic system.

### *Mobile phase*

A mobile phase in high performance liquid chromatography performs a dual function: ensures the transfer of desorbed molecules through the column and regulates the equilibrium constants, and thereby the retention as a result of an

interaction with the stationary phase (sorbing on the surface) and molecules of the substances to be separated. Thus, by changing the composition of the mobile phase in high performance liquid chromatography, one can affect the retention times of the compounds, the selectivity and efficiency of their separation.

A mobile phase may consist of a single solvent, often two, and, if needed, of three or more. The mobile phase composition is indicated as a volume ratio of its constituent solvents. In some cases, the weight ratio can be specified, which should be specifically stated. Buffer solutions with specific pH, various salts, acids, bases, and other modifiers can be used as mobile phase components.

Liquid hydrocarbons (hexane, cyclohexane, heptane), and other relatively non-polar solvents with small additions of polar organic compounds that regulate the eluting power of the mobile phase are commonly used in normal-phase chromatography.

Water or an aqueous-organic mixture is used as a mobile phase in reversed-phase chromatography. Organic additives are typically polar organic solvents (acetonitrile and methanol). In order to optimize the separation, aqueous solutions with a certain pH, in particular, buffers, and various additives to the mobile phase can be used: phosphoric and acetic acid in separation of acidic compounds; ammonia and aliphatic amines in separation of basic compounds, and other modifiers.

The chromatographic analysis is greatly influenced by the mobile phase purity, so it is preferable to use solvents manufactured specifically for liquid chromatography (including water).

When using a UV spectrophotometric detector, the mobile phase should not have an expressed absorption at the wavelength selected for detection. A transparency limit or optical density at a particular wavelength of a solvent produced by a particular manufacturer is often indicated on the packaging.

The mobile phase and analyte solutions should not contain insoluble particles and gas bubbles. Water prepared in the laboratory, aqueous solutions, organic solvents pre-mixed with water, as well as analyte solutions must be

subjected to fine filtration and degassing. For these purposes, vacuum filtration through an inert (relative to this solvent or solution) membrane filter with a pore size of 0.45 microns is usually used.

### *List of chromatographic conditions to be specified*

A pharmacopeial monograph shall indicate the following: full commercial name of a column with the catalog number and the manufacturer name, column dimensions (length and internal diameter), the sorbent type with particle size, pore size, column temperature (temperature control if necessary), sample injection volume (loop volume), mobile phase composition and its preparation method, mobile phase feed rate, detector type and detection conditions (if needed, parameters of the used detector cell), description of the gradient mode (if any), which includes a re-equilibration step to baseline conditions, chromatography time, detailed description of the method and calculation formulas, description of the preparation of standard and test solutions.

If pre-column derivatization in the autosampler is used, provide the information on the autosampler operating program. When post-column derivatization is used, specify derivatizing reagent feedrate, mixing loop volume and its temperature.

## MODIFIED FORMS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

### *Ion-pair chromatography*

One of reversed-phase high performance liquid chromatography types is ion pair chromatography that allows to determine ionized compounds. For this purpose, hydrophobic organic compounds with ionogenic groups (ion pair reagents) are added in the mobile phase in conventional reverse-phase high performance liquid chromatography. Alkyl sulphates are usually used for the separation of bases, while for the separation of acids are used tetraalkylammonium salts (tetrabutylammonium phosphate, cetyltrimethylammonium bromide, etc.). In ion-pair mode, the selectivity of separation of non-ionic components will be

limited by a reversed-phase retention mechanism, while the retention of bases and acids is markedly increased and the shape of chromatographic peaks is improved.

The retention in ion-pair mode is caused by complex equilibrium processes, competing with each other. On the one hand, due to hydrophobic interactions and the effect of displacing a polar medium from the mobile phase, hydrophobic adsorption of ions on the alkyl-substituted silica gel surface can occur so that charged groups are oriented towards the mobile phase. In this case, the surface acquires ion exchanger properties, and the retention follows the patterns of ion exchange chromatography. On the other hand, ion pairs can be formed directly in the eluent, followed by its sorption on the sorbent by the reversed-phase mechanism.

### *Hydrophilic interaction chromatography (HILIC)*

Hydrophilic interaction chromatography is used to separate polar compounds weakly retained in reversed phase high performance liquid chromatography. This type of chromatography uses acetonitrile-water mixtures with addition of salts, acids or bases as the mobile phase. Stationary phases are, as a rule, silica gels modified with polar groups (amino, diol, cyanopropyl groups, etc.). More polar compounds are retained better. The mobile phase eluent ability increases with increasing polarity.

### *Ion exchange and ion high performance liquid chromatography*

Ion exchange chromatography is used for the analysis of organic (heterocyclic bases, amino acids, proteins, etc.) and inorganic (various cations and anions) compounds. The separation of components in the mixture being analyzed by ion exchange chromatography is based on the reversible interaction between ions of analytes from the ion exchange groups of the sorbent. These sorbents are primarily polymeric ion-exchange resins (typically styrene and divinylbenzene copolymers grafted with ion exchange groups) or silica gels grafted with ion-exchange groups. Sorbents with groups —$NH_3^+$, —$R_3N^+$, —$R_2HN^+$, —$RH_2N^+$ etc. are used for the separation of anions (anionites), while sorbents with groups —

$SO_3^-$, —$RSO_3^-$, –COOH, —$PO_3^-$ etc. are used for separation of cations (cationites).

Aqueous solutions of acids, bases and salts are used as a mobile phase in the ion exchange chromatography. Typically buffer solutions are used that allow maintaining certain pH values. It is also possible to use small amounts of water-miscible organic solvents, such as acetonitrile, methanol, ethanol, tetrahydrofuran.

***Ion chromatography*** is a type of ion exchange chromatography, where a conductometric detector is used to detect analyzed compounds (ions). For a high sensitive detection of changes in the conductivity of a mobile phase passing through the detector, the background conductivity of the mobile phase must be low.

There are two main types of ion chromatography.

The first one is two-column ion chromatography, based on the suppression of the mobile phase electrolyte conductivity using a second ion exchange column or a special membrane suppression system located between the analytical column and the detector. When the mobile phase passes through the system, its conductivity decreases.

The second type of ion chromatography is single-column ion chromatography. This type uses a mobile phase with a very low electrical conductivity. Weak organic acids are widely used as electrolytes: benzoic, salicylic, or isophthalic acid.

### *Size exclusion high performance liquid chromatography*

Size exclusion chromatography (gel chromatography) is a special type of high performance liquid chromatography based on the separation of molecules according to their size. The distribution of molecules between the mobile and stationary phases is based on their molecular size and partly their shape and polarity.

There are two marginal types of a molecular interaction with a porous stationary phase. Molecules with dimensions exceeding the maximum pore diameter are not retained at all and generally elute first, moving with the mobile

phase. Molecules with sizes smaller than the minimum sorbent pore diameter easily penetrate into the pores and elute from the column the last. Other molecules with intermediate sizes are partly retained in the pores and are fractionated according to their size and, partly, shape during elution, and penetrate into the pores of the sorbent according to their size and partly their shape. As a result, substances elute with different retention times.

### *Ion exclusion chromatography*

The mechanism of ion exclusion chromatography is based on the effect by which the compounds in an ionized form are not retained on an ion exchanger sorbent, while compounds in a molecular form are distributed between the stationary and aqueous phases within the pores of the ion exchanger sorbent and the mobile phase, migrating into the space between sorbent particles. The separation is based on the electrostatic repulsion, polar and hydrophobic interactions between dissolved compounds and the sorbent.

Anionic groups on the sorbent surface act as a semipermeable "membrane" between the stationary and mobile phases. Negatively charged components do not reach the stationary mobile phase, as they are repelled by like-charged functional groups and eluted in the "dead" (free) volume of the column. Components in a molecular form are not "rejected" by the cation exchange sorbent and are distributed between the stationary and mobile phases. The difference in the retention degree of mixture components is due to a complex of polar interactions of nonionic components with functional groups of a cation exchanger sorbent and hydrophobic interactions of nonionic components with a nonpolar sorbent matrix.

### *Chiral chromatography*

The objective of chiral chromatography is the separation of optical isomers. The separation can be carried out on chiral stationary phases or conventional achiral stationary phases using chiral mobile phases. Sorbents with surfaces modified with groups or substances having chiral centers (chitosans, cyclodextrins, polysaccharides, proteins, etc. (chiral selectors)) are used as chiral stationary phases. In this case, the same phases as in the normal phase or reverse phase

chromatography can be used as a mobile phase. When using achiral stationary phases for the separation of enantiomers, the following chiral modifiers are added in mobile phases: chiral metal complexes, neutral chiral ligands, chiral ion-pairing reagents etc.

### *Ultra high performance liquid chromatography*

Ultra high performance liquid chromatography is a type of liquid chromatography, distinguished by higher effectiveness in comparison with the conventional high-performance liquid chromatography.

The specific property of ultra high performance liquid chromatography is the use of sorbents with a particle size of 1.5 to 2 microns. Dimensions of chromatographic columns generally range from 50 to 150 mm in length and from 1 to 4 mm in diameter. A sample injection volume may range from 1 to 50 microliters. The use of such chromatography columns significantly reduces the analysis time and improves the efficiency of the chromatographic separation. However, the pressure in the column may reach 80 to 120 MPa, the desired frequency of detector data collection can increase to 40-100 Hz, an extra-column volume of the chromatographic system should be minimized. Chromatographic equipment and columns used in ultra high performance liquid chromatography are specifically adapted to meet the requirements for this type of chromatography.

Equipment designed for ultra high performance liquid chromatography can also be used in the conventional high performance liquid chromatography.