**Lesson 3.**

**Fundamentals of spectrophotometry.**

Methods of spectrophotometry are methods for the study and analysis of substances based on the absorption of monochromatic electromagnetic radiation by molecules of a substance in the ultraviolet (UV), visible and infrared (IR) regions of the spectrum. The nature of the absorption bands in the UV and visible regions of the spectrum is associated with various electronic transitions in absorbing molecules and ions (electronic spectra). In the IR region, it is associated with vibrational transitions and changes in the vibrational states of the nuclei that make up the molecule of the absorbing substance (vibrational spectra).

In the case of absorption of non-monochromatic radiation by substances, photocolorimetric (colorimetric) methods of analysis are distinguished. Photocolorimetry differs from spectrophotometric analysis in that the analyte is converted (quantitatively) into a colored compound using a reagent. First, colored solutions are obtained using solutions of standard samples. Then, a calibration graph is built for the dependence of the intensity of absorption of colored solutions on the concentration of the standard solution, and the content of the substance in the test samples is calculated from the graph.

The method of absolute spectrophotometry (photocolorimetry) is based on measuring the light absorption of the analyzed solution relative to the reference solution, which can be a pure solvent or a solution containing all components of the analyzed solution, except for the analyte.

The method of differential spectrophotometry (photocolorimetry) is based on measuring the light absorption of the analyzed solution relative to a reference solution containing a certain amount of a standard sample of the test substance or its substitute. This technique leads to a change in the working area of the instrument scale and a decrease in the relative error of determination to ±(0.5–1)%, i.e., comparable to titrimetric methods. Spectrophotometric and photocolorimetric methods of analysis are based on the use of the combined Bouguer-Lambert-Beer law:



where I0 is the intensity of the radiation incident on the substance; I is the intensity of the radiation that has passed through the substance; A - optical density, absorption; k is the absorption rate of a given substance (molar absorption index ε or specific absorption index, used in pharmaceutical analysis); C is the concentration of the analyte solution, mol/l; ℓ is the length of the working layer of the cuvette, cm.

The specific absorption index (ε) is a spectrophotometric constant for each substance, independent of concentration, and represents the value of the optical density of a solution containing 1.0 g of a substance in 100 cm3 of solution, measured in a cuvette with a working length of 1 cm:



By setting the value according to the standard sample and converting this formula, it is possible to calculate the concentration of the analyte with a relative error of up to ±2%:



In case of deviations from the Bouguer-Lambert-Beer law, first, using standard solutions, the dependence of optical density on concentration is established and a calibration graph is built, and then the content of the analyte in the analyzed solution is determined from it.

The Bouguer - Lambert - Beer law reflects the linear dependence of the optical density A on the concentration C at a constant thickness of the absorbing layer l (Beer's law) and, conversely, the dependence of A on l at a constant C (the Bouguer - Lambert law).

In the second case, dependence is a rule from which there are no exceptions.

The dependence of A on C at a constant value of l should ideally be linear.



The dependence of the optical density of substance A on the concentration C

subject to the basic law of light absorption.

When Beer's law is fulfilled, the dependence of optical density on concentration is a straight line passing through the origin, and the function A = f(λ), the graphical dependence of which is called the absorption spectrum, has the same form, regardless of the layer thickness and solution concentration, and the position of the absorption maximum is preserved.

Choice of optimal conditions for carrying out photometric determinations

To carry out a photometric reaction, the component to be determined is converted into a compound with significant absorption. Most often it is associated in a complex compound.

When using photometric titration, the same reactions are used as in conventional methods, however, the content of a substance is judged not by the intensity of absorption, but by the amount of titrant consumed.

The conditions for carrying out photometric reactions should be carefully studied beforehand.

Preliminary study of working conditions includes:

1. Choice of wavelength.

In spectrophotometers, it is recommended to carry out measurements at a wavelength λ corresponding to the maximum value of A (λmax).

To do this, the dependence of A on λ is plotted (the absorption spectrum is taken) along the entire length of the scale, and the wavelength at which the absorption is maximum is selected for operation.

2. Calculation of the molar absorption coefficient ε.

The calculation is based on the observance of the basic law of light absorption. The calculation is carried out according to the formula

*А = ε* ·*l* · *C*

The optical density of a solution of the same concentration is measured in cuvettes of different thicknesses and a graph of A versus l is plotted. The straightness of the graph indicates compliance with the Bouguer-Lambert law.

The interval of compliance with Beer's law is determined by the linear dependence of A on C. To do this, at a constant value of l, the optical densities of a series of solutions with different concentrations are measured.

To calculate ε, the optical density of a solution of known concentration is measured in a cuvette of a certain thickness. In photometric analysis, preference is given to methods with a larger value of ε.

3. Study of the influence of extraneous factors on optical density (solvent nature, solution pH, temperature, presence of foreign components, etc.).

4. Choice of the optimal value l (selection of the cuvette).

First, the cuvette is selected by eye according to the color of the solution. If the solution is weakly colored, then use cuvettes with a thickness of 2 to 10 cm. If the color of the solution is sufficiently intense, use cuvettes less than 1 cm. In practice, proceed as follows: pour a solution of medium concentration from the reference series into the cuvette and measure the optical density, which should be in range from 0.4 to 0.6 (in this case, the measurement error is minimal). If A is greater than these values, then a cuvette with a smaller value of l should be taken. If A is less than 0.4–0.6, then a thicker cuvette should be used. In practice, cuvettes with a thickness of 1 cm are most often used.

5. Choice of reference solution.

As a reference solution, depending on the conditions, they most often use:

- solvent;

– reagent solution, if it itself absorbs radiation;

– solution of a blank experiment (contains all components, except for the one being determined);

– solution of the analyzed object.

7. Calculation of detection limits and determination of the minimum and maximum concentrations of this component. This is reliably established only with the use of methods of mathematical statistics.

Spectrophotometry in pharmaceutical analysis

Spectrophotometry in the UV and visible regions is one of the most widely used physical and chemical methods in pharmaceutical analysis (OFS 42-0042-07 SP RF XII). The analyzed drugs must have chromophore groups in the structure of the molecule (conjugated bonds, aromatic nucleus, etc.), which determine various electronic transitions in molecules and the absorption of electromagnetic radiation.

 Identification of drugs can be carried out by the nature of the absorption spectra in various solvents, the position of the absorption maxima and minima, or by their ratio (at different wavelengths). The absorption spectrum of a substance is its specific characteristic and is a curve of dependence of the absorption intensity (optical density) on the wavelength (l, nm).

For quantitative spectrophotometric analysis, the choice of the analytical absorption band is important. The latter should be free from overlapping absorption bands of other components of the mixture and have a sufficiently high specific absorption rate of the analyte.

One variation of differential spectrophotometry is derivative UV spectrophotometry. If in differential spectrophotometry they use the difference in optical densities at the same wavelength, then in the derivative - at two wavelengths in a small interval. This option allows you to select individual bands in the "complex" UV spectrum, which is the sum of overlapping absorption bands or bands that do not have a clearly defined maximum. At the same time, bands with distinct maxima and minima appear on the spectral curves in the derivative–wavelength coordinates (∆I – λ). Thanks to this, it is possible to identify substances similar in chemical structure, increase the selectivity of the analysis and perform the quantitative determination of two-, three-component mixtures more economically and efficiently than by titrimetric methods.

Another variant of differential spectrophotometry is the AE method based on the transformation of one of the substances that make up the analyzed sample into a tautomeric (or other) form that differs in the nature and intensity of absorption. Then the optical density of a solution of one tautomeric form is measured in relation to another, i.e., a solution of the initial analyte is used as a reference solution.

To measure the optical density and record absorption spectra, spectrophotometers are used - devices that allow the analysis of both colored and colorless compounds by selective absorption of monochromatic radiation in the visible, UV and IR spectral regions. There is a wide variety of spectrophotometers from various manufacturers on the market today. Spectrophotometers have been designed that operate in various regions of the spectrum, for example, only in the UV or only in the IR region, in the UV and visible ranges. There are devices that operate in all ranges, which allows you to conduct various studies on the same equipment. Modern equipment makes it possible to measure UV spectra in the range from 190 to 380 nm, visible spectra - from 380 to 780 nm, IR spectra - from 780 to 40,000 nm (40 μm).

The optical scheme of the spectrophotometer is shown in fig.



As a source of radiation (1) use hydrogen, deuterium, mercury-quartz, sodium, and xenon lamps. Monochromator (5) - glass or quartz prism, diffraction grating - is used to obtain monochromatic light. The photocell (8) converts the energy of the incident light into an electric current, and the amplifier makes it possible to obtain a signal that is recognized by the detector (9). The detector converts the signal into specific numerical values. An important part of the instrument is the cuvette (7), which is subject to special requirements. Permissible deviations in the layer thickness of the used cuvettes should be no more than ± 0.005 cm. The cuvettes intended for the test solution and the reference solution should have the same transmission (or optical density) when filled with the same solvent. Otherwise, this difference should be taken into account.

Benefits of using spectrophotometry in pharmaceutical analysis:

• high sensitivity (many modern drugs are extremely difficult to analyze by chemical methods due to the low content of the active substance);

• reproducibility;

the possibility of analyzing drugs that do not give chemical reactions in a stoichiometric ratio (for example, rutin);

• the possibility of analyzing multicomponent LFs for which there are no methods for quantitative determination by chemical methods (for example, complex vitamin preparations containing pyrodoxine, riboflavin and nicotinamide);

• Possibility of combination with other methods, for example, with high-performance liquid chromatography (HPLC), where the spectrophotometer is used as a detector (this combination of methods makes it possible to carry out qualitative and quantitative analysis with high accuracy in the presence of a large number of substances in a mixture with similar physical and chemical properties).