**Lesson 8**

**Nuclear magnetic resonance spectroscopy**

Nuclear magnetic resonance spectroscopy (NMR) is a method based on the absorption of radio frequency electromagnetic radiation by the nuclei of a sample with a non-zero magnetic moment placed in a constant magnetic field (B0). Non-zero magnetic moments have isotopes of nuclei of elements with an odd atomic mass (1H, 13C, 15N, 19F, 31P, etc.).

General principles

A nucleus rotating around its axis has its own angular momentum (angular momentum, or spin) P. The magnetic moment of the nucleus μ is directly proportional to the spin: μ = γ ∙ P (γ is the proportionality coefficient or gyromagnetic ratio). The angular and magnetic moments are quantized, i.e. can be in one of 2I + 1 spin states (I is the spin quantum number). Different states of the magnetic moments of nuclei have the same energy if they are not affected by an external magnetic field. When the nuclei are placed in an external magnetic field B0, the energy degeneracy of the nuclei is removed and the possibility of an energy transition from one level to another arises. The process of distributing nuclei between different energy levels proceeds in accordance with the Boltzmann distribution law and leads to the appearance of a macroscopic equilibrium longitudinal magnetization Mz. The time it takes to create Mz after turning on the external magnetic field B0 is called the time of longitudinal or spin-lattice relaxation (T1). Violation of the equilibrium distribution of nuclei occurs under the action of a radio frequency magnetic field (B1) perpendicular to B0, which causes additional transitions between energy levels, accompanied by energy absorption (the phenomenon of nuclear magnetic resonance). The frequency ν0 at which the absorption of energy by nuclei occurs (Larmor or resonant absorption frequency) varies depending on the value of the constant field B0: ν0 = γB0/2π. At the moment of resonance, there is an interaction between the individual nuclear magnetic moments and the field B1, which brings the vector Mz out of its equilibrium position along the z axis. As a result, transverse magnetization Мxy appears. Its change associated with the exchange within the spin system is characterized by the transverse or spin-spin relaxation time (T2).

The dependence of the intensity of energy absorption by nuclei of the same type on the frequency of the radio-frequency magnetic field at a fixed value of B0 is called the one-dimensional spectrum of nuclear magnetic resonance of a nucleus of this type. An NMR spectrum can be obtained in two ways: by continuously irradiating a sample with a varying frequency RF field, resulting in a direct NMR spectrum (continuous exposure spectroscopy), or by exposing the sample to a short RF pulse (pulse spectroscopy). In pulsed NMR spectroscopy, the time-decayed coherent radiation emitted by nuclei upon returning to the initial spin state (free induction decay signal) is recorded, followed by the transformation of the time scale into a frequency one (Fourier transform).

In molecules, the electrons of atoms reduce the value of the acting external magnetic field B0 at the location of the nucleus, i.e. diamagnetic shielding appears:

***B*лок = *B*0 ∙ (1 – σ),**

Where

Block is the strength of the resulting field;

σ is the screening constant.

The difference in the resonant frequencies of the signals of the nuclei, equal to the difference in their screening constants, is called the chemical shift of the signals, denoted by the symbol δ, measured in parts per million (ppm). The interaction of the magnetic moments of the nuclei through chemical bonding electrons (spin-spin interaction) causes splitting of the NMR signal (multiplicity, m). The number of components in multiplets is determined by the nuclear spin and the number of interacting nuclei. The measure of spin-spin interaction is the spin-spin interaction constant (J, measured in hertz, Hz). The values of δ, m, and J do not depend on the value of the constant magnetic field.

The intensity of the nuclear NMR signal in the spectrum is determined by the population of its energy levels. Of the nuclei with a natural abundance of isotopes, the most intense signals are produced by hydrogen nuclei. The intensity of NMR signals is also affected by the longitudinal-transverse relaxation time (larger T1 leads to a decrease in signal intensity).

The width of the NMR signals (the difference between the frequencies at half-height of the signal) depends on T1 and T2. Small times T1 and T2 cause wide and poorly interpreted spectrum signals.

The sensitivity of the NMR method (maximum detectable concentration of a substance) depends on the intensity of the nuclear signal. For 1H nuclei, the sensitivity is 10-9 ÷ 10-11 mol.

Correlations of various spectral parameters (for example, chemical shifts of different nuclei within the same molecular system) can be obtained by homo- and heteronuclear methods in 2D or 3D format.

device

High resolution NMR pulse spectrometer (NMR spectrometer) consists of:

 a magnet to create a constant magnetic field B0;

 a temperature-controlled sensor with a sample holder for applying a radio frequency pulse and determining the radiation emitted by the sample;

 an electronic device for creating a radio frequency pulse, recording, amplifying and converting the free induction decay signal into digital form;

 devices for tuning and adjusting electronic circuits;

 data collection and processing devices (computer);

and may also include:

a flow cell for NMR liquid chromatography or flow-injection analysis;

 a system for creating a pulsed magnetic field gradient.

A strong magnetic field is generated by a superconductivity coil in a Dewar vessel filled with liquid helium.

The proper functioning of the NMR spectrometer should be checked. For verification, appropriate tests are carried out, including, as a rule, the measurement of the spectral linewidth at half-height of certain peaks under certain conditions (resolution), the reproducibility of the signal position and the signal-to-noise ratio (the ratio between the intensity of a certain signal in the NMR spectrum and random vibrations in the spectral region, containing no analyte signals, S/N) for standard mixtures. The spectrometer software contains algorithms for determining S/N. All instrument manufacturers provide specifications and measurement protocols for these parameters.

NMR Spectroscopy of Samples in Solutions

Methodology

The test sample is dissolved in a solvent to which an appropriate chemical shift calibration standard may be added as specified in the regulatory documentation. The value of the relative chemical shift of the nucleus of a substance (δv-in) is determined by the following expression:

**δв-во = (νв-во– νэталон)/νприбора,**

Where

νв-в – the resonance frequency of the core of the substance, Hz;

νetalon is the resonance frequency of the etalon core, Hz;

νinstrument is the operating frequency of the NMR spectrometer (the frequency at which the resonance conditions for hydrogen nuclei are satisfied for a given B0, MHz).

For solutions in organic solvents, the chemical shift in the 1H and 13C spectra is measured relative to the tetramethylsilane signal, the position of which is taken as 0 ppm. The chemical shifts are counted in the direction of a weak field (to the left) from the tetramethylsilane signal (delta is the scale of chemical shifts). For aqueous solutions, sodium 2,2-dimethyl-2-silanepentane-5-sulfonate is used as a reference in 1H NMR spectra, the chemical shift of the protons of the methyl group of which is 0.015 ppm. For the spectra of 13C aqueous solutions, dioxane is used as a reference, the chemical shift of which is 67.4 ppm.

When calibrating the 19F spectra, trifluoroacetic acid or trichlorofluoromethane is used as the primary standard with zero chemical shift; spectra 31P - 85% solution of phosphoric acid or trimethyl phosphate; 15N spectra - nitromethane or saturated ammonia solution. 1H and 13C NMR typically use an internal reference that is directly added to the sample being tested. 15N, 19F, and 31P NMR often use an external standard, which is held separately in a coaxial cylindrical tube or capillary.

When describing NMR spectra, it is necessary to indicate the solvent in which the substance is dissolved and its concentration. Easily mobile liquids are used as solvents, in which hydrogen atoms are replaced by deuterium atoms to reduce the intensity of solvent signals. The deuterated solvent is selected based on the following criteria:

 1) the solubility of the test compound in it;

 2) absence of overlapping of the signals of residual protons of the deuterated solvent with the signals of the test compound;

 3) no interaction between the solvent and the test compound, unless otherwise indicated.

Solvent atoms give signals that are easily identified by their chemical shift and can be used to calibrate the chemical shift axis (secondary standard). The chemical shifts of the residual proton signals of deuterated solvents have the following values (ppm): chloroform, 7.26; benzene, 7.16; water - 4.7; methanol -3.35 and 4.78; dimethyl sulfoxide - 2.50; acetone - 2.05; the position of the signal of water and the protons of the hydroxyl groups of alcohols depends on the pH of the medium and temperature.

For quantitative analysis, solutions must be free of undissolved particles. For some assays, it may be necessary to add an internal standard to compare test and reference intensities. Appropriate standard samples and their concentrations should be specified in the normative documentation. After placing the sample in a test tube and capping, the sample is introduced into the magnet of the NMR spectrometer, the test parameters are set (settings, registration, digitization of the free induction decay signal). The main test parameters given in the regulatory documentation are recorded or stored in a computer.

To prevent spectrum drift over time, a stabilization procedure (deuterium lock) is performed using the deuterium signal induced by deuterated solvents, unless otherwise indicated. The instrument is adjusted to obtain the most optimal resonance conditions and maximum S/N ratio (shimming).

During the test, it is possible to perform multiple sequences of cycles "impulse - data acquisition - pause" with subsequent summation of individual signals of the decay of free induction and averaging the noise level. The delay time between pulse sequences, during which the system of nuclear spins restores its magnetization (D1), for quantitative measurements must exceed the longitudinal relaxation time T1: D1 ≥ 5 T1. The spectrometer software contains algorithms for determining T1. If the value of T1 is unknown, it is recommended to use the value D1 = 25 s.

For quantitative measurements, it is recommended to test without rotating the sample to avoid side signals.

After carrying out the Fourier transform, the signals in the frequency representation are calibrated to the selected standard and their relative intensity is measured by integration - measuring the ratio of the areas of the resonant signals. In the 13C spectra, only signals of the same type are integrated. The signal integration accuracy depends on the signal-to-noise ratio (S/N):

u(I)% = 0.25 + 100 / S/N

where u(I) is the standard uncertainty of integration.

The number of free induction decay accumulations required to achieve a satisfactory S/N ratio should be given in the regulatory documentation.

Along with one-dimensional for analytical purposes, homo- and heteronuclear two-dimensional correlation spectra are used, based on a certain sequence of pulses (COSY, NOESY, ROESY, HSQC, HMBC, HETCOR, CIGAR, INADEQUATE, etc.). In two-dimensional spectra, the interaction between nuclei manifests itself in the form of signals called cross peaks. The position of the cross peaks is determined by the values of the chemical shifts of the two interacting nuclei. Two-dimensional spectra are preferably used to determine the composition of complex mixtures and extracts, because the probability of signal superposition (cross peaks) in two-dimensional spectra is significantly lower than the probability of signal superposition in one-dimensional spectra.

To quickly obtain the spectra of heteronuclei (13C, 15N, etc.), methods (HSQC, HMBC) are used that allow one to obtain spectra of other nuclei on 1H nuclei using the mechanisms of heteronuclear interaction.

The DOSY technique, based on recording the loss of phase coherence of nuclear spins due to translational displacements of molecules under the action of a magnetic field gradient, makes it possible to obtain spectra of individual compounds (spectral separation) in a mixture without their physical separation and to determine the sizes, degrees of aggregation, and molecular weights of molecular objects (molecules , macromolecules, molecular complexes, supramolecular systems).

Areas of use

The variety of structural and analytical information contained in nuclear magnetic resonance spectra makes it possible to use the nuclear magnetic resonance method for qualitative and quantitative analysis. The use of nuclear magnetic resonance spectroscopy in quantitative analysis is based on the direct proportionality of the molar concentration of magnetically active nuclei to the integrated intensity of the corresponding absorption signal in the spectrum.

1. Establishing the identity of the active substance. The identification of the active substance is carried out by comparing the spectrum of the test sample with the spectrum of a standard sample or with a published reference spectrum. The spectra of standard and test samples should be obtained using the same methods and conditions. The peaks in the compared spectra should coincide in position (deviations of the δ values of the test and standard samples within ± 0.1 ppm for 1H nuclear magnetic resonance and ± 0.5 ppm for 13C nuclear magnetic resonance), integral intensity and multiplicity, the values of which should be given when describing the spectra. In the absence of a standard sample, a pharmacopoeial standard sample can be used, the identity of which is confirmed by independent structural interpretation of the spectral data and alternative methods.

When confirming the authenticity of samples of non-stoichiometric composition (for example, natural polymers of variable composition), the peaks of the test and standard samples are allowed to differ in position and integral intensity of the signals. The spectra to be compared must be similar, i.e. contain the same characteristic regions of the signals, confirming the coincidence of the fragment composition of the test and standard samples.

To establish the authenticity of a mixture of substances (extracts), one-dimensional NMR spectra can be used as a whole, as “fingerprints” of an object, without detailing the values of δ and the multiplicity of individual signals. In the case of using two-dimensional NMR spectroscopy in the description of spectra (spectrum fragments) claimed for authenticity, the values of cross peaks should be given.

2. Identification of foreign matter/residual organic solvents. Identification of impurities/residual organic solvents is carried out similarly to the identification of the active substance, tightening the requirements for sensitivity and digital resolution.

3. Determination of the content of foreign impurities / residual organic solvents relative to the active substance. The NMR method is a direct absolute method for determining the molar ratio of the active substance and the impurity compound (n/n impurity):

***S‘* / *S‘*примесь = *n* / *n*примесь**

where S‘ and S‘impurity are the normalized values of the integral intensities of the signals of the active substance and the impurity.

Normalization is carried out according to the number of nuclei in the structural fragment, which determine the measured signal.

The mass fraction of the impurity / residual organic solvent relative to the active substance (Xpr) is determined by the formula:

***X*пр = *M*пр x *S‘*пр  / M x *S’***

Where

Mpr is the molecular weight of the impurity;

M is the molecular weight of the active substance;

S‘pr is the normalized value of the integral intensity of the impurity signal;

S' is the normalized value of the integral intensity of the signal of the active substance.

4. Quantitative determination of the content of the substance (active substance, impurity / residual solvent) in the pharmaceutical substance. The absolute content of a substance in a pharmaceutical substance is determined by the internal standard method, which is chosen as a substance whose signals are close to the signals of the analyte, without overlapping with them. The signal intensities of the analyte and the standard should not differ significantly.

The percentage of the analyte in the test sample in terms of dry matter (X, % mass) is calculated by the formula:

***X,*% масс= 100 ∙ (*S*‘ /*S*‘0) ∙ (*M* ∙ *a*0 /*M*0 ∙ *a*) ∙ [100/(100 – *W*)],**

Where

S' is the normalized value of the integral intensity of the signal of the analyte;

S‘0 is the normalized value of the integrated signal intensity of the standard;

M is the molecular weight of the analyte;

M0 is the molecular weight;

a is the weight of the test sample;

a0 is the weight of the standard substance;

W is the moisture content, %.

The following compounds can be used as reference substances: maleic acid (2H; 6.60 ppm, M = 116.07), benzyl benzoate (2H; 5.30 ppm, M = 212.25), malonic acid (2H; 3.30 ppm, M = 104.03), succinimide (4H; 2.77 ppm, M = 99.09), acetanilide (3H; 2.12 ppm, M = 135.16), tert-butanol (9H; 1.30 ppm, M = 74.12).

The relative content of the substance as the proportion of the component in the mixture of components of the pharmaceutical substance is determined by the method of internal normalization. The molar (Xmol) and mass (Xmass) fraction of component i in a mixture of n substances is determined by the formulas:



5. NMR spectroscopy of solids Determination of the molecular weight of proteins and polymers. The molecular weights of proteins and polymers are determined by comparing their mobility with that of reference compounds of known molecular weight using DOSY techniques. The self-diffusion coefficients (D) of the test and reference samples are measured, and the logarithms of the molecular weights of the reference compounds are plotted against the logarithms of D. From the graph thus obtained, the unknown molecular weights of the test samples are determined by linear regression. A full description of the DOSY experiment should be given in the regulatory documentation.

NMR spectroscopy of solids

Samples in the solid state are analyzed using specially equipped NMR spectrometers. Certain technical operations (rotation of a powdered sample in a rotor inclined at a magic angle (54.7°) to the axis of the magnetic field B0, force depairing, polarization transfer from highly excitable nuclei to less polarizable nuclei - cross-polarization) make it possible to obtain spectra of organic and inorganic compounds with high resolution. A full description of the procedure should be given in the regulatory documentation. The main area of application of this type of NMR spectroscopy is the study of polymorphism of solid drugs.