The book “Modern Instrumental Methods in Solid Dosage Form Analysis” is focused on the description of selected modern instrumental methods widely used in the evaluation of dosage forms. It explains their main principles, provides a brief description of the instrumentation, and offers numerous examples of practical applications in both the pharmaceutical research and development, as well as in the commercial pharmaceutical manufacture. For the latter, these methods are used as valuable tools for quality assurance and quality control (QA/QC) of incoming raw materials, final medicinal products, and manufacturing processes.
MODERN INSTRUMENTAL METHODS
IN SOLID DOSAGE FORM ANALYSIS
MODERN INSTRUMENTAL METHODS IN SOLID DOSAGE FORM ANALYSIS

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Masaryk University Press
Brno 2021
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ISBN 978-80-210-9723-0 (paperback)

https://doi.org/10.5817/CZ.MUNI.M210-9724-2021
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The book “Modern Instrumental Methods in Solid Dosage Form Analysis” is focused on the description of selected modern instrumental methods widely used in the pharmaceutical technology. It explains the principles of the methods, provides a brief description of the instrumentation and offers numerous examples of practical applications in both the pharmaceutical research and development, as well as the commercial pharmaceutical manufacturing, where these methods are used as valuable tools of quality assurance and quality control (QA/QC) of incoming raw materials, final medicinal products and manufacturing processes used.

Modern instrumental methods offer numerous advantages especially for precise and fast identification of substances and quality control of final medicinal products. These methods could be used for studying and controlling quality and stability of materials: active pharmaceutical ingredients (APIs) and excipients, intermediate products (processing control) and also final products before their release to the market. They are particularly valuable for the identification of materials, determination of their solid-state properties, determination of the drug content and content uniformity, characterisation of interactions between active ingredients and excipients, determination of particle shape and size and visualization of the surface or inner structures of dosage forms.

The book does not provide a full scope of existing methods, rather describes methods with highest importance for pharmaceutical technology. Therefore, readers can familiarize themselves with infrared spectroscopy, Raman spectroscopy, thermal analysis, X-ray powder diffraction analysis, solid-state nuclear magnetic resonance and dissolution methods. Other described instrumental methods, especially optical and electron microscopy and scanning probe microscopy, have a specific role in the evaluation of the size, surface and shape of particles of pharmaceutical products. The book also describes the basic principles of process validation and patent protection of pharmaceuticals.
1 INTRODUCTION

Pharmaceutical technology is a pharmaceutical scientific discipline dealing with the composition, formulation, manufacturing and quality control and assurance of medicinal products. It studies conditions under which active pharmaceutical ingredients (APIs) and pharmaceutical auxiliary substances (excipients) are transformed into a final drug. It describes rules, standards and principles controlling these processes. The main objective of the pharmaceutical technology is to produce a dosage form that is effective, stable and safe, delivers the correct dose of an API in the specified time to the specific area and allows a comfortable administration of the drug into a human or animal body to diagnose, cure, mitigate, treat and prevent a disease or to relief a discomfort.

To achieve these objectives, pharmaceutical technology utilizes modern instrumental analytical methods that are able to analyse the dosage form and provide critical information about its state, composition and other physicochemical properties that determine the outer appearance, efficacy and stability of the drug; and significantly influence its pharmacokinetic behaviour and in vivo bioavailability. Instrumental analytical methods play an important role at all stages of pharmaceutical manufacture, from the drug development to marketing, from the API production through formulation to packaging.

The purpose of this book is to give a basic understanding of the principles and instrumentation of the selected instrumental methods suitable especially for solid dosage form assessment and to provide an overview of their practical applications in different aspects of pharmaceutical research and industry.

1.1 QUALITY CONTROL OF RAW MATERIALS (APIs AND EXCIPIENTS)

Testing of raw materials (APIs and excipients) is one of the main tasks of the quality control departments within the pharmaceutical industry. It must be ensured that the necessary tests are conducted and the raw
Materials are released for use only after verifying their quality. Raw material testing carried out by the pharmaceutical manufacturer can prevent costly production problems and delays by confirming that the raw materials contained in the pharmaceutical products are suitable for their intended use. The current regulatory framework on medicinal products requires APIs and excipients to be carefully selected: from the development throughout the product lifecycle. A great effort is spent on establishing specifications for raw materials and that of the finished product during the development cycle. Failing to do so can result in manufacturing problems and production lot failures attributable to raw materials used in the product. The drug manufacturer must firstly understand and identify critical factors that influence the stability of the pharmaceutical product, its \textit{in vitro} and \textit{in vivo} performance and processability. Once these factors are understood, the manufacturer should identify those factors that are impacted by the properties of raw materials, e.g. particle size and shape, polymorphism, crystallinity and presence of known impurities, such as by-products of synthesis, catalysts, moisture, residual solvents, degradation products etc. Understanding ingredient variation can also help to prevent future defects in final products. While a lot-to-lot variation of the raw materials is expected, the level of variation should be assessed and controlled.

Raw material analysis requires a wide range of analytical expertise. To expand the number and variety of APIs and excipients, a modern quality control laboratory must contain a wide spectrum of instrumentation exceeding beyond the most commonly used instruments, such as pH meters, instruments for gas or high performance liquid chromatography, ultraviolet/visible (UV/Vis) spectrophotometers, melting point apparatus, polarimeters, refractometers, dissolution apparatus, viscometers etc. Although the challenges associated with raw material testing are extensive, using appropriate advanced analytical methods, such as X-ray diffraction, laser diffraction, mid- and near-infrared spectroscopy or Raman spectroscopy can help to successfully meet the challenges.

Currently, much attention is paid to the evaluation of physical/crystalline form of APIs and excipients. Due to the influence of external physical and/or chemical conditions (type of solvent used, temperature, pressure etc.), many pharmaceutical substances can crystallise in different forms, so called polymorphic modifications (polymorphs). Amorphous
forms or various polymorphs of one chemical substance can significantly differ in important physicochemical parameters, such as solubility, dissolution rate, melting point, true density, compressibility, powder flow properties and solid-state stability. Therefore, it is important to characterise the solid state (crystalline or amorphous structure) of each raw material and subsequently select the modification with the most favourable properties in terms of product stability, bioavailability and processability. In some instances, a change of the solid state of a substance can occur spontaneously (recrystallization) due to the small changes in external conditions during manufacturing or storage. This can ultimately result in changes of dissolution profiles and altered bioavailability. Amorphous forms that are thermodynamically metastable and with higher molecular mobility exhibit the highest solubility and thus increased drug release rate and faster bioavailability. On the other hand, they are less physically and chemically stable and have shorter shelf-life. Also, if used in the pharmaceutical product, more stringent control of external conditions during manufacture and storage is required. Solid-state properties of raw materials can be easily characterised by modern instrumental methods, especially X-ray diffraction, thermal analysis, infrared and Raman spectroscopy, solid-state nuclear magnetic resonance or thermal analysis, and optical microscopy.

The physicochemical properties of raw materials are also influenced by other phenomena, such as formation of molecular complexes (co-crystals) in which the drug substance retains its chemical identity but exhibits different physicochemical properties, mainly dissolution rate, solubility, melting point and chemical reactivity, or solvates/hydrates where the drug substance is bound together with molecules of a solvent/water. Materials prepared by various strictly controlled crystallisation methods also differ in the crystalline size and size distribution.

With respect to the formulation of dosage forms, particle size and surface area of raw materials, shape, structure and porosity of particles, as well as their density, moisture content and electric charge are also very important properties. These parameters influence, among others, dissolution and drug release rate, homogeneity of powder mixtures, powder flow properties critical in tableting and hard capsule filling, stability of suspensions, etc. Particle size, shape and structure can be optimised by various methods such as grinding, milling, controlled
crystallisation or by sophisticated technological procedures like spray drying. Sieve analysis, laser diffraction and sedimentation techniques are the most common methods used for determination of particle size and size distribution. The selection of the suitable method depends on the expected size range and physicochemical properties of measured materials (solubility etc.). Particle shape, surface morphology, inner structure and porosity can be evaluated by microscopic methods, such as light and electron microscopy, often coupled with the image analysis technology.

1.2 QUALITY CONTROL IN FORMULATION DEVELOPMENT AND PHARMACEUTICAL MANUFACTURE

Quality control together with quality assurance (QC/QA) is an integral part of formulation development and pharmaceutical production. Formulation development is focused on finding the best way of how to incorporate APIs and excipients into dosage forms of desired properties suitable for their intended application. For all dosage forms, their physicochemical properties, such as mass and content uniformity, disintegration time, hardness or friability, their dissolution profile, application comfort and long-term stability are the crucial factors determining the efficacy, safety and stability of the final medicinal product.

Modern instrumental methods became valuable tools for an in-line and at-line monitoring of pharmaceutical processes, process analytical technology (PAT) thus supporting innovation and efficiency in pharmaceutical development, manufacturing and quality control/quality assurance. Formulation of most solid dosage forms starts with powder mixtures of APIs and excipients with a strict requirement for the uniformity of the blend. To improve the efficiency of the homogenisation process, PAT methods as for example near-infrared or Raman spectroscopy can be employed for the in-process monitoring of the blend homogeneity, determination of the blending end-point, as well as content uniformity assessment of final medicinal products (e.g. tablets). The same methods can be utilised during a drying phase of a granulation or pelletisation processes for the in-process measurement of moisture content.
Many dosage forms are coated, i.e. a layer of a coating material is applied to their external surface to protect the core (e.g. tablet, pellet, capsule, granule) from the acidic environment of the stomach or vice versa to protect the stomach from irritant drugs present in the core, to control the drug release or to improve the appearance and stability of the product. A number of factors such as the thickness and morphology of the coating layer, interactions between the coating materials and the substances within the core, can significantly influence the behaviour of the final dosage form and its dissolution profile. Many methods including infrared and Raman spectroscopy or microscopic methods can be useful in their evaluation and understanding.

In the formulation development, dissolution testing represents an important tool to study the influence of various process and formulation variables on the drug release rate of the product. Based on the information obtained during dissolution studies, a formulation with the optimal drug release profile is selected. Dissolution tests are also used during the initial step of bioequivalence studies necessary for the medicinal product approval. On a routine basis, dissolution tests are carried out by QC labs as part of the batch release testing of finished products or stability testing.

Several other parameters can be successfully investigated using modern instrumental techniques. These include interactions between the individual substances of a powder mixture, its inner structure (porosity, specific surface area), particle size or residual moisture. The knowledge of these parameters, especially interactions between APIs and excipients is beneficial for explaining and understanding the behaviour of the final product and it is essential for predicting its long-term stability and dissolution profile. Besides chemical interactions that can cause a degradation of an API, physical interactions occur frequently during the pharmaceutical manufacture. These interactions, such as formation of complexes, solid-phase changes, surface interactions, adsorption etc., can be either beneficial or detrimental to the product performance. The beneficial physical interactions can be used for modification of drug dissolution, poorly water-soluble drugs bioavailability increase, improvement of dose uniformity or more comfortable administration. However, many of these interactions are unwanted and can cause problems ultimately leading to an incomplete or too fast drug release or lack of system stability.
It can be concluded, that in pharmaceutical development, manufacturing and quality control, modern instrumental methods described in this book play a vital role in characterisation of APIs and excipients, investigation of their possible interactions during manufacturing and storage and also in characterisation of physicochemical properties of final dosage forms. They are widely used throughout the product lifecycle, from the initial formulation development up to the quality control during commercial manufacture. For the above-mentioned purposes, all procedures and methods must be validated (for more details see chapter 10).

1.3 REFERENCES


2 INFRARED SPECTROSCOPY

Infrared spectroscopy is the analysis based on infrared (IR) light interaction with a molecule of a sample. This can be analysed in three ways: measuring absorption, emission or reflection. Nowadays, infrared spectroscopy is a popular analytical method commonly used by numerous research and control laboratories. It is based on the ability of individual molecules of a sample to absorb a part of the infrared radiation and change their rotational-vibrational movements. Infrared spectra are records of such changes.

Infrared radiation is a part of the electromagnetic radiation with a wavelength typically ranging between 800 nm and 1000 µm (or 12500 and 10 cm$^{-1}$, if expressed as a wavenumber). The infrared radiation can be further distinguished as far infrared (FIR) with wavenumbers below 200 cm$^{-1}$, mid infrared (MIR) with wavenumbers ranging between 200 and 4000 cm$^{-1}$ and near infrared (NIR) with wavenumbers between 4000 and 12500 cm$^{-1}$.

Infrared spectroscopy is one of the most common and widely used spectroscopic techniques. Infrared spectroscopy is very useful in the identification of substances and structure analysis of a variety of substrates, including both organic and inorganic compounds. Also, it can be used for both qualitative and quantitative analysis of complex mixtures.

The MIR spectroscopy is used for decades. It is employed in pharmaceutical analysis mainly for identification of pure substances and analysis of the structural organisation of individual molecules. MIR spectroscopy found its new applications with the development of the chemical imaging technology.

A major breakthrough in the use of the NIR spectroscopy came in the 1990's in connection with the implementation of effective mathematical methods of statistical analysis necessary for the interpretation of the NIR spectra. This came coupled with the development of fibre optic probes since then used by the industry in NIR quality control of manufacturing processes. The NIR spectroscopy has found its place mainly
in the quantitative analysis of different samples and in the identification of substances.

2.1 **Theoretical background**

Infrared radiation can change the rotary-vibration state of a molecule by interacting with it. A change of the vibrational state can be viewed in a way of classical physics as an increase in the amplitude of bond vibration (Fig. 2.1) or molecule vibration, and a change in the rotary state as an acceleration of molecule rotation. Under normal conditions, atoms in molecules are tied by bonds while oscillating around their equilibrium position at a certain frequency which is given by the atoms participating in the bond. When exposed to infrared radiation, bonds in the molecule absorb a part of radiation of such energy (wavelength, wave number) which equals the frequency of bond vibration. Molecules then transit from their basic vibrational state to the excited state. Vibrational states of a molecule do not change continuously, but in jumps: they are quantized. As the energy of the absorbed radiation depends on the frequency of the bond vibration (thus on the type of atoms), the absorbed wavelengths will differ for various molecules. This phenomenon is a cause for the formation of characteristic vibration bands of the given molecule or function group. The intensity of molecule vibrations in the spectrum depends on the change of the dipole moment during the prolongation or shortening of the bond. A critical condition for the IR absorption to occur is an existence of a net change in a molecule dipole moment as it vibrates and rotates. Using the HBr molecule as an example, the charge distribution between hydrogen and bromine is not even since bromine is more electronegative than hydrogen and has higher electron density. The dipole moment is determined by the magnitude of the charge difference and the distance between the two centres of the charge. It is defined as the ratio of $d\mu/dr$, where $\mu$ is the dipole moment of the bond and $r$ is the length of the bond (inter-nuclear distance). As the molecule vibrates, there is a fluctuation in its dipole moment; this creates a field that interacts with the electric field associated with the radiation. If there is a match in frequency of the radiation and the natural vibration of the molecule, absorption occurs, and this alters the amplitude of the molecular vibration. This also
happens when rotation of asymmetric molecules around their centres results in a dipole moment change, which permits an interaction with the radiation field. Homo nuclear molecules, such as H₂, O₂, N₂ do not have a changing dipole moment when they undergo rotational and vibrational motions and as a result they cannot absorb IR radiation and provide any IR spectra.

![Fig. 2.1 Anharmonic oscillator.](image)

At room temperature, most molecules are at ground vibrational level \((n = 0)\). When infrared radiation is absorbed, molecules transit to higher vibrational levels. The behaviour of a real diatomic molecule can be described by using the model of an anharmonic oscillator (Fig. 2.1). A harmonic oscillator obeys Hook's Law and is an idealised expression that assumes that a system displaced from its equilibrium responds with a restoring force whose magnitude is proportional to the displacement. On the other hand, anharmonic oscillator description says that the restoring force is no longer proportional to the displacement. The lines in the Fig. 2.1 represent the quantum number \((n)\) which

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INFRARED SPECTROSCOPY

terminates at the top line corresponding to the dissociation limit. The energy spacing is not equal between energy levels. With an increasing quantum number, the vibrational levels become more condensed until the dissociation limit is reached when the bond is broken and the molecule dissociates. Due to the anharmonic character, so-called multiple overtones occur, which correspond to transitions when the vibrational quantum number \( n \) is changed by more than 1. The wavenumber of overtones is approximately an integer multiple of the wavenumber of the fundamental vibrations \((n_0 \rightarrow n_i)\). Overtones occur approximately in the wavenumber range of 14000–4800 cm\(^{-1}\) and can therefore be observed in NIR spectra. On contrary, MIR spectra exhibit vibrational bands corresponding to the fundamental transitions when the vibrational quantum number \( n \) is changed only by 1.

Vibrational spectra of polyatomic molecules are even more complicated as every atomic nucleus can influence the others. Besides fundamental transitions and overtones, combination transitions can therefore appear in a spectrum of polyatomic molecules. They usually appear at frequencies corresponding to sums of fundamental transition frequencies. Combination transitions occur in the near-infrared region between 5200 cm\(^{-1}\) and 4000 cm\(^{-1}\) as a result of vibrations interaction. The complexity of a spectrum of polyatomic molecules is further increased by the fact that more types of vibrations occur at the same time. Valence vibrations (stretching) cause a change in the length of the bond during vibration, whereas deformation vibrations (bending) cause a change in the angle of the bond (Fig. 2.2). Valence vibrations are further classified as a symmetric or an asymmetric stretching. During a symmetric vibration, the length of the bond in an atomic group is concurrently prolonged or shortened while during an asymmetric vibration, a bond of one atomic group is shortened as the bond of the other atomic group is prolonged. Deformation vibrations are further classified as in-plane (scissoring and rocking) and out-of-plane (wagging and twisting) vibrations. Given the complexity of the infrared spectra of a polyatomic molecule, it is usually impossible to perform a full analysis of all vibrational bands present in the spectra. Characteristic vibrations, i.e. vibrations of specific bonds or function groups occurring at wavenumbers characteristic for the given bond, are the most important ones. Characteristic vibrations used for an identification of a molecular
structure occur at wavenumbers from 4000 cm\(^{-1}\) to 1500 cm\(^{-1}\) in the mid-infrared region. Also, the IR spectra can be used for identification of compounds by comparing their spectra in the mid-infrared region from 1300 cm\(^{-1}\) to 900 cm\(^{-1}\) (fingerprint region) with a spectrum of a reference substance.

Spectra in the NIR region are formed by overtones and combination transitions. Most of these vibrations originate from C–H, O–H, S–H and N–H bonds. The probability of the occurrence of overtones and combination transitions in the NIR region is much lower than the probability of the occurrence of fundamental transitions in the MIR region. That is why the intensity of the bands in the NIR spectrum exhibit 10–1000 times lower intensity than the bands corresponding to the fundamental transitions in the mid-infrared region. Due to a high number of overtones and combination transitions, the individual absorption bands in the NIR spectrum overlap and only the envelope curve of these absorption bands is observed. Therefore, it is impossible to identify substances by matching absorption bands to individual functional groups. Spectra in the NIR region are less characteristic compared to the MIR spectra and their evaluation is more complicated. To obtain analytically significant information from the NIR spectrum, it is necessary to apply methods of applied mathematical statistics: chemometrics. The near-infrared spectroscopy provides possibilities which the analysis of MIR spectra simply does not. Thanks to lower intensity of absorption in the near-infrared region, radiation can permeate several millimetres deep into the sample, thus an analysis of even complicated non-homogeneous samples (coated tablets, capsules, dispersion, etc.) directly without a sample modification (dissolving or crushing of solid samples, diluting of liquid samples, etc.)
INFRARED SPECTROSCOPY

is possible. The comparison of characteristic features of the NIR and MIR spectroscopy is shown in Table 2.1.

Table 2.1 Comparison of characteristic properties of MIR and NIR spectroscopy

<table>
<thead>
<tr>
<th>Characteristic properties</th>
<th>MIR</th>
<th>NIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural analysis</td>
<td>Yes</td>
<td>Difficult</td>
</tr>
<tr>
<td>Substance identification</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Measuring directly in a transport package</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Sometimes necessary</td>
<td>No</td>
</tr>
<tr>
<td>Non-destructive analysis</td>
<td>Not always possible</td>
<td>Yes</td>
</tr>
<tr>
<td>Direct quantitative analysis</td>
<td>Liquids and thin films</td>
<td>Yes</td>
</tr>
<tr>
<td>PAT method</td>
<td>Liquids</td>
<td>Yes</td>
</tr>
</tbody>
</table>

2.2 INSTRUMENTS AND MEASURING TECHNIQUES USED IN IR SPECTROSCOPY

Spectrometers for measuring spectra in infrared area generally consist of a light source radiating continuous radiation in the infrared region, a monochromator or an interferometer, a sample holder, a detector and a computer with suitable software for mathematical data processing. Older types of spectrometers (dispersion spectrometers) are based on the principle of dispersion of infrared radiation to individual wavelengths by passing the beam through a monochromator. Individual wavelengths are detected one by one after passing through the sample. This technique, however, is disadvantageous due to time, energy and technical demands. In the newer type of spectrometers, the monochromator is substituted by an interferometer and a Fourier transformation (FT-IR, FT-NIR) is applied. They are most often based on a Michelson's interferometer (Fig. 2.3). The result of the measurement is an interferogram showing the dependency of a detector response on time; the resulting spectrum is only obtained after a mathematical (Fourier's) transformation is performed.

The source of near-infrared radiation is usually a tungsten or halogen lamp. The detector consists of photodiodes (photoconducting detector) made from various materials (InGaAs, Si, Ge). Photodiodes are semiconductor devices that convert light into an electrical current. The current is generated when photons are absorbed in the photodiode.
The source of mid-infrared radiation is an inert solid rod (e.g. silicon carbide rod—Globar®) which is electrically heated to a temperature ranging from 1500 K to 2000 K. The heated material then emits infrared radiation. There are three categories of detectors: thermocouple, pyroelectric and photoconducting.

Thermocouple is a type of a thermal detector that places two different metals (e.g. bismuth and antimony) together. When the metals are heated by infrared radiation, a small voltage proportional to the temperature at the junction between the two metals is sent out.

A pyroelectric detector consists of a pyroelectric material which is an insulator with special thermal and electric properties. Triglycine sulphate is the most common material for pyroelectric infrared detectors. Pyroelectric detectors have a fast response time. They are used in most Fourier transform IR instruments.

A photoconducting detector has better response characteristics than pyroelectric detectors and is used in FT-IR (FT-NIR) instruments. For mid-infrared radiation, the mercury cadmium telluride detector is used.

Fig. 2.3 Diagram of NIR spectrometer with Michelson's interferometer.
2.2.1 MICHELSON'S INTERFEROMETER AND FOURIER'S TRANSFORMATION

When radiation from the source hits a semi-permeable beam splitter one half of the beam is transmitted onto a movable mirror and the second half is reflected towards a fixed mirror. Beams are reflected back from both mutually perpendicular mirrors and are either summed up or deducted on the beam splitter according to the position of the moving mirror. If the optical trajectory of the beams is identical in both arms of the device, a constructive interference occurs for all wavelengths. If the optical trajectories in both arms are different, either a constructive or destructive interference occurs for waves with different wavelengths (Fig. 2.4). This resulting modulated radiation is passed on a sample and, subsequently, on a detector. An interferogram containing all spectral information is formed. However, in this form, the interferogram cannot be directly interpreted. Data are further transformed by means of mathematical operation so-called Fourier transformation that associates the frequency domain representation to a function of time (domain of time).

![Fig. 2.4](image)

**Fig. 2.4** Formation of modulated radiation after passage through the interferometer.²

2.2.2 Measuring Techniques

Infrared absorption or emission spectra can be obtained by measuring substances in their solid, liquid and gaseous phase. The absorption of infrared radiation is observed in most cases. The three following measuring techniques are the most frequently used in the NIR spectroscopy: transmittance, transflectance and diffuse reflectance. Besides procedures when a sample is situated in the holder of the device, NIR spectra are often measured by various types of contact probes which can be situated directly in the production equipment. Thanks to fibre optics that ensures connection to the spectrometer for even tens of meters, this technique enables light energy to enter the sample and scanning at the same time. This setup finds its application in the operational and output inspection and in industrial process control, i.e. where real time availability of results is crucial.

In the MIR spectroscopy the three basic measuring techniques include: transmittance, diffuse reflection method and attenuated total reflection (ATR, this technique is very often used in MIR spectroscopy, it allows a direct measurement of powder samples). Moreover, nowadays infrared microscopy can be used. It allows chemical mapping of final dosage forms and thus it is very useful technique during formulation development.

The selection of a suitable measuring technique is based mainly on the optical properties of the sample and the sample form. From the sample viewpoint, it is possible to use more than one method to measure an infrared spectrum for a single sample. However, besides the choice of measuring technique, it is also necessary to consider a series of factors influencing the precision and accuracy of the measurement e.g.:

- Sample temperature (a difference of several degrees can cause significant changes in obtained spectra).
- Humidity and residual solvents (humidity as well as solvents exhibit distinct absorption bands in the IR region).
- Sample thickness.
- Physical properties of the sample (compactness, particle size and their shape, air area, porosity of powdered substances, smooth or glossy surface of the sample can significantly change the spectrum due to the scattering of radiation).
• Polymorphism.
• Sample age.

2.2.2.1 Transmittance

Transmittance \((T)\) measures the absorption of radiation after its passage through a sample (Eq. 2.1 and 2.2):

\[
T = \frac{I}{I_0} \quad (2.1)
\]

\[
A = -\log_{10} T = \log_{10} \frac{1}{T} = \log_{10} \frac{I_0}{I} \quad (2.2)
\]

where \(I\) is the intensity of the passed radiation and \(I_0\) is the intensity of the incident radiation (Fig. 2.5 (a)). A sample is situated between the source of radiation and the detector which detects \(T\) or absorbance \((A)\). This method is usually used for measuring of diluted or undiluted liquids and for solutions of solid substances. In the NIR spectroscopy, samples are measured in a cuvette of a suitable thickness (usually from 0.5 mm to 4 mm) made of a special glass which shows a high permeability in the near infrared region. The transmittance technique is sometimes used for tablet analysis. The condition is that NIR radiation has to pass through the tablet, and thus this technique is not suitable for strongly absorbing samples. In the MIR spectroscopy, samples are commonly measured by the KBr method or Nujol method. KBr method includes preparation of a tablet from approximately 200 mg to 250 mg of KBr well mixed with 0.1 % to 1.0 % of the sample. In Nujol method, the sample (approx. 10 mg) is dispersed in non-volatile liquid paraffin (Nujol) that has low absorption in the infrared region.

2.2.2.2 Transreflectance

Transreflectance \((T^*)\) uses a special steel cuvette with a reflective surface or mirrors situated behind a sample on which radiation not absorbed by the sample is reflected (the absorption double layers). A part of the radiation is reflected and absorbed in the bulk of the sample (Fig. 2.5 (b)). Reflected radiation is directed to a detector and \(T^*\) can be calculated (Eq. 2.3 and 2.4):

\[
T^* = \frac{I}{I_T} \quad (2.3)
\]

\[
A^* = \log_{10} \frac{1}{T^*} \quad (2.4)
\]
where $I$ is the intensity of the passed and reflected radiation measured with the sample and $I_T$ is the intensity of the passed and reflected radiation without the sample.

Transflectance is mostly used in the NIR spectroscopy for measuring diluted and undiluted liquids, for liquid samples of dissolved or suspended solid substances or for semi-solid dosage forms.

2.2.2.3 Diffuse reflectance

Diffuse reflectance ($R$) measures the changes that occur in an infrared beam when the infrared radiation interacts with the sample by alternately passing through the sample and reflecting from it. After the interaction with the sample, the reflected and diffusely scattered radiation (Fig. 2.5 (c)) is transmitted to the detector of the spectrometer (Eq. 2.5 and 2.6):

$$R = \frac{I}{I_R}$$

$$A^* = \log_{10} \frac{1}{R} = \log_{10} \frac{I_R}{I}$$

where $R$ is the diffuse reflectance, $I$ is the intensity of radiation diffusely reflected by the sample and $I_R$ is the intensity of radiation reflected by the background or a reference reflective surface.

The diffusion reflectance technique is mainly used for measuring solid substances with an uneven or irregular surface or for powdered substances. In pharmaceutical applications, this technique is usually used in connection with the NIR spectroscopy.

![Fig. 2.5 Measuring techniques. Transmittance (a), Transflectance (b), Diffuse reflectance (c).](image)

2.2.2.4 Attenuated total reflection

The attenuated total reflection (ATR) method is used in the MIR spectroscopy and involves pressing the sample against a high-refractive-index prism and measuring the infrared spectrum using infrared light that
is totally internally reflected in the prism. A ZnSe or Ge prism are used in the ATR accessory. In comparison with the other measuring techniques used in the MIR spectroscopy (KBr method or Nujol method), the ATR method is an excellent method for obtaining IR information for the powder or liquid samples.

2.2.2.5 Infrared microscopy

Another possibility how to use the IR spectroscopy is a technique of so-called chemical mapping (IR mapping). Chemical mapping couple the spectrometer to an infrared microscope and allows digital image processing. The infrared microscope is focused on a specific spatial position of the sample and a pre-defined area of the sample is gradually scanned in this way. Obtained image contains an IR spectrum for every spatial position. The result is a 3D image, where $x$ and $y$ axes represent spatial information and $z$ axis provides spectral information. Chemical mapping thus enables visualization of individual components present in individual parts of the sample because sample areas with similar spectral properties have similar chemical composition. This technique is used in pharmaceutical analysis during quality control and formulation development.

2.3 Evaluation of IR spectra: chemometrics

The field of chemometrics is defined as a chemical discipline using mathematical methods for the evaluation of chemical data. Chemometric processing of results often facilitates explanation of a certain chemical phenomenon and its causes, especially because it enables obtaining information from data which would not be obvious without such processing. The main goal in spectroscopy is to describe relationships between a set of measured data (spectrum) and calibration model quantities, for example concentrations of substances in the mixture. Chemometric processing is necessary for evaluation of spectra especially in the near-infrared spectroscopy.

2.3.1 Multi-component analysis

A multi-component analysis is used in the cases when the analysed sample is a mixture of two or more substances whose absorption bands
mutually overlap. Methods of multi-component analysis seek relationships between the measured parameter and the desired information. They include the creation of a calibration model and its use in the sample identification or in the analysis of samples with unknown substance concentration. Methods of multi-component analysis include e.g. multiple linear regression or dimension reduction methods.

Multi-component quantitative analysis is carried out using standards with known concentration. Standards can be both external and internal. External standards are used independently of the analysed sample; in fact, they represent independent samples analysed in a series with samples of unknown composition. Internal standards are added to an unknown sample and the resulting mixture is measured. It is necessary to know the identity and quantity of the substance added to the unknown sample. This substance must be chemically stable and must not interact in any way with the content of the unknown sample.

When preparing standards, it is necessary to determine where standards will originate at first. There are two possibilities:

- Standards can be pure substances, or simple multi-component systems, or proportional parts of pure materials. They can be mixed to form standards.
- Standards can be complex mixtures.

The exact concentration of an analyte in the standard must be determined by primary (reference) method (e.g. titration, gravimetric analysis etc.). The second step is the determination of the number of standards for the analysis. For this purpose, the rule of $2n + 2$ is used, where $n$ stands for number of components in the system. This equation states the minimum number of standards necessary to obtain a calibration model for the given number of variables. It is always suitable to use more standards. For dimension reduction methods, there is a general rule where $3n$ is a minimum number of standards; however, the ideal number is somewhere between $5n$ and $10n$. It is also important to achieve a balance between having a wide set of standards and the time spent by measuring them. Another consideration in preparing standards is the range of used concentrations. For example, knowing that analysed samples should contain 5–15% of the analyte, standards containing the analyte at the concentration below 5% and above 15% should
be prepared. The calibration model can only be applied in the concentration range for which the calibration data were obtained. As the calibration model is more precise in the range of concentrations where the majority of calibration samples is situated, the distribution of concentrations across the concentration range should also be considered when the standards are prepared. It is important whether most measured concentrations of the standards are grouped around 5 % or 15 % or are situated in the middle of the concentration range. When selecting concentrations of analytes in the standards, it is important to set concentrations of two or more analytes, so they are not co-linear. This means concentrations must not be mutual multiples. To obtain all sources of variability in the spectrum, the number of spectra taken for each standard should be considered as well. Generally, it is recommended to take two or more spectra per standard and use them in the model.

2.3.1.1 Multiple linear regression

The multiple linear regression (MLR) method only requires the knowledge of an analyte in calibration samples (standards). Calibration samples can be complex mixtures with a complicated matrix and unknown interfering substances (natural, food, petrochemical materials, etc.). Absorbance spectra are recorded for the samples and a calibration model is constructed by appropriate software. The calibration model describes the relationship between the concentration of the analyte $c$ and the absorbance $x$ (Eq. 2.7):

$$
\begin{bmatrix}
c_1 \\
\ldots \\
c_i \\
\end{bmatrix} =
\begin{bmatrix}
x_{11} & \ldots & x_{1j} \\
\ldots & \ldots & \ldots \\
x_{i1} & \ldots & x_{ij} \\
\end{bmatrix}
 \times
\begin{bmatrix}
\beta_1 \\
\beta_j \\
\end{bmatrix}
+ 
\begin{bmatrix}
e_1 \\
\ldots \\
e_i \\
\end{bmatrix}
$$

(Eq. 2.7)

where $c_i$ is the concentration of the analyte in the calibration sample $i$, $x_{ij}$ is the absorbance of the calibration sample $i$ at $j^{th}$ wavelength, $\beta_j$ is the regression coefficient for the wavelength $j$ and $e_i$ is the concentration residuum of the sample $i$ (the difference between the concentration determined by the reference method and the concentration calculated from the calibration model).

The estimate of model parameters is given by the equation $\beta = (X^T \times X)^{-1} \times X^T \times c$. The solution can be found only in the case when the number of calibration samples $i$ is higher than the number
of wavelengths $j$ of the spectrum. Otherwise, it is impossible to invert the product of multiplication $X^T \times X$ into the invertible matrix, and therefore to estimate parameters $\beta$. Modern analytical devices are able to measure spectra at hundreds of wavelengths, however, it is not possible to use them all for the calculation of a calibration model. This is the main limitation of the MLR.

### 2.3.1.2 Dimension reduction methods

Unlike the MLR, in the case of dimension reduction methods the number of standards is not limited by the number of wavelengths at which the sample absorbance is measured. The dimension reduction methods enable to obtain a calibration model without knowing specific wavelengths absorbed by the analyte. Another advantage is that the dimension reduction methods tolerate a spectral noise and interferents. To create a calibration model using dimension reduction methods, it is necessary to know only the concentration of analytes in standards or sample identity for identification methods. A disadvantage is the difficulty of calibration processes because higher number of calibration samples is required.

**Principal component regression**

In principal component regression (PCR) method, a huge number of spectral data is reduced to a limited number of independent factors. These principal components (factors) express main deviations in spectra.

It is a two-step process; a spectrum is used to determine factors in the 1st step. In the next step a calibration model is created as a function of factors on the analyte concentration. Using a calibration model created in this way an analyte concentration in an unknown sample can be calculated. A disadvantage of the two-step process is that the information about the analyte concentration is not used in the calculation of factors, which may be a source of deviations as all changes in the spectrum are attributed to the changes of an analyte concentration only. Due to this reason, other methods of dimension reduction were developed (e.g. PLS).
Partial least squares

A method of partial least squares (PLS) is based on the decomposition of the original measurement (spectrum) and response (e.g. concentration) to a new smaller set of new variables, so-called factors, by which all data variability is described in the best way possible. The first factor describes most variability and contains the majority of common information contained in the data. Other factors describe more specific information. However, too many factors can impair the calibration model by insignificant noise-describing information. A calibration model which allows to analyse an unknown sample is compiled from new variables: factors. This is a single-step process where information on both spectrum and concentration is included in the calculation of factors.

An advantage of the PLS method is that it allows to create a calibration model using even largely overlapping absorption bands and that it also addresses the interference of unknown analytes (e.g. impurities). On the other hand, the PLS method requires a large number of standards to be prepared and measured.

Linear discriminant analysis

Identification methods are used to build classification rules for a number of pre-specified subgroups of samples (i.e. the training set). The classification rules are later used for allocating unknown samples to the most probable subgroup. Identity or good/bad quality are, thus, defined as belonging to a group with known properties. Linear discriminant analysis (LDA) is typically used for constructing spectral libraries for near-infrared spectra. Common approach is to classify samples by measuring the distance between points (representing exactly one sample) in a multidimensional space (e.g. Mahalanobis distance).

2.4 APPLICATION OF IR SPECTROSCOPY IN PHARMACEUTICAL ANALYSIS

MIR spectroscopy is widely used in the industry as well as in the research. It is a simple and reliable technique for identification of raw materials and quality control. It is also employed in the forensic analysis in civil and criminal cases. The major advantage of the MIR spectroscopy is that it provides a lot of insights into the functional groups present
in a compound and therefore the major application of the MIR spectroscopy is in a structure investigation or substance identification.

NIR spectroscopy finds its practical application in numerous industrial fields. An advantage of this method is the possibility to analyse even complex samples where determination of more parameters, both chemical and physical, from a single spectrum is possible. Other advantages include a non-destructive character of the analysis, i.e. the NIR spectrum is obtained directly without a sample modification. The possibility to perform the analysis at the distance of several tens of meters using fibre optics makes the NIR spectroscopy one of the PAT methods where speed of the analysis including the possibility of direct continuous in-line control during the production process is emphasised. NIR spectroscopy can directly analyse almost all types of samples: solutions, suspensions, emulsions, powders and strongly absorbing substances.

2.4.1 APPLICATION OF MIR SPECTROSCOPY

Mid-infrared spectroscopy has been introduced to the pharmaceutical industry as a solution of various specific problems such as drug identity, purity, structure, polymorphism, interactions between APIs and excipients or quantification. The indisputable importance of the MIR spectroscopy in pharmaceutical sciences and industry has been proven by its recent successful classification as an obligatory/guiding analytical method.

2.4.1.1 Identity and purity of raw materials

The need of efficient and reliable methods for identification of pharmaceutical substances has caused the MIR spectroscopy to become very popular. The MIR spectroscopy has an obligatory character in all Pharmacopoeias in the world. Samples can be identified by comparing their mid-infrared spectra to the spectra of known compounds (reference standard). MIR spectroscopy allows a simultaneous verification of drug identity and examination of drug purity. Usually, the drug identification precedes the drug purity test. The drugs are the finest products of the pharmaceutical manufacture (industry). Nevertheless, they can contain minimal amounts of pollutants, intermediates and/or degradation products, and each one of them can cause unwanted alteration in drug therapeutic activity. Eventual
impurities are reflected as absorption bands not observed in the MIR spectrum of the standard. As well as drug identity, the identity of excipients can be verified by MIR (Fig. 2.6).

**Fig. 2.6** MIR spectra of dihydrate (---) and anhydrate (---) of dicalcium phosphate.

### 2.4.1.2 Identification of functional group and structure elucidation

MIR spectroscopy can be used for the purpose of identification of structure and composition of a chemical compound because functional groups give rise to characteristic bands (Table 2.2) both in terms of intensity and position (frequency). These characteristic bands can be observed at wavenumbers from 4000 cm\(^{-1}\) to 1500 cm\(^{-1}\). However, in the identification of the structure of complex organic molecules, it is usually necessary to use a combination of several methods of structure analysis, e.g. MIR, nuclear magnetic resonance (NMR), X-ray or mass spectrometry (MS).
### Table 2.2 Characteristic MIR absorption bands

<table>
<thead>
<tr>
<th>Bond</th>
<th>Compound type</th>
<th>Frequency (cm(^{-1}))</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>–C–H</td>
<td>Alkanes</td>
<td>2800–3100</td>
<td>Strong</td>
</tr>
<tr>
<td>≡C–H</td>
<td>Alkenes and arenes</td>
<td>3000–3100</td>
<td>Medium</td>
</tr>
<tr>
<td>≡C–H</td>
<td>Alkynes</td>
<td>3200–3350</td>
<td>Strong, sharp</td>
</tr>
<tr>
<td>–C–C</td>
<td>Alkanes</td>
<td>750–1200</td>
<td>Weak to medium</td>
</tr>
<tr>
<td>≈C≡C</td>
<td>Alkenes</td>
<td>1600–1680</td>
<td>Variable</td>
</tr>
<tr>
<td>–C≡C</td>
<td>Alkynes</td>
<td>2050–2260</td>
<td>Variable</td>
</tr>
<tr>
<td>–C≡N</td>
<td>Nitriles</td>
<td>2200–2400</td>
<td>Variable</td>
</tr>
<tr>
<td>–C–O</td>
<td>Alcohols, ethers</td>
<td>980–1250</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>Carboxylic acids</td>
<td>1350–1440</td>
<td>Weak to medium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1210–1320</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>Esters</td>
<td>1035–1300</td>
<td>Strong (two bands for unsaturated esters)</td>
</tr>
<tr>
<td>≈C≡O</td>
<td>Aldehydes</td>
<td>1690–1740</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>Ketones</td>
<td>1650–1730</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>Carboxylic acids, esters</td>
<td>1710–1780</td>
<td>Strong</td>
</tr>
<tr>
<td>–O–H</td>
<td>Alcohols, phenols</td>
<td>3400–3700</td>
<td>Variable, sharp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000–1450</td>
<td>Strong</td>
</tr>
<tr>
<td>–NH(_2)</td>
<td>Primary amines</td>
<td>3200–3600</td>
<td>Medium (double peak)</td>
</tr>
<tr>
<td>–NH</td>
<td>Secondary amines</td>
<td>3100–3500</td>
<td>Medium (single peak)</td>
</tr>
</tbody>
</table>
2.4.1.3 **Drug solid-phase identification**

The MIR spectroscopy has a role of a guiding method in solid-phase identification (Fig. 2.7). The method is usually combined with other analytical methods investigating drug polymorphism/pseudopolymorphism (e.g. X-ray diffraction or thermal analysis). The analysis of polymorphism/pseudopolymorphism can be performed only in a solid state. For this purpose, the Nujol method is often used. Also, the KBr disk method or the ATR method can be used, but in some cases, these techniques can alter the crystalline structure of the drug.

![MIR spectra of API in two solid-phase state: acid form (—) and sodium salt (---).](image)

**Fig. 2.7** MIR spectra of API in two solid-phase state: acid form (—) and sodium salt (---).

2.4.1.4 **Investigation of drug-drug and/or drug-excipient interactions**

The MIR spectroscopy offers the possibility to investigate the interactions between APIs and excipients. These interactions, mainly complexation a hydrogen bonding, can modify the physicochemical, pharmacological and pharmacokinetic behaviour of active substances. Pharmaceutical technology and pharmaceutical industry can use this knowledge for development of new dosage forms or to improve the current once by modifying the product's solubility, stability, therapeutic activity or decreasing the number of unwanted side effects. Combination of the MIR spectroscopy with methods such as the X-ray diffraction (XRD), Raman spectroscopy or thermal analysis seems to be a particularly successful application for analysis of the abovementioned interactions.
2.4.1.5 Quantitative analysis
Quantitative analysis of a component of interest in a solution can be successfully carried out in cases where a suitable band in the spectrum of the component exists. The band chosen for the analysis should have a high molar absorptivity, it should not overlap with other peaks from other components in the mixture or the solvent, it should be symmetrical, and it should give a linear calibration plot of absorbance versus concentration. Simple solid mixtures may also be quantitatively analysed. These are more susceptible to errors because of the radiation scattering. Such analyses are usually carried out with KBr method or Nujol method. The determination of a component in a complex mixture is possible with the use of dimension reduction methods (see chapter 2.3.1).

2.4.2 APPLICATION OF NIR SPECTROSCOPY
The near-infrared spectroscopy has extensive applications in the pharmaceutical industry. One of the reasons is the effort of regulatory authorities, such as the American Food and Drug Administration (FDA) or the European Medicinal Agency (EMA) in Europe, to increase quality of authorised medicinal products. This goal can be achieved by continuous monitoring of critical production parameters directly during production. The continuous control leads to better understanding of production processes, and to increase in production capacity and efficiency, thus reducing operational costs and increasing quality of products. Thanks to the advantages, such as distant measurement by using fibre optics without sample modification and availability of analysis results in real time, the near infrared spectroscopy became one of the main methods in the implementing of process analytical technology.

2.4.2.1 Identification of raw materials
In compliance with the good manufacturing practices, the pharmaceutical industry requires initial inspection of all incoming raw materials intended for the production of medicinal products. The raw material inspection employs special tests which should ensure, that the identity and quality of the incoming substance is satisfactory and meets the pre-set
INFRARED SPECTROSCOPY

requirements. The NIR spectroscopy is an approved and common alternative to traditional time-consuming analytical methods. The NIR methods used for purposes of pharmaceutical quality control can be validated for the identification of large quantities of raw materials without the necessity for further chemical analysis (e.g. chromatography). The NIR probe is simply brought to a close contact with the analysed material; a NIR spectrum is scanned and immediately compared with a library of reference spectral data. The evaluation is performed by a computer and methods of mathematical statistics (Fig. 2.8). The identification of substances by means of the NIR spectroscopy is more suitable and efficient than the identification using the mid-infrared spectroscopy which is primarily focused on the chemical structure of a substance. NIR spectra are influenced by physical properties of analysed materials, such as the shape and size of particles of the analysed sample.

![Fig. 2.8 Discriminant analysis. Mahalanobis distances ($D_M$) are calculated on the basis of the NIR spectra of cellulose derivatives. CMC—carboxymethyl cellulose, EC—ethylcellulose, HEC—hydroxyethyl cellulose, HPC—hydroxypropyl cellulose, HPMC—hydroxypropylmethyl cellulose, MCC—microcrystalline cellulose.]

2.4.2.2  Content and content uniformity analysis

Another field where the NIR spectroscopy found its practical use is the APIs content determination. It is used extensively in the analysis of tablets, but can also be used in API quantification in other dosage forms such as capsules, gels, syrups etc. The analysis is almost immediate as the sample preparation is omitted and the whole process can be automated. Thanks to this fact, NIR spectroscopy partly substitutes established methods such as liquid chromatography. The comparison of characteristic features of the NIR spectroscopy and liquid chromatography is shown in Table 2.3. Methods of multi-component analysis (see chapter 2.3.1) are usually necessary for a quantitative analysis of complex mixtures such as medicinal products.

Table 2.3  The comparison of characteristic features of the NIR spectroscopy and liquid chromatography on an example of tablet analysis

<table>
<thead>
<tr>
<th>Characteristic properties</th>
<th>NIR</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>Non-destructive</td>
<td>Crushing and solving</td>
</tr>
<tr>
<td>Time of analysis (1 tablet)</td>
<td>Approximately 30–60 s</td>
<td>Approximately 20–30 min</td>
</tr>
<tr>
<td>Use of chemicals</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Information on physical properties</td>
<td>Possible (e.g. strength, package thickness)</td>
<td>No</td>
</tr>
<tr>
<td>Moisture content</td>
<td>Possible</td>
<td>No</td>
</tr>
<tr>
<td>Information on chemical properties</td>
<td>Determination of content, content uniformity</td>
<td>Determination of content, content uniformity</td>
</tr>
<tr>
<td>Determination of impurities</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

2.4.2.3  Evaluation of blend uniformity

Homogeneity of starting materials used in the production of medicinal products influences the final content uniformity of the final product, and therefore it is one of the basic parameters carefully monitored throughout the production phase. The possibility to identify the point when the mixture is homogeneous increases process efficiency (by reducing operating costs) and guarantees the required content uniformity (higher products quality). The NIR spectroscopy is suitable for monitoring of homogenisation processes and finding an optimum
I N F R A R E D  S P E C T R O S C O P Y

blending time, especially in cases where it is difficult to achieve the desired content uniformity (e.g. due to a blend sampling error). By using a direct NIR spectrometer connection to homogenisation equipment, the whole process can be automated, and mixing can be stopped immediately after the required degree is reached.

A simple way of assessing the blend uniformity is to scan the NIR spectra in short time intervals (e.g. after each turn of the homogenisation vessel). A suitable count of time intervals is connected to a block. A standard deviation is consequently calculated from the reflectances found in the individual time intervals and the average reflectances in the defined blocks. If a mixture is homogeneous, the individual spectra are similar to each other and the standard deviation reaches its minimum and does not change with continuing mixing (Fig. 2.9 (a)). This procedure allows determination of the overall mixture uniformity, but not of the uniformity with regards to the distribution of the active substance in the mixture. In the pharmaceutical industry, it is necessary to meet the limits set by the regulatory authorities (e.g. EMA, FDA) that specify uniformity of mixtures based on the determination of the active substance (e.g. relative standard deviation < 5 %, the individual drug contents in the interval from 90 % to 110 % of the average content). Therefore, multi-component quantitative analysis is usually used for determination of the active substance content in individual time intervals (e.g. after each turn of the homogenisation vessel) (Fig. 2.9 (b)).

![Fig. 2.9 Blend content uniformity determined on base NIR spectra. Total content uniformity (a), Active substance content uniformity (b).](image)
2.4.2.4 Drug-solid phase identification

The NIR spectroscopy enables observing changes in the crystalline structure, i.e. the formation of an amorphous form or various polymorphic forms both in pure substances, and in mixtures or dosage forms (Fig. 2.10). As various polymorphic forms of the same substance differ in physicochemical properties (e.g. solubility, size and shape of crystals, reactivity, hygroscopicity, thermal stability etc.), they can influence the stability of a dosage form or its physicochemical properties (e.g. the hardness of tablets, flow properties of powdered mixtures or granulates) or final bioavailability. Most APIs can occur in more than one form and the crystalline structure can also change throughout the production (milling, granulation, pressing) or during the storage. Every manufacturer is therefore obliged to check polymorphism of its substances. Studying polymorphic transformations is an important part of pre-formulation studies. The simplicity and rate of NIR spectra measurement enables checking the polymorphism continuously during the production process, which is an advantage compared to commonly used methods, such as the MIR spectroscopy, X-ray powder analysis or differential scanning calorimetry.

Fig. 2.10 The comparison of NIR spectra obtained for API occurring in two crystalline forms (a) and Tablets containing such API (b); form I (——) and form II (---).4

2.4.2.5  *Moisture determination*

Water gives rise to strong absorption in the NIR area and therefore changes in the moisture content of a sample can be easily observed. This property can be used in numerous technological processes. In addition to drying, it is mainly useful in the wet granulation and pelletisation, i.e. processes where the water content is a highly important parameter influencing the resulting product quality. Ideally, the NIR spectrometer is connected directly to the production equipment, enabling continuous monitoring of the moisture degree, thus optimising the drying time and formation of the required product. NIR spectroscopy is currently approved as a primary method for the determination of an optimum time of drying in production processes.

2.4.2.6  *Monitoring of drug layering or coating operations*

Besides the moisture content, many other parameters can be monitored in real time by means of the NIR spectroscopy. One of them is the quantity of drug layered on inactive nuclei in the fluid-bed equipment (Fig. 2.11). Management of the production processes by employing the in-process analysis increases the content uniformity and overall product quality. Similarly to the drug content, the quantity of a polymeric coat applied on pellets or tablets during the production process can be observed. The coat thickness is very important for reaching an optimal therapeutic effect, especially in dosage forms with controlled release. By selecting a suitable coating material and the thickness of the coating layer, it is possible to target the drug release to a certain part of the gastrointestinal tract. Continuous monitoring of the coating material amount already applied results in higher quality of the finished product.
Fig. 2.11 Diagram of pellet production inspection in fluid-bed equipment by NIR spectroscopy.

2.5 REFERENCES


3 Raman spectroscopy

Raman spectroscopy is a method of vibrational molecular spectroscopy (similar to IR spectroscopy), which has been going through enormous progress in the recent years. Raman spectroscopy is becoming one of the most popular analytical measurement tools for pharmaceutical applications, ranging from the verification of raw materials to process monitoring of drug production to quality control of products. Raman spectroscopy allows to obtain a set of information that is not apparent from the infrared spectrum and vice versa. As infrared and Raman spectroscopy complement each other, their combination enables a complex analysis of the samples. Used not only in the pharmaceutical industry, both methods are widely employed in research and control laboratories.

The principle of Raman spectroscopy is the irradiation of the sample by monochromatic radiation (most commonly Vis or NIR) from a laser source, and subsequent measurement of the radiation scattered by the sample. Backscattered radiation is generated by the interaction of the radiation with the sample molecules with simultaneous changes in the vibrational and rotational states of these molecules. Raman bands can be assigned to characteristic vibrations corresponding to chemical bonds or functional groups. Combined with other methods, such as confocal or electron microscopy, Raman spectroscopy offers new possibilities for identifying samples with a number of benefits. Raman spectroscopy enables analysis of a wide range of solids (crystalline and amorphous materials, polymers), liquids (pure substances, aqueous and non-aqueous solutions), gases, surface analysis (e.g. sorbents), or analysis of biological systems (from biomolecules to organisms).

3.1 Theoretical basics of Raman's scattering

Raman spectroscopy is based on the interaction of photons of monochromatic radiation (emitted from a laser source) with sample molecules. Most of the scattered radiation is of the same energy and thus of the same wavelength as the original radiation (perfectly elastic
collisions, so called Rayleigh scattering). Yet a small fraction of the scattered radiation (approximately 1%) has wavelengths shifted in both directions compared to the original wavelength—the so-called Raman scattering (Fig. 3.1), first described by the Indian physicist Raman (1930 Nobel Prize for Physics). Raman scattering corresponds to the situation where the scattered photons have either higher or lower energy than the incident photons. This can be viewed as the transition of the sample molecule from the basic vibrational energy state \( n_0 \) to the virtual energy state and the subsequent transition to the vibrational energy state \( n_1 \) (Stokes transition). Parallel transition from the \( n_1 \) state to the virtual one and the subsequent transition to the basic vibrational state can occur (anti-Stokes transition). Frequency difference between the incident radiation and the measured Raman line equals the energy difference between the vibrational states of the molecule (Fig. 3.1). If the given vibration mode is active in the Raman spectrum, it is generally possible to observe two spectral lines corresponding to the Stokes and anti-Stokes transition. These lines are symmetrically distributed around the Rayleigh scattering line. In a number of practical cases, however, the spectra are measured only in the area of Stokes scattering, due to the need to filter out the Rayleigh scattering, whose intensity is several orders of magnitude higher than that of the Raman lines. In addition, anti-Stokes transitions are less intense (as the number of excited states \( n_1 \) is smaller) than Stokes transitions.

**Fig 3.1** Energy-level diagram showing the states involved in Raman spectra.
As outlined by previous facts, Raman scattering changes the vibrational state of the molecule. Raman line described by a wavenumber lies in the range of 50 cm\(^{-1}\) to 4000 cm\(^{-1}\), which corresponds to the absorption bands of the infrared spectra. However, the change in the vibrational state of the molecule occurs in a different way than in infrared spectroscopy, where the vibrational state changes directly by absorption of radiation. For the formation of Raman lines, it is essential that the vibrational motion leads to a change in polarizability, as the intensity of such lines is proportional to the change in polarizability. This rule is principally different from the observation of vibrational transitions in infrared spectroscopy, where the basic condition is that the vibrational movement leads to the change of the dipole moment. The example of the valence vibrations in carbon dioxide (Fig. 3.2) shows that the symmetrical valence vibration does not change the dipole moment, but there is a deformation of the molecular orbital and thus a change in the polarizability (change of the electron density distribution). Therefore, this vibration does not appear in the infrared spectrum, but it does appear in Raman spectrum. The opposite phenomenon can be observed when asymmetric valence vibration changes the dipole moment. But, as the shortening of the bond between oxygen and carbon on one side is balanced by lengthening of the bond on the other, the polarizability does not change. The most intensive bands in Raman spectra arise out of the vibrations of non-polar bonds with symmetrical charge distribution (the largest polarizability changes), especially C=C, C–C, C≡C, C–S, S–S bonds. On the contrary, the water (polar bonds) is weak Raman scatterer, therefore Raman exhibits minimal sensitivity towards interference by water. A major problem for Raman measurements can be seen in the high levels of fluorescence (intrinsic or impurity caused) overlaying the Raman bands. To solve this problem, various techniques of Raman spectra measurement were developed (e.g. FT-Raman, chapter 3.2.1).

![Symmetrical stretch](O=C=O)

Symmetrical stretch
No change in dipole moment
therefore IR inactive
There is change in polarizability
therefore Raman active

![Asymmetrical stretch](O=C=O)

Asymmetrical stretch
There is change in dipole moment
therefore IR active
No change in polarizability
therefore Raman inactive

**Fig 3.2** Examples of Raman active and inactive vibrations in CO\(_2\).
3.2 INSTRUMENTATION AND DATA ANALYSIS TECHNIQUES

Since its prediction and discovery in 1928, through numerous technological innovations easy use of Raman spectroscopy has been made possible. The first major breakthrough came with the development of the laser, which provided considerably more photons to generate Raman scattered photons and therefore improved method sensitivity. The 2D charge-coupled device (CCD) detectors reduced the measurement time from hours to minutes. These detectors also allowed replacement of strip-chart recorders with x-y plotters. In the 1980s, fiber optic probes were introduced, allowing the first in-process measurements. Interferometer-based systems that employed 1064 nm lasers were also introduced during this time. Because very few chemicals have electronic absorptions at this longer wavelength, the generation of fluorescence that could obscure the Raman signal was virtually eliminated. The 1990s introduced two new optical elements that simplified the design of Raman spectrometers. Notch filters eliminated the need for large or multi-stage spectrometers in order to physically separate the excitation laser Rayleigh scattering from the Raman scattering, and sharp optical cut-off filters allowed the design and use of 180° backscattering single-ended probes. In addition, power-efficient and stable diode lasers were developed. Computing power also allowed the application of statistics to large spectra data sets and chemometrics has also become an important part of Raman spectral analysis.

3.2.1 RAMAN MEASURING TECHNIQUES

Two major techniques are used to collect the Raman spectra: dispersive Raman and Fourier transform Raman (FT-Raman). The differences between both techniques are the laser used and the way the Raman scattering is detected and analysed. Each technique has unique advantages and the method that suits the sample should be preferred. Generally speaking, probability of fluorescence decreases with increasing laser wavelength, however intensity of obtained absorption bands decreases as well. For this reason, at higher wavelengths it is necessary to use laser with higher power, which in turn can cause sample destruction. The comparison of characteristic features of the dispersive and FT-Raman spectroscopy is shown in Table 3.1.
### Table 3.1 Comparison of dispersive and FT-Raman spectroscopy

<table>
<thead>
<tr>
<th></th>
<th>Dispersive Raman</th>
<th>FT-Raman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser</td>
<td>Vis (455 nm, 532 nm, 633 nm, 785 nm)</td>
<td>NIR (1064 nm)</td>
</tr>
<tr>
<td>Detector</td>
<td>Silicon based CCD</td>
<td>InGaAs or Ge</td>
</tr>
<tr>
<td>Advantages</td>
<td>Higher spatial resolution for microscopy applications (&lt; 1 μm)</td>
<td>Limited fluorescence</td>
</tr>
<tr>
<td></td>
<td>Higher sensitivity</td>
<td>Higher spectral resolution and an invariant x axis</td>
</tr>
<tr>
<td></td>
<td>Lower laser power</td>
<td></td>
</tr>
</tbody>
</table>

Several other variations of Raman spectroscopy for pharmaceutical solids analysis have been developed (Fig. 3.3). A traditional backscattering Raman analysis acquires data from a small spot of a sample. Thus, backscattered Raman is a good approach for measuring surface (commonly used geometry to Raman microscopy, chapter 3.2.2). However, the resulting spectral output may fail to entirely represent the static and heterogeneous sample. Sample rotation during spectral acquisition and the temporal averaging of the acquired data, the spatial averaging of the data acquired by scanning different regions of sample, and simultaneous wide area illumination (WAI) are configurations available to overcome the issues related to sub-sampling. Furthermore, the configuration enabling the collection of signals from locations laterally offset away (hundred micrometers to centimeters in some cases) from the illuminated area is called spatially offset Raman spectroscopy (SORS). In SORS geometry the laser irradiates center of the ring and Raman collection from the circumference, the radius being the spatial offset. The avoidance of surface interference with this technique can facilitate the depth profiling of the sample. Spectral acquisition representing a bulk sample can be performed using transmission Raman spectroscopy (TRS), wherein the incident and the collection beam path are separated to the extreme on opposite sides of sample. TRS potentially avoids the sub-sampling problem of heterogeneous samples and yields (semi) averaged spectral data of the bulk composition for turbid or opaque materials.

Simultaneous wide-angle illumination, SORS and TRS are methods for analysing bulk turbidly scattering materials such as powders, tablets, suspensions and other chemical mixtures, and provides non-invasive measurements with high selectivity and sensitivity. They can be effectively used for non-invasive probing of the content.
of pharmaceutical capsules and tablets and provide an analytical technique ideally suited for fast on-line process control monitoring applications in pharmaceutical industry where rapid, chemically specific bulk analysis is required.

**Fig. 3.3** Schematic comparison of sample excitation (solid line) and signal collection (dashed lines) variants used in Raman spectroscopy in measuring turbidly scattering materials. Backscattered Raman (a), Wide area Raman (b), SORS (c), Transmission Raman (d).

### 3.2.2 RAMAN MICROSCOPY

Raman's spectrometer coupled to a confocal microscope possessing high numerical aperture lenses enables high spatial resolution. This instrumental arrangement, combined with possibility of moving the sample on x-y or x-y-z axes using scan stage allows the acquisition of individual spectra or generation of detailed chemical images based on a sample's Raman spectrum (Raman imaging and mapping). Such imaging is rapid, but it has low resolution and it is limited in a way that only particular region of the spectra can be surveyed at a time. Mapping allows the whole spectrum to be recorded and stored digitally but it is time consuming process. With single-point Raman microscopy, information about a specific location on the sample is provided. Raman's microscopy is particularly suited to be used in the pharmaceutical formulation development. With Raman imaging/mapping, an expanded view of the sample is provided, allowing for a more thorough analysis of the homogeneity, spatial distribution of components and variations in chemical structure throughout the sample. Raman microscopy can, for example, evaluate the homogeneity of the substances in the tablet, the coat quality of the coated pharmaceutical forms, the API solid-phase change in the final dosage form, the distribution of the substances in the matrix during the dissolution, or the understanding and consideration
of all the circumstances that affect the release of the substances. Raman microscopy also allows the identification of impurities in tablets, for example impurities caused by local overheating during tabletting (dots on the surface of tablets caused by elemental carbon).

### 3.2.3 Raman Data Analysis

There are two main approaches to analysing Raman data: univariate and multivariate. A univariate approach uses Raman band features of area, intensity, or centre of gravity to understand the sample chemistry. Most univariate Raman data are reported as band ratios, where band intensities or areas are expressed in ratios. Band ratios are correlated to a material's mechanical properties, chemical composition, or a pharmaceutical solid's crystal form. Although a univariate data analysis is straightforward to employ, it requires that the components of interest have distinguishing and unique Raman bands. If band overlap is observed, use of multivariate data analysis techniques is required. Multivariate data analysis, or chemometrics (chapter 2.3), is widely used in chemical imaging and process analytical technology.

Raman spectroscopy is also commonly used in qualitative analysis to provide a structural fingerprint by which molecules can be identified. The fingerprint region of organic molecules is in the wavenumber range of 500–1500 cm\(^{-1}\). Qualitative analysis based on fingerprint region is employed not only in the pharmaceutical industry (e.g. API identification, counterfeit detection) but also, for example, in forensic analysis or in civil and criminal analysis, where it is used for identifying substances (narcotics, drugs, alcohol, explosives, etc.).

### 3.3 Application of Raman Spectroscopy in Pharmaceutical Analysis

Raman spectroscopy is suitable for analysis in the pharmaceutical industry as a part of formulation development, process analytical technology and quality control. Yet several factors have prevented its wider employment in the past, especially the high cost of instrumentation. The devices were large, heavy and required a stable environment. The analysis was considerably lengthier than the MIR or NIR spectroscopy. Great improvement came upon the arrival of highly efficient CCD detectors, a new generation of filters for removing
scattered laser radiation and new generations of semiconductor lasers. These innovations have enabled the construction of high-sensitive tabletop sized disperse Raman spectrometers that have opened the way for Raman spectroscopy into the pharmaceutical industry and development. The advantages of Raman spectroscopy to the pharmaceutical community have come largely from the ease of use, minimal sample handling and strong differences in relative scattering strengths of packaging materials, tablet excipients and the active agents. These strengths combined with the use of microscopes or fibre optics have seen a large growth of use in the pharmaceutical industry. Raman spectroscopy is now implemented in all major standards (e.g. the United States Pharmacopoeia and the European Pharmacopoeia) and, together with NIR spectroscopy, it is one of the main methods for the implementation of PAT procedures in the pharmaceutical industry, particularly in the manufacture of solid dosage forms.

3.3.1 IDENTIFICATION OF RAW MATERIALS

Chemical and physical properties of APIs and pharmaceutical excipients are typically a critical process parameter because they affect manufacturability, bioavailability, and risk of process-induced API transformations. The most widespread instrumental technique used for the chemical identity testing of pharmaceutical materials is infrared spectroscopy (chapter 2.4). IR spectroscopy offers several advantages, but also comes with some limitations that have arisen recently, as demands on the analysis speed and complex method validation increased. The main problem of the MIR spectroscopy is higher requirements for sample preparation. NIR spectroscopy, on the other hand, does not require sample preparation, but most devices still require sampling (direct measurement through packaging is limited) or an optical probe must be employed. As sampling means breaching the original packaging, it calls for analysis in clean rooms. Due to the inherently low sensitivity and selectivity of NIR spectroscopy, it is also necessary to use relatively complicated chemometric procedures to generate identification patterns.

Raman spectroscopy is more suitable technique for the identity testing of pharmaceutical materials thanks to its high selectivity (Fig. 3.4) and virtually no need for sample preparation. Furthermore, non-contact
measurement (Raman probes), measurement of water solutions (no water signal interference) or measurements directly through transparent and semi-transparent packaging materials, is possible. This eliminates the need for sampling of materials in clean rooms or requirements for their further treatment. Analysis can be performed in input stores of pharmaceutical products or throughout manufacturing facilities, to carry out identity testing directly through original packages. Still, as outlined earlier, the main limitation of Raman spectroscopy lies in the measurement of samples exhibiting high fluorescence at the wavelength of the excitation laser used.

**Fig. 3.4** Identification of an API in two solid state forms (amorphous/crystalline) used in tablet production; API in the crystalline form exhibited three narrow separated peaks of 1605, 1630 and 1660 cm\(^{-1}\), while the spectrum of the amorphous form revealed a strong-narrow peak at 1605 cm\(^{-1}\) with a wide and not fully separated peak at 1621 cm\(^{-1}\).

### 3.3.2 CONTENT AND CONTENT UNIFORMITY ANALYSIS

Drug content assays and impurity profiles are two critical quality attributes of any finished dosage form. As an off-line PAT, Raman spectroscopy offers rapid and nondestructive API measurements in tablets or capsules (the most common pharmaceutical formulation). Raman PAT tools provide representative sampling therefore pharmaceutical applications of Raman spectroscopy consistently report rapid API quantification with suitable prediction model error. For the quantification of pharmaceutical tablets with Raman spectroscopy the chemometric methods are usually used to analyse data (e.g. PLS, PCR; chapter 2.3).
The choice between Raman spectroscopy and microscopy depends on the type of investigation. Raman spectroscopy is better for quantitative analysis of a large number of samples (e.g. quality control of final product). Raman microscopy is suitable for analysis of a small portion of the tablet blend or a single tablet (e.g. during formulation development). The high spatial resolution of the Raman microscopy is suitable for the identification of small particles, such as dosage form contamination, API distribution, or identification of the solid state of the drug (Fig. 3.5).

**Fig. 3.5** Image of a pharmaceutical tablet. Optical microscopy image (a), Raman spectral image showing the distribution of API (black) and excipients (shades of gray) (b).

### 3.3.3 Drug Solid Phase Identification

API polymorphic forms can affect solubility, efficacy and shelf life of an API, and can provide active patent protection. The highly specific spectral information provided by Raman spectroscopy can elucidate polymorphic form, pseudo-polymorphic phases, crystallinity and hydration of a sample and thus giving characterization and understanding of API physical state. Raman microscopy can provide qualitative and quantitative information on polymorphic state with spatial resolution of 1 μm which allows API solid-phase identification directly in pharmaceutical solid samples.

Drug release from solid dosage forms, such as tablets and capsules, is a complex process which is accompanied by concomitantly occurring physicochemical, solution-mediated solid-state transformations at the surface and in the bulk of solids in contact with the aqueous media. The lack of water signal interference and inherent capability of data collection in aqueous systems have led to the increasing popularity of fiber optic Raman probes and Raman microscopy as powerful tools for in situ monitoring of various solution-mediated phase transformations, including anhydrate to hydrate transformation, crystallization kinetics
of amorphous pharmaceuticals, disproportionation of API salt forms (Fig. 3.6) and polymorphic transformations.

![Raman spectra of an API in the salt form (before dissolution test) and acid form (after dissolution test in pH 1.2).](image)

**Fig. 3.6** Raman spectra of an API in the salt form (before dissolution test) and acid form (after dissolution test in pH 1.2).

### 3.3.4 INVESTIGATIONS OF DRUG-EXCIPIENT INTERACTION AND DOSAGE FORM STABILITY

The interactions between APIs and excipients can have an effect on the drug stability, solubility, bioavailability and therapeutic safety. Raman imaging is useful to study the physicochemical interaction of an API and excipients or the changes in the API solid-state form as it interacts with the excipients. During the shelf life, variations in the dosage form quality may also occur as a result of changes in the physicochemical properties of the API due to external conditions (temperature, humidity). These changes include the solid-state transformations such as recrystallization/transition to amorphous, change of crystalline structure, formation of hydrates or formation of decomposition products. Specific case of drug dosage deterioration can occur in the formulations with controlled drug release, as possible changes in the coat quality may be directly related to changes in the API release rate from the dosage form during the stability tests. The Raman spectra can be processed to give unambiguous identification of both APIs and excipients (Fig. 3.7), and the relative intensities of API and excipient bands can be used for quantitative or at least semi-quantitative analysis. The spectra can even be used to distinguish between chemically similar substances, such as the geometric isomers and between different
polymorphic/hydrated forms of the same API. Moreover, these differences can be found even in the directly recorded spectra of pharmaceutical samples (e.g. tablets, blends) giving a rapid and non-destructive alternative for drug-excipient interaction identification.

![Fig. 3.7 Penetration of API from the coated pellet core to the pellet surface; SEM image of coated pellet cross-section and pellet surface (top) and Raman spectra of individual pellet components (bottom). In the spectrum of crystalline object on the surface of the coated pellet, the Raman signatures characteristic for the API were found (dashed rectangles).](image)

3.3.5 ENANTIOMERIC COMPOSITIONS ANALYSIS

In the pharmaceutical industry, currently more than two thirds of the APIs on the global market are chiral. Shortening timelines for chiral API

---

discovery and development usually depends on the efficiency of asymmetric synthesis, enantiomeric separation and determination of absolute configuration. The prerequisite for optimizing the production of enantiopure substances is the development and implementation of suitable analytical techniques for process monitoring. However, a major problem in this context is that the enantiomers are virtually identical in terms of molecular structure. This implies that methods that provide structural information and allow for enantioselective discrimination are rare. Raman optical activity (ROA), measuring the difference in Raman scattering intensity for right and left circularly polarized incident radiation, has emerged as a technique for investigating the solution structure of chiral molecules in aqueous solution. The basic principle of Raman optical activity is that there is interference between the light waves scattered by the polarizability and optical activity tensors of a chiral molecule, which leads to a difference between the intensities of the right- and left-handed circularly polarised scattered beams. The spectrum of intensity differences recorded over a range of wavenumbers reveals information about chiral centres in the sample molecule.

The experimental setup very much builds on the Raman technology, but the ROA is intrinsically a weak phenomenon, the differential circular scattering being several orders of magnitude weaker than the corresponding vibrational Raman scattering. The choice of the excitation frequency strongly affects the intensity and the signal to noise ratio. Raman intensity is proportional to the fourth power of the frequency, and Raman optical activity even to its fifth power. Scattering geometry, i.e. the angle between the incident and scattered radiation is another critical experimental factor affecting the intensity and detectable signal to noise ratio of Raman scattering and ROA. The Raman optical activity is maximal in the back-scattering geometry. The ROA setups may also differ by so-called modulation scheme, i.e. a selection of the polarization states of the excitation and scattered radiation. The simplest and historically first was the incident circularity polarization (ICP) scheme. The sample is sequentially illuminated by right- and left-circularly polarized radiation, whereas the total scattered radiation is detected. Other ROA setups, so called in-phase and out-of-phase dual-circular polarization modulation schemes (DCPI, DCPII), were developed, where both the incident and scattered radiations
are circularly polarized. Finally, in the scattered circular polarization (SCP) ROA scheme, the sample is irradiated by essentially unpolarized radiation, and the right- and left-circular component is detected in the scattered signal. The right- and left-circularly polarized components can thus be detected simultaneously. This is a great advantage over the other modulation schemes, as artefacts due to fluctuation in the laser power and sample absorption, otherwise causing a problematic flicker noise, can be reduced. By now, the ROA technique allows the determination of the absolute configuration of small molecules, which is very important for the pharmaceutical industry, but also it has much potential for biomolecules (proteins, polysaccharides, DNA) structure determination in solution.

3.3.6 COUNTERFEIT DETECTION

A new area of use, which is gaining importance, is the identification of drug counterfeits by the verification of chemical identity. Complementary, as chemical identity may not be the only evaluated parameter, an array of methods is usually used to monitor diverse parameters. The issue of massive entry of counterfeit medicines on the market is no longer a problem solely for developing countries, as it is becoming a serious problem even in economically developed ones. The World Health Organization defines a counterfeit medicine as “one which is deliberately and fraudulently mislabelled with respect to identity and source”. Counterfeit drugs range from those employing incorrect ingredients, no active substances (e.g. sugar pills), or insufficient amount of active substance. The latter are the most challenging since a simple compositional analysis may pass the sample as the genuine product. Authentic and counterfeit products can be examined by many analytical methods, including MIR, NIR, and Raman spectrosopies.

3.3.7 OTHER PHARMACEUTICAL APPLICATIONS

Tablets and capsules are still considered as the preferred dosage forms. The manufacture of tablets involves several operations such as milling, powder blending, granulation, drying, compaction and coating. It is crucial to control the quality of the intermediate products generated during various unit operations to manufacture the final quality product.
Raman spectroscopy has been found to be a very useful PAT tool for in-process monitoring to test for the desired intermediate product after each unit operation. Raman spectroscopy can be used e.g. as a PAT tool for the end-point control of a powder blending process or as a valuable tool for monitoring the solid dosage forms coating process, irrespective of the coating apparatus or the core type (tablets or pellets).

3.4 REFERENCES


4 THERMAL ANALYSIS

Term “Thermal analysis” summarises a group of experimental methods in which certain physical or physicochemical properties of the studied system are observed depending on temperature. It is possible to observe processes occurring in the system while heating it up or cooling it down (dynamic) or at a constant temperature depending on time (static).

Methods of thermal analysis are an important source of information about properties of solid substances. The observed processes (phase transformations, polymerization, oxidations, dehydrations etc.) can be accompanied by a change in weight, volume, release or consumption of energy, a change in conductivity or magnetic properties. The type of thermal analysis is designated according to the system property observed. There are several dozens of thermo-analytical methods, three of which are the most important in pharmaceutical analysis. They are the differential scanning calorimetry (DSC), the differential thermal analysis (DTA) and thermogravimetry (TGA).

4.1 THERMOGRAVIMETRY

Thermogravimetry observes changes in sample weight \((m)\) depending on the change of temperature \((T)\). The temperature is most frequently changed at a controlled rate according to the selected program and the temperature of the weighed sample is measured. The obtained record (a thermogravimetric curve) gives information about thermal stability of the sample, decomposition temperatures and/or about the interval of temperatures which correspond to the existence of intermediate products. The example of a thermogravimetric curve shows some reactions connected with a change of weight (Fig. 4.1). If weight changes caused by temperature increase appear close to each other, it is more suitable to record the weight change rate of the sample depending on the temperature (the first derivation of the function \(m = f(T)\), derivation thermogravimetry).

The device which enables determining the weight and temperature of the observed sample at any moment of the heating process is called the thermobalance. Basic parts of a thermobalance are devices for sample
heating and cooling according to the pre-set temperature program, a sample holder (usually a crucible or a boat) in controlled atmosphere, an electrobalance and a recorder. The material used for sample holder is e.g. corundum ceramics, graphite or platinum.

In pharmaceutical technology TGA is used especially for characterizing solvatomorphism (certain molecule can crystallise from different solvents in the form of different solvates) and for determining the presence of a residual solvent in API (e.g. after crystallising).

![Fig. 4.1 Example of a thermogravimetric curve (----) and its first derivation (---).](image)

### 4.2 Differential Thermal Analysis

Differential thermal analysis can be used to observe all processes occurring during uniform increase or decrease of the examined sample temperature which are accompanied by a change in energy content (exothermic and endothermic processes). It is therefore possible to observe more reactions than in thermogravimetry. These processes manifest with a sharp increase or decrease in the examined sample temperature compared with the ambient temperature or a temperature of the reference sample which is heated up under the same conditions as the examined sample. The DTA is thus an analytical method based on measuring a difference in temperatures between two samples: the measured one and the reference one. Both samples are identically heated up, while the reference sample is selected to be perfectly inert in the measured interval of temperatures (an empty sample container is often used instead of a reference sample). All actions associated with heat exchange cause a difference in temperatures between the measured and the reference sample, which is shown in a DTA curve.
by a temperature extreme called the peak. According to the position and the shape of the peak it is possible to assess the temperature under suitable conditions, at which the transformation occurs (characteristics of the examined substance), its reaction heat (proportionate to the heat effect of the reaction and the sample quantity) and the rate of the occurring process. The released or consumed reaction heat \( H \) can be calculated from the peak area according to the following equation (Eq. 4.1):

\[
P = \frac{m \times \Delta H}{k \times k_k}
\]

where \( P \) is the peak area, \( m \) is the sample weight, \( k \) is the thermal conductivity of the sample and \( k_k \) is the shape constant of the crucible.

Instrumental equipment consists of a furnace (an electrical resistive heater), a measuring head, a temperature regulator and a recording device. The measuring head is an assembly of holders for measured and reference samples, including temperature sensors. A sample temperature is most frequently measured at the bottom of the sample container using a thermocouple. Materials used for sample holder are e.g. Pt, Cu or corundum ceramics.

The DTA applications in pharmaceutical technology are similar to those of differential scanning calorimetry (chapter 4.3).

When an analysis is performed by means of thermal analysis, it is necessary to consider a lot of factors influencing the precision and accuracy of the measurement. Some factors influencing the resulting curves are:

- Heating rate (it is highly important for a separation of consequent actions/peaks).
- Sample weight (a small quantity of the sample shows a good heat gradient but observed actions are less intensive).
- Geometry of the sample container (influences the heat gradient).
- Atmosphere in the sample chambers (vacuum, inert gas, reduction or oxidation atmosphere).
- Sample particle size.
- Compacting of the sample (influences heat conductivity of the sample).
4.3 DIFFERENTIAL SCANNING CALORIMETRY

In the DSC it is possible to observe all processes which occur during an increase or decrease of temperature of the examined sample (as in DTA) and which are accompanied by a change of energy content. However, the differential scanning calorimetry differs by the fact that it observes changes in heat capacity of the sample \((C_p)\) as a temperature function. In practice, the heat flow \(q\) (the difference in heat delivered to the measured and reference samples in the given time) depending on temperature is most frequently plotted on a DSC curve. The heat capacity of the measured sample can be calculated as a ratio of the heat flow and the heating rate, respectively as a ratio of the heat given off or received and the difference of temperature between the initial and final value \(T\) (Eq. 4.2):

\[
\frac{q}{t} = \frac{q}{\Delta T} = C_p
\]

where \(q/t\) is the ratio of heat/time = heat flow, \(\Delta T/t\) is the difference in temperatures/time = heating rate and \(C_p\) is the heat capacity.

As in DTA, the peak area \(P\) is proportional to the reaction heat \(H\) given off or received and the weight of the measured sample \(m\) according to the following equation (Eq. 4.3):

\[
P = K \times \Delta H \times m
\]

where \(K\) is the instrument constant.

4.3.1 INSTRUMENTATION

In DSC, measured and reference samples undergo linear heating (usually 5–20 °C/min) and subsequent measurement of the heat flow (or the heat capacity) is based on the difference in heat received by the measured and the reference samples or the difference in temperatures between the measured and the reference samples. According to the instrumentation, the DSC devices are divided to the power compensation DSC and to the heat flux DSC (chapters 4.3.1.1 and 4.3.1.2). A temperature range for the use of DSC is usually from the laboratory temperature to 800 °C (some devices enable measuring even at higher temperatures, up to 1600 °C). The temperature range of measurement can be also extended to negative values (−170 °C) when liquid nitrogen is used for cooling. Aluminium pans (usable up to 550 °C) are most frequently used as sample containers but other
materials, such as stainless steel, gold or platinum, are also available. The quantity of the measured sample is usually in milligrams (the advantage is small sample consumption and a good heat gradient). An empty sample container is often used instead of a reference sample. Measuring is carried out in the atmosphere of a recommended gas (most often nitrogen or inert gases). Temperature program, signal acquisition, data storage and analysis are handled by computer software. The DSC technique exists in numerous modifications, such as hyper-DSC, temperature modulated DSC or isothermal microcalorimetry (IMC). Hyper-DSC is more sensitive than conventional DSC and enables measuring changes obtained at rapid heating of a sample (up to 500 °C/min). The advantage of this method is that certain phenomena, such as water evaporation or re-crystallising, occurring during slow heating, are eliminated. It is used in the precise temperature determination of a glass transition or in studying the substance polymorphism. The principle of temperature modulated DSC is a periodic change of the heat flow to which the sample is exposed. The temperature modulated DSC is used as an additional method to the conventional DSC in cases when it is difficult to interpret some changes observed by means of the conventional DSC. Temperature modulated DSC allows a separation of complex transitions (phase changes, chemical reactions, etc.) into more easily interpreted components, more accurate determination of the crystallinity or detection of weak transitions. The isothermal microcalorimetry, which has a higher sensitivity compared to the conventional DSC, enables observing processes in which only small changes of $H$ occur. The IMC differs from the conventional DSC in instruments, measurement conditions and obtained calorimetric curve. The main differences between the IMC and the conventional DSC are as follows:

- The temperature of measurement ranges from $-20$ °C to 120 °C.
- The sample weight is approximately 150 mg.
- The heating rate is 0.001–1.2 °C/min (most often 1 °C/min).
- Special closable cells are used instead of a sample container (they can also have a mixer for analyses of mixtures of liquid and solid substances).
- Analysis lasts dozens of hours (slow heating to the required temperature and consequent observation of thermal phenomena at a constant temperature).
- Obtained calorimetric curves are usually without marked peaks, a deviation from the basic line (so-called drift) and the noise are usually the only evaluated output results.

4.3.1.1 Power compensation DSC

The principle of the method is to maintain the same temperature in the measured and in the reference samples throughout the controlled temperature program. An electric power input, which has to be supplied either to the measured or the reference sample to keep the null temperature difference, is measured. The measured and the reference samples are placed in sample pans in two separated furnaces. Holders of both samples have a built-in resistance thermometer and heaters which enable control of the sample temperature by external input voltage. The samples are perfectly isolated from each other to prevent a heat flow between them and measurement is carried out in the recommended atmosphere (e.g. N₂).

The equipment for power compensation DSC (Fig. 4.2) consists of two separated control circuits. One circuit measures an average temperature of the samples and automatically balances the heat flow so that the average temperature of the samples is increased linearly. The second circuit measures differences of the temperatures between the measured and the reference samples and determines which of the samples has higher temperature and automatically compensates these temperature differences.

4.3.1.2 Heat flux DSC

Heat flux DSC measures the difference of temperatures between the measured and the reference samples (as in DTA). Knowing the heat resistance between the furnace, the sample and the reference, can be considered, the heat flow from the sample or to the sample proportionate to the difference in temperatures. The measured and the reference samples are put in similar sample containers (usually flat pans), which are placed on individual thermally conducting bases. The thermocouple junctions are attached to these bases and are thus not directly in the sample or reference material.
The equipment for the heat flux DSC (Fig. 4.2) consists of a furnace, a measuring head with a holder for the measured and the reference samples and a gas source.

**Fig. 4.2 DSC instrumentation.**

### 4.4 APPLICATION OF THERMAL ANALYSIS METHODS IN PHARMACEUTICAL TECHNOLOGY

The differential scanning calorimetry provides quality and quantity information on physicochemical properties of APIs or their mixtures with excipients. Samples are analysed in the solid phase thanks to which DSC can provide information on crystalline structure changes (polymorphism) or e.g. on interactions between the API and excipients. A DSC curve (Fig. 4.3) shows endothermic processes (e.g. melting, dehydration), exothermic processes (e.g. crystallisation) and changes in the heat capacity of the sample (e.g. glass transition temperature). It is possible to determine temperatures at which these processes occur and to determine the process reaction heat on the basis of the observed peaks on the DSC curve. A DSC curve provides further information in relation to stability (e.g. crystallisation of amorphous substances, changes in the crystalline structure or degradation), purity (a change in melting temperature) or compatibility of an API and excipients (a different shape of calorimetric curves obtained for dosage forms with various excipients). For these reasons, DSC and other methods of thermal analysis are among the main methods used in formulation development and quality control.
Glass transition temperature ($T_g$) is a temperature which can be observed on a DSC curve of amorphous materials and polymers. The heat capacity of a sample increases at the glass transition temperature and therefore a higher heat flow needs to be delivered to the sample. This change appears as an S-shaped curvature on the DSC curve. Molecules below $T_g$ have a relatively slow mobility; polymers and amorphous substances are in the solid and fragile phase. In technological processes, such as film coating of pellets or tables, to achieve the required quality it has to be ensured that the polymer coat does not crack or break. Polymeric materials used in the pharmaceutical technology contain excipients (plasticisers) which reduce the glass transition temperature. The lowest temperature at which latex, emulsion or adhesive will uniformly coalesce when laid on a substrate as a thin film is called minimal film-forming temperature. In practice, coating with polymeric materials is carried out at temperatures ranging from 10 °C to 20 °C above the minimum film-forming temperature. At such conditions, polymer chains slide over each other, polymer becomes elastic and forms a film of the required quality. The knowledge of the $T_g$ value of polymers and the minimum film-forming temperature of their mixtures with excipients are important parameters which influence the film-coating of dosage forms.

Determination of the glass transition temperature is essential for the assessment of amorphous APIs stability because spontaneous crystallisation often occurs at a temperature close to $T_g$ (see chapter 4.4.2).
4.4.2 Crystallisation

Molecules of amorphous substances and polymers have higher mobility at the temperature above $T_g$. When further heated, molecules gain sufficient energy to re-arrange to a highly organized form and thus crystallisation may occur. A crystalline form has a lower content of energy than the amorphous material, therefore heat is released during this process (exothermic peak on a DSC curve). A crystalline form has different physicochemical properties than the original amorphous substance, therefore, to be able to predict the stability of a medical preparation, it is important to know whether the crystallisation of an amorphous substance occurs and under what conditions (the influence of temperature, humidity) and what the rate of this process is. These data are very important in pre-formulation studies.

4.4.3 Polymorphism and Pseudo-polymorphism

Polymorphism is a property of a substance to crystallise in more crystalline forms. These crystalline forms differ in physicochemical properties e.g. the melting temperature (Fig. 4.4). In pseudo-polymorphs, the crystals differ in the presence of solvent molecules. If the solvent is water, hydrates are formed; other solvents form solvates. Different polymorphic/pseudo-polymorphic forms of an API can differ e.g. in solubility, dissolution rate or stability, so they show different pharmacokinetic properties and they can thus influence the resulting treatment effect. Polymorphic behaviour may be also classified as monotropy (an irreversible change of a metastable form to the stable one) and enantiotropy (a reversible change of one form to another depending on the temperature). Studying the polymorphic behaviour of a given substance is important for understanding and influencing the stability of APIs and dosage forms (e.g. if an API is monotropic, it can be transformed to a more stable form by heat treatment).
4.4.4 MELTING TEMPERATURE

Melting temperature is an important physicochemical parameter. Melting of crystalline substances is associated with energy consumption. All the heat is consumed for melting, therefore the measured sample must be heated more to keep the temperature of the reference and the measured samples identical. This difference in the received heat is visible on the DSC curve. The melting temperature is characteristic for a given API and is also a criterion of its purity. Even small impurities are manifested as a measurable change of the melting temperature and as the broadening of the peak. A decrease in melting temperature of a sample enables the determination of impurities according to Van't Hoff's equation (Eq. 4.4):

\[
T_0 - T_m = \frac{R \times T^2 \times X}{\Delta H_0} \times \frac{1}{F}
\]

where \(T_m\) is the melting temperature of the measured sample, \(T_0\) is the melting temperature of the pure substance, \(R\) is the gas constant, \(X\) is the impurity (molar %), \(\Delta H_0\) is the molar heat of melting of the pure substance and \(F\) is the melted fraction.

The broad shape of the peak indicates a higher distribution of API crystallites. The melting heat (area under the peak) reflects the crystallinity of API. The DSC curves can also indicate the interaction (physical or chemical) of the API and the excipient. Such interaction results in a change of API melting temperature after the incorporation into a dosage form (Fig. 4.5) or in changes in the heat flow (see chapter 4.4.5).
4.4.5 INVESTIGATIONS OF DRUG-EXCIPIENT INTERACTION AND DOSAGE FORM STABILITY

Stability tests are necessary for the registration of medical preparations. One of the methods suitable for stability evaluation during pre-formulation studies is the differential scanning calorimetry. During a thermal stability studies the observed changes of $H$ are small, therefore the isothermal microcalorimetry is especially used for these purposes. Using this method during the stage of formulation development can help identify critical factors which directly influence the quality and stability of the formulation. A measured sample is usually kept at a constant temperature for a period of several hours or days and during this period changes in the heat flow are observed (the deviation of an IMC curve from the baseline). Conditions during the thermal analysis rank this procedure among the group of stress stability tests, in which a formulation or an API are subjected to an extreme physical stress. An advantage of the IMC is that the information on stability of the preparation is available very quickly compared to the long-term or even accelerated stability studies which take several years or months, respectively. Figure 4.6 shows the isothermal microcalorimetry curves of two different dosage forms containing the same API. Different thermal behaviour caused by the instability of preparation 1 is obvious.
Fig. 4.6 An example of the isothermal microcalorimetry curves of two dosage forms containing the same API.

Besides thermal stability, the IMC is applied in the observation of API-excipients compatibility. The selection of excipients is an important step in the formulation of a new medical preparation. Excipients must meet numerous criteria and one of them is that no chemical interactions should occur between the APIs and excipients. In some cases, however, the interaction of the APIs with the excipient is used for stabilisation. These are especially stabilisations of amorphous substances or less stable polymorphic forms (e.g. by the formation of a solid dispersion in a suitable polymer). The IMC enables determination of the interactions between APIs and excipients because they are expressed as changes in the heat flow. When an interaction occurs, these changes are observed as a deviation of the calorimetric curve from the baseline, while the IMC curves of a placebo, an API or a dosage form with no interactions, do not show such changes.

4.5 REFERENCES


5 X-RAY DIFFRACTION ANALYSIS

X-ray diffraction (XRD) analysis is unique technique which can determine the crystal structure and the chemical composition of a sample, which are critical parameters of the drug. For pharmaceutical applications, it is possible to perform raw materials identification (excipients), polymorph screening and it is possible to do stability studies as function of temperature and humidity. XRD is used as a part of quality control in pharmaceutical production process. XRD is technique for understanding the molecular and physical structure of the crystals. The principle of X-ray diffraction analysis is the irradiation of the sample with a beam of X-rays and subsequent measurement of the radiation diffracted by the crystal lattice of the sample. The signal coming from the sample is recorded in graph (diffractogram) on which peaks related to the atomic structure of the sample can be observed. The particle arrangement in a solid material can be random or ordered with repeated short and long-order. The ordered particles can be defined as a crystalline solid but if the solid has low degree of long-range order, it is called amorphous. The nature of materials structure is given by the conditions in which they were formed. The type of order is given by the type of interaction with a certain periodicity and symmetry. Different bonding causes different periodicity in their crystal structure. The exact structure analysis can be performed by previously mentioned spectroscopic methods as Raman or IR and further discussed with NMR but the analysis of three-dimensional arrangement of whole molecule would still be missing. This opens the possibilities for XRD utilization. The analysis of crystalline state, symmetry and exact determination of atom position in the structure (i.e. lattice, unit cell parameters, defects) can be performed by methods based on X-ray diffraction analysis.

5.1 THEORETICAL BASICS OF X-RAY DIFFRACTION

X-ray radiation is a high energy electromagnetic radiation with wavelengths shorter than UV radiation and particles of energy called photons. It is usually defined by the wavelength (in the interval between
0.001 nm and 10 nm), constant frequency and varied photon energy (0.1–100 keV).

5.1.1 SOURCES OF X-RAY RADIATION

For X-Ray analysis, monochromatic radiation with the wavelength of 0.05–0.25 nm is the most frequently used. X-ray radiation is produced most often by an X-ray tube when heated cathode emits high speed electrons which hit a metal target (anode). The X-ray tube is evacuated ceramic tube with two electrodes between which there is a high voltage of 20–60 kV. A tungsten filament heated to a very high temperature serves as a cathode. The cathode produces electrons which are accelerated in the electrical field and then they impact on the anode with high energy. On impact, the kinetic energy of electrons is mainly transformed into heat (therefore an X-ray tube needs to be intensively cooled down) and only about 1% of energy is used for the emission of X-ray radiation. The X-ray emission histogram (Fig. 5.1) consists of several intense peaks, the so-called characteristic lines, superimposed over a continuous background (continuous radiation). The continuous part of the histogram is generated by electrons decelerating rapidly and unpredictably and the distribution of the wavelengths depends on the accelerating voltage but not on the nature of the anode material. The continuous radiation is highly undesirable in XRD analysis applications. Characteristic radiation is formed by accelerated electrons penetrating to sub-valence electron levels of the anode where they eject an electron out of the electron shell. Electrons from a higher energy level fill the place of the ejected electron, emitting X-ray radiation with quantized (precise) energies, determined by the respective electron energy levels. The wavelength of the X-ray radiation is therefore given by the difference in energies between both electron levels and depends on the anode material. The selection of the X-ray tube (anode material) depends on the required wavelength of the incident X-ray radiation and therefore on the nature of the analysed material. Excessive absorption of X-ray radiation or generation of fluorescent radiation by the sample is undesirable. The most commonly used anode materials in X-Ray diffraction analysis are copper, cobalt or molybdenum.
5.1.2 Principles of X-ray Diffraction

The X-ray analysis normally works with the wavelengths between 0.05 and 0.25 nm, i.e. wavelengths comparable with interatomic distances in crystals. Diffraction phenomena occur when the wavelength of X-ray radiation is close to the interatomic distances and possess the atoms observation by its diffraction pattern as a function of crystal symmetry and atomic structure. When X-Ray's interact with the matter the various situation can occur:

- No interaction: i.e. resonance absorption.
- Coherent scattering: produces beam with the same wavelength as the primary one.
- Incoherent (Compton) scattering: the wavelength of the scattered beam increases due to the loss of photon energy in collisions of core electrons.
- Absorption: some photons lose their energy by ionization or photoelectric effect (i.e. fluorescence—Fluorescence analysis, Auger electron production—Auger spectroscopy).

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Incoherent scattering and absorption effect are legible within the standard X-ray interaction. Only if they are in significant range, then they are processed as a separate effect. The interactions are described in Fig. 5.2.

![Fig. 5.2 Scheme of the X-ray, photoelectric and Auger effects.]

Thus, only the coherent (i.e. elastic) scattering coming from the photon interaction with the material is taken to account in X-ray diffraction of the crystal structure. The XRD results from the coherent scattering of radiation on various atoms (more precisely the electrons periodically distributed in the crystal lattice) and its consequent interference, which occurs due to the differences in lengths of trajectories from various scattering atoms to the place of detection. Each electron which interacts with the incident X-ray wave produces a spherical elastically scattered wave. Thus, the scattering of X-rays by a single electron yields an identically scattered intensity in every direction. When more than one point is affected by the same incident wave, the overall scattered amplitude will be the result of the interference among multiple spherical waves. The result of the interferences is a formation of a diffraction pattern with characteristic maxima and minima of diffraction circle intensities. By analysing diffraction circles

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it was found out that a crystal behaves as if it contained a large quantity of equispaced planes (Bragg's description of diffraction). A diffracted beam is formed by “reflection” from this system of parallel planes of atoms in the crystal structure. The incident as well as diffracted beam forms the same angle $\theta$ with the considered system of planes, corresponding to the Bragg's Law of reflection (Fig. 5.3). Although every plane reflects only a small quantity of radiation, the multiple reflections (resulting from the large number of planes) provide intensity sufficient for observation.

![Fig. 5.3 Geometrical illustration of Bragg's Law of reflection.](image-url)

Structural information about the positions of atoms in a crystal can be derived from the positions of diffraction maxima. The X-rays are diffracted by every crystalline substance depending on the type of atoms of which the crystal of the given substance consists, and their layout. When an X-ray impacts and is diffracted on a sample, it is possible to measure the distance between the individual planes of atoms according to the Bragg's Law. If we have a beam of parallel X-rays with the wavelength $\lambda$ impacting on any parallel planes at distance $d$, the rays are in phase on impact. Considering that ray 1–1' is diffracted by plane A and ray 2–2' is diffracted by plane B (Fig. 5.3), with both rays being in the phase after the diffraction, the path-length difference between the ray 2–2' and ray 1–1' must equal the whole $n$ multiple of the impacting

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radiation wavelength $\lambda$. The interference maximum of diffracted rays can be detected only under this condition. The path-length difference of rays 1–1' and 2–2' equals $2d \times \sin \theta$, where angle $\theta$ is an angle formed between the impacting ray and the plane of the crystal. By uniting the aforementioned equations, it is possible to get Bragg's Law (Eq. 5.1):

$$2d \times \sin \theta = n \times \lambda$$

(5.1)

Bragg's Law is a crucial equation for X-ray diffraction analysis. If the wavelength of X-ray radiation $\lambda$ is known and if the respective Bragg angle $\theta$ at which the diffraction occurs is measured, the distance $d$ between the crystalline planes can be determined. The integer $n$ is order of diffraction and it reflects the crystallographic planes with Miller indices ($d_{hkl} = nd_{nh \cdot nk \cdot nl}$). The Bragg equation simplifies the mathematical description of real diffraction of crystal geometries in three-dimensional space and restricts the analysis to two-dimensional plane within the lattice. To simplify the relationship between the planes in the lattice, another method is taken to account. The vector $d_{hkl}$ describes each of the two-dimensional planes so the real unit cell is represented by the sheaf of vectors projecting out from the origin in all directions. The visual representation of planes in the unit cell is given on Fig. 5.4. The Ewald's method (application of Ewald sphere) introduces the reciprocal vectors and reciprocal lattice/space which makes the visualisation of the Bragg planes easier. The reciprocal lattice is described on Fig. 5.4; where the concept of full three-dimensional lattice prolonged in all directions to fulfil the reciprocal space is given. The so-called reciprocal lattice is very close to the real lattice with the same properties. This way the XRD analysis through the recording of reciprocal lattice which gives the possibility to observe full crystallographic information about the analysed material (size, shape and symmetry).
The X-ray diffraction from a single crystal or powders should be done through various techniques which visualize the reciprocal lattice. Two basic techniques namely the single crystal diffraction and the powder diffraction is typically used. The special methods as Grazing Incidence Diffraction (GIXS, analysis of thin films or thin multilayers) or Small Angle X-Ray Scattering (SAXS, measuring under the 10°: the determination of the structure of nanoscale materials by the means of size, macromolecule arrangement etc.) are not so common and need special apparatus. Single crystal diffraction enables a complete structural analysis of a substance (crystal) and it is therefore one of the main methods for characterizing the atomic structures of new substances. The X-ray powder diffraction (XRPD) is used especially as a quality control method and for the characterisation of raw crystalline material. Its main advantages include the high speed of measuring, unneeded preparation of a single crystal, and the ability to analyse mixtures of substances.

5.1.3 SINGLE CRYSTAL DIFFRACTION

This technique requires adequately crystallized single crystalline material sized between 50 to 250 μm with no crystal twinning to assure the quality of the obtained results but it is not easy in real systems,

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as the occurance of single crystals without defects is in nature exceptional. The measured material is rotating or oscillating around its axis, the diffracted ray from each of 0kl points of the reciprocal lattice is recorded on the film. The recorded intensity of nkl point to the pattern of spots should be related to the unit cell description (atom location etc.). Fig. 5.5. shows the typical diffraction pattern from rotation photograph and CCD device.

![Diffraction pattern](image)

**Fig. 5.5** Diffraction pattern from single crystal obtained by Rotation photograph (a) and CCD detector (b).

The film technique is quite archaic; the modern diffractometers use automated data detection/collection by charge coupled devices (CCD) and multiplayers with high resolution. The typical instrument arrangement for a single crystal is shown on Fig. 5.6. The measured crystal is supposed to be un-fractured, optically clear; therefore, the special growing procedure is necessary. The mounting of the crystal for measurements is crucial for data acquisition. There are several methods how to mount it: glass capillary technique or to affix it by epoxy or some other glue to the end of glass fibre or nylon loop. The various detecting principles of diffracted signal have been used throughout the historical development. The point detectors can be seen as counters producing the discreet pulses. There are various types of counters: ionization counters (i.e. gas, solid-state, proportional and Geiger), scintillation counters, semiconductor detectors (intrinsic-barrier counters, diffused junction counters, surface-barriers counters and lithium-drifted

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counters). Nowadays the area detectors are used (position sensitive, CCD, image plate detectors). Typically, the CCD-type detector can convert photons at very high speed but it needs to be cooled for accurate working (around −70 °C). The image plate scanners are circular plates from X-Ray sensitive material where the intensities are recorded. Relatively newly developed CMOS/CPADS detectors are based on complementary metal-oxide semiconductors. Although they are not the newest, a lot of manufacturers still use them for small-type equipment. Nowadays detecting technology innovation is developed by individual manufacturers, for example Rigaku is using the hybrid photon counting, Bruker Instruments use detection based on mixed photon counting and Malvern PANanalytical uses 2D solid-state hybrid pixel detectors.

Fig. 5.6 Schema and real arrangement of single crystal diffractometer.\textsuperscript{11}

5.1.4 X-RAY POWDER DIFFRACTION

Many materials are in the form of powders, usually a polycrystalline material (differently oriented small crystals—crystallites) takes various

5 X-RAY DIFFRACTION ANALYSIS

positions with regards to the impacting photons. At every moment, at least some sets of planes are oriented so that Bragg's Law is met for a set of lattice planes. Diffraction always occurs under the characteristic diffraction angle $\theta$, so the angle $\theta$ can be changed to $2\theta$ and all possible diffraction from differently oriented crystallites can be detected. From each of crystallites the reciprocal $d_{hkl}$ forms a vector cone which is repelled as a cone of diffractions (Fig. 5.7).

Detection of diffracted X-ray radiation is based on several principles such as exposure of the photographic film, the ionisation of gases, luminescence of certain materials, and a change in conductivity etc. From historical point of view the first method was the Debye-Scherrer camera. A photographic film was spread along the perimeter of the oval chamber and the recorded circles corresponded to diffractions. This technique possessed poor resolution, so the innovation took place as Gandolfi and Gunier camera, respectively. The development of powder diffractometer was the boom from instrumental point of view. Generally, XPRD consists of radiation source, detector/counter and diffractometer (system of goniometer, slits and monochromator).

A typical representative of the powder diffractometer uses the Bragg-Brentano geometry. The mechanical assembly that makes up the sample holder, detector arm and associated gearing is referred to as a goniometer. The working principle of a Bragg-Brentano reflection goniometer is shown in the Fig. 5.8. The X-ray beam leaves the X-ray tube through the slits and hits the sample in the centre of the goniometer. The distance from the X-ray focal spot to the sample is the same as from the sample to the detector. The angle between the incident beam and the surface of the sample is $\theta$. The detector has an angle of $2\theta$ with respect

![Fig. 5.7 Scheme of powder diffraction.](image-url)
to the incident beam. With the simplest form of this setup, the X-ray source can be fixed and the detector is turned with the double angular velocity as the sample, so that the angle between the incoming beam and the surface equals the angle between the diffracted beam and the surface. A typical diffraction pattern consists of a plot of reflected intensities versus the detector angle.

The detection is similar as in single crystal: point or area detectors (gas filled/ionization transducers, scintillation counters, semiconductor transducers etc.) and again each of the manufacturers developed their own technology of data collecting. The crucial component of instrumentation is monochromator (i.e. \( \beta \)-filter) which cover that one of the planes meet the Bragg condition. The automatization of separate processes in diffractometer give the higher sensitivity and increased velocity of the measurements and possibility to full control of all components. The main impact of automatization take place in the accuracy of the diffraction angle measurement and using more intuitive algorithms to process digitized step scan data.

Fig. 5.8 Bragg-Brentano parafocusing geometry.
To work with the obtained data, the scattered intensity is plotted as a function of Bragg angle ($2\theta$) and the final form is called diffraction pattern or histogram. In some cases the plotted intensity can be used as a function of interplanar distance. Intensity is plotted as total number of counts or arbitrary units. To understand all information from the collected data is to see a pattern as a set of discrete peaks over a background. Background should be extracted from a pattern, but with respect to the information which it could provide, especially the crystallinity of the sample material. Pattern is simply consisted of intensities/peaks, positions and shapes which contain information about the measured sample and its crystal structure. The peak position is connected with the unit cell parameter information and sample absorption; the peak intensity is talking about atomic parameters or preferred orientation and finally the peak shape is connected with crystallinity, defects or disorders and sample grain size/stress/stain. The instruments parameters, for example coming from the algorithm calculations, could also influence the collected pattern. The unique peak positions appear due to scattering on periodic lattice and provide the information about interplanar distances and wavelength as a function of unit cell parameters. The peak intensities are related to many factors (structure, scale, multiplicity, Lorentz polarization, absorption, extinction, temperature factors etc.), which can give information about qualitative or semi-quantitative analysis. The calculated mathematical model (synonymically “fit”) of the measured peaks (shape, intensity) can give the quantitative information and also exact structural data which will be discussed further.

The quality of measured data is given by the measurements conditions, as type of scanning (step or continuous), scan rate, step size, scan range and type of divergence or Soler slits and sample preparation. The quality of the powdered sample preparation is also crucial. Ideally prepared sample is fixed in holder with circle geometry and radius 10 mm in 0.1 mm thick layer filled by particles sized around 10 μm. Powders are due to this restriction milled in automatic mills or ground manually in an agate mortar. In Fig. 5.9 typically used holders from stainless steel is shown. In the case of the low sample mass, the holders from silicon wafer should be also used.
5.2 DATA PROCESSING

Collected data is in raw form and the operator have to process them to obtain desirable information as phase composition (both qualitative and quantitative), precise lattice parameters and crystal structure (i.e. atom distribution and position in the unit cell, atomic displacement). Obtained data could be processed within various software given by manufacturer or commercially available (i.e. EVA, DQUANT, PolySNAP, HIGHSCORE, TOPAS, PDF ANALYSIS, PDXL). Firstly, the pre-processing before the real data analysis has to be done:

- Data are usually collected in more scans, so they need to be summarized in one pattern.
- The determination of background and its substraction.
- Data smoothing to suppress the statistical noise.
- $K\alpha_2$ stripping to remove the doublet peaks coming from single point scattering.
- Peak search: automatic or manual.
- Profile fitting (fit all marked peaks to form real envelope of experimental data).

Processed data are then compared with pattern databases: ICDD PDF databases from International central of diffraction data, NIST, ICSD, open crystallographic databases from COD or RRUFF Project and others. The database should be part of the installation packet from the instrument manufacturer. Within the pattern comparison phase composition can be found and in EVA or HIGHSCORE the semi-quantitative analysis is proceeded. But the information about crystal structure is still missing.

Quantitative analysis may be proceeding by several approaches: the absorption-diffraction method, method of standard addition (spiking), internal standard addition (typically used also for amorphous part determination), the reference intensity ratio, full pattern decomposition by Le Bail or Pawley techniques and Rietveld refinement.

Nowadays, the Rietveld refinement method is commonly applied for determination of crystal structure. The software as HIGHSCORE or TOPAS possesses the Rietveld refinement process in separated or complex calculation process for beginners and also advanced users.

5.3 APPLICATION OF XRPD IN PHARMACEUTICAL TECHNOLOGY

The X-ray powder diffraction is far more used in pharmaceutical manufacturing (formulation development, quality control) than the single crystal X-ray diffraction. The reason is, for instance, a high-speed rate of measurement, no need for single crystal preparation and the possibility of substance mixtures analysis. On the other hand, single crystal diffraction has a non-substitutable role in the precise determination of chemical structures of unknown substances. Usually, the XPRD is applied due to the heterogeneity of the measured substances.

5.3.1 IDENTIFICATION OF RAW MATERIALS

The commonly XRD application is the identification and verification of raw materials used in drug manufacturing. Simple and fast qualitative analysis provides a powerful tool in quality control. The identification is based on the principle that there are no two different substances having an identical diffraction record. Every crystalline phase is characterized by a unique number of diffraction lines, represented in the diffractogram by a specific position and intensity. If a sample consists of more phases, the diffractogram of the mixture will be a superposition of diffraction records of all components in the sample. There are several most intensive lines of the powder diffractogram, called “characteristic lines”, which are usually used for the identification of raw materials. Certain computer programs can be currently used for the identification facilitation (see chapter 5.2).
5.3.2 Drug Solid Phase Identification

From the diffractogram it is possible to determine whether the substance is crystalline or amorphous because the diffractograms of crystalline substances contain only peaks (Fig. 5.10). The XRD therefore cannot be used for amorphous samples. In practice, there are often mixtures which contain both crystalline and amorphous phases. In such case, it is possible to determine their relative representation or crystallinity of the sample (e.g. the determination of the content of amorphous filler and a crystalline component in a matrix forming a dosage form). The limitation of this method is that it is impossible to differentiate whether one of the substances (e.g. an API) is dissolved in the carrier or whether it is amorphous. Monitoring the crystallinity of APIs and excipients is important because a change in crystallinity of input substances or substances in the final medicinal product can influence the bioavailability of the API.

![Image of X-ray powder diffractograms](image)

**Fig. 5.10** X-ray powder diffractograms of an API in the crystalline and amorphous forms.

The XRPD is the most frequently used method for identifying crystalline modifications. Various polymorphic and pseudo-polymorphic forms and their mutual transformations play an important role in the development and production of dosage forms (see chapters 2.4.1.3, 3.3.3 and 4.3.2.3). Monitoring the crystalline state of APIs is very important because any change of the polymorphic form in the final product can influence the stability and bioavailability of the API. The control of the polymorphic form is necessary because transformations
can occur during the production (a change of crystallisation conditions, mechanical stress—tablet production, milling etc.) but also during storage (the effect of temperature and humidity). The XRPD is used in quality control of the produced crystalline phase and it enables checking the presence of undesirable crystalline impurities (mainly undesirable polymorphic forms). These crystalline modifications can be also in the final solid dosage form. The limitation in the analysis of mixtures is that in the diffractogram there must be an area where separate peaks occur, or a peak of one of the studied substances (polymorphic forms) occurs exclusively (Fig. 5.11).

![Diffractogram](image)

**Fig. 5.11** X-ray powder diffractograms of an API in two polymorphic forms.

### 5.3.3 Investigations of Drug-Excipient Interaction and Dosage Form Stability

The XRPD is an ideal tool for evaluating the compatibility of APIs and excipients during pre-formulation studies. In case of interaction between the individual components, the formed product will cause the sum of peaks on the diffractogram of the mixture to differ from the sum of peaks on diffractograms of pure components. A careful

---

selection of excipients is absolutely necessary for achieving the required release of an API or for preventing unexpected stability complications during the development of a new dosage form. The XRPD measurements expressed as the function of time, temperature and relative humidity allow controlling the stability of APIs, the presence of hydration/dehydration processes and characterisation of the entire formulation (e.g. correlating the XRPD data to the observed stability and drug release profiles). This analysis can be performed at any stage of the development.

5.3.4 QUANTITATIVE ANALYSIS

There are numerous analytical methods for the content determination that was mentioned above. Quantitative analysis is thus possible in a complex matrix (pharmaceutical formulations) containing even insoluble excipients. Methods which require dissolving of the analyte enable the determination of a total drug concentration in the dosage form but they do not differentiate the state of the drug in the dosage form, for example if the substance is dissolved or dispersed in the carrier, whether it is a crystalline or amorphous substance, etc. The commonly used technique to quantify some specific substances is spiking; the exact amount of substance’s pure form is added and a calibration curve is made to determine the substance's concentration in sample. The XRPD have also been paid attention to as important tools for PAT. When two or more components with broad and overlapping XRD peaks are present in one formulation, the use of chemometrics (see chapter 2.3) is useful to evaluate multi-components in pharmaceutical formulation.

5.4 REFERENCES


6 SOLID-STATE NMR SPECTROSCOPY

Currently, solid pharmaceutical dosage forms (tablets, capsules, granules, etc.) represent approximately 80–90% of the drug market. Similar to other dosage forms, solid dosage forms can also be described in simple terms as an API-excipient two-component system, predominately of solid particles. Dosage form formulation, properties and eventual in vivo performance may be influenced by many factors, including interactions between substances (API-excipient, API-API; excipient-excipient), miscibility of the individual components, their rates of solidification and crystallization, and the synthetic and manufacturing procedure (freeze-, vacuum- and spray-drying). The majority of the aforementioned factors comprises particle properties, which are closely related to their structure. These properties (mechanical, electrical, optical and magnetic) often differ considerably depending on whether the materials are amorphous or crystalline and/or what type of crystallographic structure they possess. It is therefore of great importance to examine the structural characterization of the components used.

One of the most potent reasons for the detailed structural characterization of drug products results from the existence of the unpredictable polymorphism or extent of crystallinity of APIs or excipients because an undesirable form of the solid phase may be ineffective or even detrimental to the patient. The resulting solid phase structures considerably influence the incorporation and crystal structure of APIs in drug formulations. In addition, the stability and bioavailability of the APIs in drug formulations often depend upon the selection of excipients and their concentration levels and different combinations as well as their structures and interactions between APIs and excipients. Detailed structural characterizations of drug formulations are, thus, in the interest of protecting the patients.

Because solid APIs and excipients often exist at the borderline between the crystalline and amorphous phases and/or API content in the solid dosage form is usually low (often less than 5%), high-quality XRD, IR and Raman spectral data are not often suitable for precise structural analysis. Therefore, describing the structures of these materials
at the atomic resolution is extremely difficult and requires the development of new and effective approaches. One of the most promising approaches to resolving the problem of structural characterization of APIs and the corresponding drug formulations lies in using solid-state nuclear magnetic resonance spectroscopy (ssNMR).

In general, NMR is a spectroscopic absorption method based on the interactions of radio frequency electromagnetic radiation (tens of hundreds of MHz) of atomic nuclei (with magnetic moments) of the analyzed molecules in a strong homogeneous magnetic field. The method is very sensitive to the features of molecular structure because neighboring atoms considerably influence the NMR signals of individual nuclei. This sensitivity is a key parameter for determining not only the composition and primary structure of the investigated substances but also the reconstruction of their complete 3D structure and crystal arrangement. Consequently, NMR spectroscopy is a noninvasive, nondestructive, atom/isotope selective method that provides information on a microscopic, atomic-level scale. Currently, NMR has been used in all scientific disciplines, i.e. chemistry, biology, medicine, physics and material research, and the basic principles of NMR spectroscopy are the same for solution- and solid-state measurements. However, to obtain high-resolution solid-state NMR spectra with well-resolved signals, the standard methods of solution-state NMR have been modified to overcome two major obstacles that considerably complicate the measurement of high-resolution spectra in solid-state NMR. First, severe line broadening results from the very strong anisotropic interactions of nuclear spins with static magnetic fields, such as chemical shift anisotropy (CSA) and dipole-dipole interactions. Second, relatively low sensitivity of the applied experiments results from very slow relaxation of heteronuclei. This fact results from hindered segmental and molecular mobilities in the solid state.

Nevertheless, ssNMR spectroscopy, with its advanced techniques, is a powerful method capable of providing information both about the structure of materials and about the dynamics of processes occurring within them. In principle, a wide range of different experimental methods within ssNMR spectroscopy can be used. In particular, for pharmaceutical research, a range of experimental strategies, including analysis of solid forms (polymorphs, solvates), hydrogen bonding, crystal packing and/or solid-solid interactions (phase transformations, activation energies
of molecular motions), have been developed and successfully applied. Solid-state NMR spectroscopy is also a technique that complements X-ray diffraction crystallography in many respects. This is given by the fact that the ssNMR has the unique ability to probe electron environments of specific nuclei in the solid state over a large timescale without the requirement of single-crystal substrates or even homogeneous samples. Thus, ssNMR spectroscopy is suited not only for identifying different solid forms of drugs but also for providing detailed structural information useful for rationalizing the physical properties of drug forms in terms of molecular and crystal structures.

6.1 THEORETICAL BASICS OF NMR SPECTROSCOPY

NMR-detectable nuclei are only those with nonzero nuclear spin quantum number \( I \) \((I \neq 0)\), which can be either half-integers \((\frac{1}{2}, \frac{3}{2}, \frac{5}{2} \text{ etc.})\) or integers \((1)\). The principle of quantum mechanics determines that a nucleus of spin \( I \) will have \(2I + 1\) possible orientations; thus, a nucleus with spin \( \frac{1}{2} \) will have two possible orientations. In the absence of an external magnetic field, these orientations are of equal energy. If a homogenous magnetic field is applied, then the energy levels split in accordance with its magnetic quantum number \((m)\). The most easily measurable nuclei have a spin of \( \frac{1}{2} \), such as \(^1\text{H}, \, ^{13}\text{C}, \, ^{15}\text{N}, \, ^{31}\text{P}, \, ^{29}\text{Si}, \) and \(^{119}\text{Sn}\). For the nuclei with spin \( I = \frac{1}{2} \), \( m = \pm \frac{1}{2} \), these nuclear spins occupy two orientations (parallel and antiparallel with the direction of a static magnetic field), and the originally degenerated energy level splits into two energy levels \((m = \pm \frac{1}{2})\). The spins with the antiparallel orientation relative to the external magnetic field have higher energy and are somewhat less populated than the spins with the same (parallel) orientation. This difference in the populations of nuclear spin on energy levels follows the Boltzmann distribution and generally increases with decreasing temperature and increasing strength of the magnetic field. The existence of a nonzero nuclear spin is associated with another significant nuclear property, namely, the nuclear magnetic moment \( \mu \), which is a vector given by gyromagnetic ratio \( \gamma \), a constant characteristic for each nucleus. Different nuclei, therefore, have different magnetic moments. Because these differences are very large, the resonance frequencies \( v \) of distinct
nuclei differ considerably. The relationship between the resonance frequencies \( v \) and the external magnetic field \( B_0 \) is (Eq. 6.1):

\[
v = \frac{\gamma \times B_0}{2\pi}
\]  

(6.1)

For instance, at a strong magnetic field, \( B_0 = 11.7 \) T (Tesla, unit of magnetic field strength), the resonance frequency of \(^1\)H is 500 MHz, while the corresponding resonance frequency of \(^{13}\)C is 125 MHz. Consequently, the NMR signals of different isotopes (nuclei) do not overlap, and the corresponding NMR spectra can be measured separately.

For applications of NMR spectroscopy in structural chemistry, it is quite significant that binding and nonbinding electrons placed in the strong external magnetic field induce additional local magnetic fields \( B_{loc} \). Consequently, the observed nucleus is exposed to the influence of a magnetic field that is slightly larger or smaller in comparison with the external magnetic field \( B_0 \). The resulting magnetic field is the so-called effective magnetic field \( B_{eff} \). The relationships between \( B_0 \) and \( B_{eff} \) can be expressed by using the shielding constant \( \sigma \) (Eq. 6.2). The shielding constant of a given nucleus strongly depends on the chemical structure, which is related to the electron density.

\[
B_{eff} = B_0 - B_{loc} = B_0 \times (1 - \sigma)
\]

(6.2)

\[
\sigma = \sigma^{dia} + \sigma^{para} + \sigma^i
\]

(6.3)

The total effect of chemical shielding can be divided into three contributions (Eq 6.3): i) a diamagnetic component \( \sigma^{dia} \) that originates in \( s \) orbital electrons and is in the opposite direction to the static magnetic field; ii) the paramagnetic component \( \sigma^{para} \) (generated by the electrons of the \( p \) orbitals and the \( \pi \) electrons) which is in the identical direction to the static magnetic field; and iii) the contribution \( \sigma^i \) reflecting the influence of nearby atoms and functional groups (induction or mesomeric effects). This effect can be both positive and negative. Consequently, this contribution \( \sigma^i \) is extremely important because it causes the same nuclei in one molecule to have different chemical shifts. The chemical shift \( \delta \) (ppm) is defined as the resonance frequency of a nucleus relative to a standard in a magnetic field (Eq. 6.3) and used for describing the positions of NMR signals in the recorded spectra and is expressed in parts per million (ppm). In practice, a resonance frequency shift is caused by shielding. In equation (Eq. 6.4), \( v_{sample} \) is the absolute resonance frequency of the sample, and \( v_{ref} \) is the absolute
resonance frequency of a standard reference compound, measured in the same applied magnetic field $B_0$:

$$\delta = \frac{\nu_{\text{sample}} - \nu_{\text{ref}}}{\nu_{\text{ref}}}$$  \hspace{1cm} (6.4)

As a standard sample, a chemically stable substance is used. Usually, it is tetramethylsilane (TMS) with the chemical shifts of $^1\text{H}$, $^{13}\text{C}$ and $^{29}\text{Si}$ defined as $\delta = 0$ ppm. The position and number of signals in the NMR spectrum reflects the structure of a molecule. In other words, due to the induction contribution ($\sigma^i$), one can clearly resolve signals of various functional groups and units, such as $-\text{CH}_3$, $-\text{CH}_2-$, $>\text{CH}-$ or $>\text{C}<$ in an NMR spectrum. It is worth noting that these local magnetic fields are very weak, which is the reason for the corresponding chemical shielding $\sigma$ having been expressed in parts per million (ppm) of the basic carrier frequency (for example, 500 MHz for $^1\text{H}$ nuclei at 11.7 T).

### 6.1.1 Nuclear Interactions in ssNMR Spectroscopy

In general, nuclear spin interactions with the static magnetic field generated by a superconducting magnet are anisotropic and depend on the orientation of the molecule relative to the direction of the external magnetic field. Very fast molecular motions and reorientations of the molecules in the solution-state lead to the averaging of all possible orientations and measurable values during the time window of the NMR experiment (several milliseconds up to seconds). Therefore, the only measurable NMR quantity in the solution-state is the isotropic contribution of the NMR chemical shift. However, even small peptides that are associated with the phospholipid membrane are so immobilized that the molecular orientations in the time window of the NMR experiment are not averaged. Consequently, the resulting NMR spectrum contains broad and often asymmetric signals. The observed broadening and signal asymmetry reflect the anisotropic nature of nuclear spin interactions.

The most important anisotropic interaction in the solid state is the chemical shift anisotropy, which can be described by the 2nd rank tensor and can be represented by an ellipsoid, as demonstrated in Fig. 6.1. Theoretically, for a single nucleus and the given orientation of the tensor, the chemical shift value recorded in the NMR spectrum corresponds to the cross-section that is created by the vector of static magnetic field through this ellipsoid. In the resulting spectrum, a single signal is detected
(this situation can occur when a single crystal is subjected to NMR spectroscopy measurements), and the frequency position of this signal depends on the tensor orientation. In reality, however, in powdered, non-oriented solid samples, all possible tensor orientations exist, and each orientation produces its own specific NMR signal. As a result of the solid character of the measured sample, the generated signals mutually overlap and form a frequency continuum. Thus, the resulting $^{13}$C NMR spectrum recorded even for a very simple system, such as crystalline glycine, is difficult to interpret because it consists of broad and asymmetric signals (Fig. 6.2). The signal centered at approximately 170 ppm corresponds to the C=O unit, whereas the CH$_2$ group resonates at approximately 50 ppm. The observed anisotropic broadening of the signals (powder pattern) then reflects the existence of multiple orientations of the crystallites and molecules in the static magnetic field. His spectrum thus represents an envelope of individual signals of the corresponding crystallites. It is clear, that for substances consisting of a large number of carbon atoms, the resulting powder pattern in NMR spectra is beyond the direct structural interpretation. Therefore, the primary aims of the advanced techniques of solid-state NMR spectroscopy are the removal and suppression of the effect of chemical shift anisotropy and the simplification of ssNMR spectra.

Fig. 6.1 Graphical presentation of chemical shift tensor.
Fig. 6.2 Static $^{13}$C NMR spectrum of glycine as an example of spectral inhomogeneous broadening. The shapes of the signals are created by the overlap of many signals as each crystallite has individually contributed its own NMR frequency to the resulting spectrum.

Other types of anisotropic interactions, which cause broadening of solid-state NMR spectra, are represented by *dipole-dipole interactions* between atomic nuclei. These interactions are observed in spin pairs and larger spin clusters. In principle, each nucleus represents a nuclear magnetic moment that produces a weak magnetic field. Neighboring nuclear spins up to a distance of approximately 5 Å will then interact with this magnetic field. Subsequently, their resonance frequencies are more or less modified, modulated and changed. This fact is demonstrated for two orientations of the $^{13}$C–$^1$H spin pair in Fig. 6.3. If the orientation of the $^{13}$C–$^1$H spin pair is parallel with the direction of the static magnetic field, the frequency modulation is the strongest, whereas if the orientation is perpendicular, the frequency modulation is opposite, and its amplitude is halved. The zero-frequency modulation is reached when the internuclear vector adopts an angle of 54.7° relative to the direction of the static magnetic field. As mentioned above, the powdered solid samples consist of a wide range of randomly oriented crystallites, in which the internuclear vectors of the spin-pairs (e.g., $^{13}$C–$^1$H in the –CH group) remain invariant. Consequently, each crystallite and each spin-pair orientation contributes to the resulting NMR spectrum by its own signal. The signal is then considerably broadened and basically featureless (Fig. 6.3, right side). When considering dipole-dipole interactions, homonuclear interactions between the nuclei of the same type, usually $^1$H–$^1$H, and heteronuclear interactions...
between different nuclei, e.g. between $^1\text{H}$ and $^{13}\text{C}$, $^1\text{H}$ and $^{15}\text{N}$, and $^1\text{H}$ and $^{29}\text{Si}$, can be distinguished. Many techniques have been developed to suppress the interactions in solids that provide high resolution ssNMR spectra (chapter 6.2.).

![Graphical representation of the heteronuclear dipole-dipole interaction between nuclear magnetic moments $^{13}\text{C}$ and $^1\text{H}$.

**Fig. 6.3**

**6.2 INSTRUMENTS AND MEASURING TECHNIQUES USED IN ssNMR SPECTROSCOPY**

Three experimental techniques have been developed and optimized to remove the effects of the abovementioned anisotropic interactions, to reach the high-resolution ssNMR spectra and to enhance the sensitivity of NMR. The techniques are magic angle spinning (MAS), high power heteronuclear decoupling (HPHD), and cross-polarization (CP). All these techniques can be combined together into an experiment known as cross-polarization magic angle NMR experiment, e.g. $^{13}\text{C}$ CP/MAS NMR and correlation spectroscopy.

**6.2.1 MAGIC ANGLE SPINNING**

During a classical solution-state NMR experiment, Brownian motion leads to the averaging of all molecular orientations. Consequently, the corresponding anisotropic interactions are averaged to their isotropic values. In such cases, the anisotropic interactions can be neglected on the timescale of the NMR experiment. However, anisotropic interactions have a substantial influence on the behaviour of a spin system in a solid-state NMR experiment. As it is impossible to achieve isotropic molecular motion in the solid state, an alternative approach has been developed. One possible way to compensate for the missing molecular
motion is mechanical uniaxial rotation. The anisotropic interactions, such as dipolar coupling between a pair of nuclei or chemical shift anisotropy, can be described by the 2nd rank tensor as mentioned above. As the sample rotates around the axis, which is inclined at an angle of 54.7° (magic angle = diagonal of a cube) with respect to the static magnetic field, anisotropy interactions and the corresponding spectral broadenings are averaged to zero (Fig. 6.4). As the anisotropic interaction is refocused at the end of each rotor period (i.e. CSA), the originally broad static NMR signal is easily broken up into a sharp central signal reflecting the isotropic chemical shift and a series of spinning sidebands separated by the rotation frequency $v_r$ (Fig. 6.5 and 6.6).

**Fig. 6.4** Graphical representation of MAS, which has the dimensions of diagonal of cube. The sample (cylinder) is rotating at a high frequency inside the main magnetic field ($B_0$). The axis of rotation is tilted at the magic angle, $\theta_m = 54.7°$, with respect to the direction of $B_0$.

**Fig. 6.5** Comparison of static $^{13}$C NMR and $^{13}$C MAS NMR (the data were measured using MAS).
Fig. 6.6 $^{13}$C NMR (left) and $^1$H NMR (right) spectra of $^{13}$C selectively labelled (C=O) glycine measured at various MAS spinning frequencies.$^{14}$

6.2.2 HIGH-POWER HETERONUCLEAR DECOUPLING

Similarly, the elimination of dipolar interactions may be achieved by fast magic angle spinning, but this requires a very fast rotation frequency of the sample to exceed several times the magnitude of these interactions, whose typical value in the chemically bound $^1$H–$^{13}$C pair is nearly 30 kHz. Thus, to remove the dipolar effect of $^1$H atoms on low isotopic nuclei (e.g. $^{13}$C, $^{15}$N), the irradiation of the $^1$H spin system must be carried out by a continuous radiofrequency field during the detection of the NMR signal. This dipolar decoupling (DD) techniques is based on the fact that the $^1$H nuclei with spins oriented parallel and antiparallel to the external magnetic field have exactly opposite effects on the frequency of other nuclei, i.e. the $^{13}$C nuclei. The application of the radio frequency field causes very rapid jumps between these orientations; thus, the time-average orientation of the $^1$H magnetic moment is close to zero, and the effect of the dipolar interactions is eliminated.

6.2.3 CROSS POLARIZATION

The problem of low sensitivity of dilute spin $\frac{1}{2}$ nuclei such as $^{13}$C (low isotopic content and low gyromagnetic ratio) is resolved by the CP technique. The given pulse sequence was introduced by Pines, Gibby

and Waugh. The basic CP pulse sequence for $^{13}$C–($^1$H) is shown in Fig. 6.7 (a). With this technique, the $^1$H and $^{13}$C spins are brought into resonance by the application of two spin-locking fields, the magnitude of which will satisfy the Hartmann-Hahn matching condition ($\gamma_H B_{1H} = \gamma_C B_{13C}$). When the correct spin-locking fields are chosen, thermal contact is established, and a polarization transfer will occur between the abundant spins ($^1$H) and the rare spins ($^{13}$C) to which they are coupled. The polarization transfer process allows the rare spins ($^{13}$C) to take on the magnetization and relaxation behaviour of the abundant spins ($^1$H), leading to a sensitivity enhancement (up to four times based on the ratio of the $^1$H and $^{13}$C gyromagnetic ratios) and a reduction of the pulse repetition time.

![Fig. 6.7 Block representation of a CP pulse sequence and a model $^{13}$C spectrum (a) and a 2D pulse sequence showing heteronuclear correlations between nuclei $^1$H and $^{13}$C (b). In the representation of pulse sequences in NMR spectroscopy ▀ is commonly used for DD, ▼ for illustrating pulses and ▶ for detecting (acquisition (AQ)) FID.](image)

**6.2.4 CORRELATION SPECTROSCOPY**

High resolution one-dimensional (1D) NMR spectra (e.g. $^{13}$C CP/MAS NMR) give information on isotropic chemical shifts. However, the removal of anisotropic interactions is accompanied by the loss of important structural information. Therefore, experimental techniques that allow the separation or correlation of isotropic and anisotropic features were designed. For this reason, the measurement
of two-dimensional and multidimensional spectra is not just a fashionable matter it is a necessity, as multidimensional spectra significantly increase the spectral resolution and information pithiness of NMR experiments. The principle of 2D spectrometry can be illustrated by a simple example of a heteronuclear correlation between the $^1\text{H}$ and $^{13}\text{C}$ nuclei, which originates from a pulse sequence designed to measure the cross-polarization spectra (Fig. 6.7 (b)). Consider a system in which the $^1\text{H}–^1\text{H}$ dipolar interaction is very weak (hydrogen atoms are very distant from each other). When measuring the classical one-dimensional spectra, the response of the sample after the excitation is detected, e.g. after the CP. This response is the free induction decay (FID) that is induced in the detection coil; following Fourier transform, these oscillations provide a classical 1D frequency spectrum. Fourier transform is a function of frequency. To obtain a 2D NMR spectrum, several FIDs with time increments between two pulses are detected, as shown in Fig. 6.7 (b) as parameter $\Delta t_1$. Several FIDs provide the same number of 1D spectra with different signal intensities of peaks related to $\Delta t_1$. The final 2D spectrum is obtained using double FT processing of raw data, which provides the advantage information about the investigated system (chemical bonds, structural angles and distance).

6.2.5 NMR INSTRUMENT COMPOSITION

The basic elements of a typical NMR spectrometer consist of the main parts: i) a superconducting magnet, which generates a powerful magnetic field that is tens of thousands of times stronger than the earth's magnetic field (from 5 T to 30 T) (Fig. 6.8 (a)), ii) a spectrometer (console), which transmits and receives radio-frequency waves used to make the NMR measurements (Fig. 6.8 (b)), and iii) a computer, which is an instrument to control and process data (Fig. 6.8 (c)).
The measured sample is placed into a ZrO$_2$ rotor (Fig. 6.9). The choice of the ZrO$_2$ rotor type is dependent on the type of measurement, spinning rate and amount of accessible sample. After filling, the sealed rotor is placed into the probehead with a detecting coil, and the probe head is inserted into the superconducting magnet. After tuning the measurement frequency and setting the pulse program, it is finally possible to collect data. The sample is placed within this magnetic field and exposed to radio waves.

6.2.6 THE BASIC SPECIFIC FEATURES OF SSNMR SPECTRA
The result of the measurement is the NMR spectrum, which is necessary to interpret. The primary pieces of the information available from the NMR spectrum are the chemical shift, number of signals
and area under each signal (Fig 6.10). The chemical shift is dependent on the local electronic environment of the molecule and is very sensitive to slight changes in the molecular conformation. The number of signals (peaks) reflects how many kinds of atoms are present in the sample. The area under each signal tells how many atoms of each kind are in the molecule.

The NMR line width also provides additional information about the system. Amorphous line widths are approximately an order of magnitude broader than those of crystalline lines. The widths of the crystalline line can further specify the morphology of the system. The distortions from the symmetrical shape of the spectral peak can also be significant. For example, $^{13}$C lines will be asymmetric due to the coupling to $^{14}$N. Relaxation times reveal information about the dynamics of the system, especially as a function of temperature. In addition, relaxation times of crystalline materials have been correlated with particle size and may have implications for predicting functional properties, such as chemical stability and dissolution rate. Finally, two-dimensional NMR techniques are used to show connectivity between drugs and excipients as well as assigning peaks in the solid-state, which is useful for determining the miscibility of amorphous dispersions.

![13C CP/MAS NMR spectrum of glycine](image)

**Fig. 6.10** $^{13}$C CP/MAS NMR spectrum of glycine; the two narrow signals correspond to the two types of C atoms in the molecule of crystalline glycine: $\delta$(CH$_2$) = 43.39 ppm and $\delta$(COOH) = 176.03 ppm.

### 6.3 Application of ssNMR Spectroscopy in Pharmaceutical Technology

Currently, ssNMR spectroscopy has an important position as a technique for the structural characterization of APIs and pharmaceutical systems. Structural analyses are required by pharmaceutical regulatory authorities
(i.e. EMA, FDA). Furthermore, when a new API is discovered, it is important to characterize the solid-state properties of both the bulk API and potential formulations as these properties can have a dramatic effect on dissolution rate, bioavailability, and stability (both chemical and physical).

In pharmaceutical development, ssNMR spectroscopy is mainly used:

- To reveal a difficult predictable polymorphism.
- To determine the bioavailability vs. the crystal modification (patient protection).
- To solve and prevent patent disputes (manufacturer's protection).
- To analyze crystals with difficult preparations for X-ray structure analysis.

Advantages of ssNMR spectroscopy:

- Nondestructive method (minimum requirement for sample preparation is the only careful crushing (approximately 10–50 mg) and minimal effect on the devastation of sample).
- Selective measurement of each nucleus ($^{13}$C, $^{15}$N, $^{31}$P, $^{19}$F, $^1$H).
- Measurements are independent of the crystallinity or amorphousness of the sample.
- Measurements are quantitative.
- Measurements provide information about the structure, mobility and degree of disorder.
- Separate characterization of both APIs and excipients and their mutual relationships.

Levels of uses of ssNMR spectroscopy:

- Standard analysis of API: $^{13}$C CP/MAS NMR includes i) the qualitative identification of crystalline and amorphous forms of API and excipient (polymorphs), as well as solvates and hydrates and ii) the quantitative determination of the proportion of solid forms, for example crystalline and amorphous material, within a sample or the quantitative determination of phase impurities.
- Comprehensive API analysis: identification and resolution of salts, bases or cocrystals; localization of $\text{H}^+$ ions; and temperature characteristics.
6.3.1 REPRESENTATIVE CASES

Important and basic applications of ssNMR spectroscopy in pharmaceutical development, such as the identification of crystal and amorphous phases in drug substances and solid oral dosage forms, are demonstrated in representative cases below. Generally, XRD is regarded as a basic and usually cost-effective technique for studying polymorphisms. However, the small amount of APIs and the presence of excipients in drug formulations complicate the interpretation of their X-ray patterns and the determination of their crystal structure. The X-ray record is quantitative, and signals of APIs are often overlapped by the signals of predominating excipients. In contrast with X-ray diffraction, a suitable ssNMR method can be chosen and adjusted to find a relevant part of the spectrum, which relates either with APIs or with excipients. According to this part of the spectrum, it is then very easy to determine the given crystal form of APIs or crystal phase of excipients. For this reason, the main advantage of ssNMR spectroscopy over other techniques is its selectivity.

6.3.1.1 Specific example 1

Spectra in Fig. 6.11 (a) and Fig. 6.11 (b) show significant differences between crystalline and amorphous forms of simvastatin. The narrow and broad signals in $^{13}$C CP/MAS NMR spectra are typical of crystalline and amorphous forms of simvastatin, respectively. Each signal in the spectrum corresponds to one chemically nonequivalent carbon labeled by the individual number (letter) in the given molecular scheme. Considerable structural changes of simvastatin upon the formation of a solid dispersion with poly(N-(2-hydroxypropyl)methacrylamide) (pHPMA) are observed in the $^{13}$C CP/MAS NMR spectra (Fig. 6.11 (c)). Predominantly, the observed broadening of the simvastatin signals clearly
indicates it is completely amorphized, and no residues of crystalline simvastatin were found in the simvastatin/pHPMA solid dispersion.

Fig. 6.11 $^{13}$C CP/MAS NMR spectra of Neat crystalline (a) and Amorphous simvastatin (b). The Sim_pHPMA solid dispersion (c) and Neat pHPMA (d). The rectangle shows part of the spectra that unambiguously determines the API in solid dispersion (drug formulation).$^{15}$

6.3.1.2  **Specific example 2**

The next illustrative case is a study of the olanzapine polymorphism (Fig. 6.12). There are observed differences in the $^{13}$C CP/MAS NMR spectra of typical polymorphs and solvates. The spectra of several crystal forms of olanzapine, including an anhydrate (Form I), polymorphic dihydrates (B and D), and an EtOH–H$_2$O solvate, are shown in Fig. 6.12. The weak peaks are spinning sidebands, which, as previously mentioned (see chapter 6.2), arise from insufficient sample spinning relative to the CSA of the $^{13}$C nuclei. The remaining peaks reflect the isotropic resonances of the drug, and in the case of the EtOH–H$_2$O solvate, ethanol. Polymorphism (or solvate formation) may be inferred by the different isotropic chemical shifts of equivalent $^{13}$C nuclei. Organic solvents, such as ethanol, can usually be detected by $^{13}$C ssNMR spectroscopy; however, water is observed only indirectly by its influence on the $^{13}$C environments of the drug.

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**Fig. 6.12** $^{13}$C CP/MAS NMR spectra of neat crystalline forms of olanzapine: anhydrous Form I, dihydrate B, dihydrate D and EtOH–H$_2$O solvate.$^{16}$

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6.3.1.3 Specific example 3

Solid-state NMR spectroscopy is also sensitive to the size of the particles (domains) in the sample being analyzed. In the literature, it has been shown that a decrease in the particle size of several compounds may lead to a decrease in their proton spin-lattice relaxation ($T_1^1H$). The experimental approach is based on the measurement of $^{13}C$-detected $^1H$ spin-lattice relaxation times ($T_1(^1H)$). The $^1H$–$^1H$ spin diffusion is a fast magnetization transfer over large distances that occurs during the relaxation periods and induces equilibration of the $^1H$ magnetization behaviour of different nuclear spins representing different molecules or components. Very simply, if the recorded $T_1(^1H)$ relaxation times of different components in a multicomponent system differ considerably, then the system is phase-separated from the large domains, the size of which usually exceeds 100–500 nm. Conversely, if the recorded $T_1(^1H)$ relaxation times of these components are similar or identical, then the system is rather homogenous, with sizes of domains smaller than 10 nm. In general, the rate of magnetization equilibration reflects the extent of phase separation in multicomponent and multiphase systems.

6.3.1.4 Specific example 4

The instance of comprehensive structural research is the study of mucoadhesive buccal films, where the active substance was ciclopirox olamine (CPX), which has been incorporated into a polyether oxide (PEO) matrix. An unusual behaviour of the CPX/PEO systems was observed. Systems containing a small amount of active ingredient (CPX) show rapid drug release and loss of mechanical properties, whereas high CPX systems show the desired delayed drug release. The elasticity and plasticity of the material with high CPX content is surprisingly high. By combining different types of solid-state NMR experiments, materials with low and high CPX concentrations are dramatically different in their structure. The results of ssNMR spectroscopy are schematically summarized in Fig. 6.13. Low CPX content leads to two-phase nano-heterogenic films. In one phase, the active substance is molecularly dispersed in an amorphous PEO matrix. The second phase consists of a small amount of polymer crystals seeded in the amorphous phase (Fig. 6.13 (a). On the other hand, systems with a high CPX content exhibit a pseudo-cocrystalline solid dispersion architecture, where the drug crystallizes in the interlayer space of the polymer matrix crystals. These
films also contain a very small amount of the amorphous phase, which, in combination with the surface interactions of the crystals of the active substance and the polymeric matrix cause both an increase in mechanical resistance and a delay in the release of the drug. It is therefore a combination of two factors that define the structural and physicochemical properties of these systems: a) the accessibility and affinity of suitable molecular sites for forming a polymer-drug interaction with hydrogen bonding and b) the tendency of polymer chains to form a highly crystalline phase. Thus, it is clear that the bioavailability of the drug can be effectively controlled by the targeted structuring of mucoadhesive buccal films.

Solid-state NMR spectroscopy has many applications in the characterization of pharmaceutical solids. Recent publications have highlighted the capability of ssNMR to identify polymorphs, provided quantitative data in both API and dosage form, and studied complicated formulations such as amorphous dispersions.
Fig. 6.13 Schematic representation of two distinct types of CPX/PEO mucoadhesive buccal films: The low-CPX-loaded film CPX0.2/PEO (a) and The high-CPX system CPX1.8/PEO (b).\textsuperscript{17}

6.4 References


7 DISSOLUTION TESTING

A dissolution test is used for the determination of a drug release from the tested dosage form in a prescribed liquid medium during a time course (so called in vitro testing). The result of this test is a drug dissolution profile which belongs among one of the most significant characteristics of a dosage form.

The principle of dissolution is a transfer of particles from a solid substance to a liquid medium. At the beginning of the process, dissolving particles are solvated by a thin layer of a solvent. Consequently, the solvated particles leave the solid phase and cumulate in the marginal layer of the solvent where a small layer with saturated concentration \( C_s \) is created. Particles are further transported through the diffusion layer to the whole volume of the dissolution medium. The concentration in the diffusion layer gradually decreases to the value of \( C_t \), which corresponds to the concentration in the whole solvent volume. This is a principle of a concentration gradient.

The first formal description of dissolution was given by Noyes and Whitney (1897) who compiled so-called Noyes-Whitney equation (Eq. 7.1) for the calculation of the dissolution rate of solid particles in a liquid solvent based on Fick’s First Law:

\[
\frac{dW}{dt} = \frac{D}{h} \times S \times (C_s - C_t) \tag{7.1}
\]

where \( dW/dt \) is the dissolution rate, \( D \) is the diffusion coefficient, \( S \) is the area of the interphase of a solid and liquid phase, \( h \) is the diffusion layer thickness and \( (C_s - C_t) \) is the concentration gradient of the diffusion layer.

The concentration gradient remains stable in open systems (a dissolution apparatus with flow-through cell). Therefore, it is possible to speak about so-called sink conditions under which the dissolving substance is only in contact with a pure solvent. Such conditions best correspond to in vivo conditions where the dissolved drug is absorbed. The concentration gradient gradually decreases by dissolution of the drug in the closed systems under so-called batch conditions (dissolution apparatus with a paddle, basket and a reciprocating cylinder). To get as close as possible to the sink conditions, the closed system should...
be sufficiently distant from the equilibrium ($C_t < 0.1 \ C_s$). The volume of the dissolution medium should be at least 3 to 10 times the volume of saturated solution.

The procedure and the basic rules of the dissolution test are described in the current European Pharmacopoeia (Ph. Eur.) for the following dosage forms: single solid dosage forms (tablets, capsules, coated tablets, coated capsules; it can be applied also to modern multiple dosage forms, such as pellets, microparticles, etc.), transdermal patches, chewing gums and lipophilic dosage forms. The dissolution test can be used for both immediate- and modified-release dosage forms to reveal a type and a manner of drug release. According to Ph. Eur. three different types of modified-release dosage forms are distinguished (Fig. 7.1): prolonged-release dosage form showing a slower drug release than a conventional-release dosage form administered by the same route, pulsatile-release dosage form showing a sequential, intermittent drug release, and delayed-release dosage form showing the drug release onset adjusted to take place after a specific time or at a specific location in the gastrointestinal tract.

Dissolution test parameters, such as bath temperature, pH of a dissolution medium, its composition and ionic strength or a type and a concentration of used enzymes, should be selected to mimic real conditions in an organism (in vivo). The dissolution test provides the first information about potential behaviour of the dosage form in the human and animal organism and reveals whether the theoretical presumptions reflecting a dosage form concept are correct or not.

![Types of controlled drug release.](image)

**Fig. 7.1** Types of controlled drug release.
7.1 **Dissolution Test Equipment and Conditions**

According to the Ph. Eur., a compendial apparatus for solid dosage forms testing with paddle, basket and reciprocating cylinder can be used, or in special cases, a device with a flow-through cell is employed. The first two mentioned methods are the most frequently used. The type of method is chosen based on properties of the tested dosage form. The dissolution test by the paddle method is often used for tablets and the basket method can be used for multiple dosage forms. The methodology of dissolution test has been harmonized with the valid version of the U.S. Pharmacopoeia (USP I—basket, USP II—paddle, USP III—reciprocating cylinder, USP IV—flow-through cell). The Japanese Pharmacopoeia does not contain the use of the reciprocating cylinder and the flow-through cell.

For completeness, other three USP compendial methods: USP V—paddle over disc, USP VI—cylinder, USP VII—reciprocating holder were developed mainly for evaluation of transdermal patches. Similarly, the disk assembly method, cell method and rotating cylinder method are specified in Ph. Eur. for these dosage forms.

7.1.1 **Instrumentation**

7.1.1.1 *Basket apparatus*

The assembly consists of a cylindrical vessel with a hemispherical bottom with a volume of 1000 ml which can be covered with a lid made of glass or another transparent inert material, a motor, a stainless-steel drive shaft which ensures a fluent rotation without vibrations, and a stainless-steel cylindrical basket (stirring element). The vessel is partially immersed in a suitable water-bath of any convenient size or can be heated by a suitable device such as a heating jacket. The equipment permits maintaining the temperature inside the vessel during testing at 37 ± 0.5 °C and a permanent fluent movement of the dissolution medium. No part of the equipment should prevent a fluent movement of the mixing unit. A speed-regulating device is used to allow the shaft rotation speed to be selected and maintained at a specified rate, within ± 4 %. An apparatus that permits observation of the dosage form and stirring element during the test is preferable.
7.1.1.2 **Paddle apparatus**
The equipment is identical with the one mentioned above, except that a paddle formed from a blade and a shaft is used as the stirring element. A floating dosage form is kept at the bottom of the vessel using a suitable device called sinker, such as a wire or glass helix. A dosage form with a content of swelling polymer has often a tendency to stick on the bottom of the dissolution vessel. This fact can significantly influence the drug dissolution profile. In this case, it is also suitable to place this dosage form into the wire helix.

7.1.1.3 **Reciprocating cylinder**
The assembly consists of a set of glass cylindrical vessels with a flat bottom, a set of glass reciprocating cylinders, inert fittings and screens that are designed to fit the tops and bottoms of the reciprocating cylinders. It contains a motor and a drive assembly to reciprocate the cylinders vertically inside the vessels, and if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The vessels are partially immersed in a suitable water-bath of any convenient size that permits holding the temperature at 37 ± 0.5 °C during the test. Fluent movement of reciprocating cylinders, setting of mutual velocity and maintaining a reciprocation rate at the range of ± 5 % must be ensured. An apparatus that permits observation of the preparations and reciprocating cylinders is preferable.

7.1.1.4 **Flow-through cell**
The assembly consists of a reservoir and a pump for the dissolution medium which is pumped through a flow-through cell, and a water bath which maintains the dissolution medium at the temperature of 37 ± 0.5 °C throughout the test. The size of the cell is precisely specified. The pump should ensure a constant flow. The flow-through cell made of transparent inert material is vertically connected with a filtration system preventing the escape of unsolved particles. The bottom cone is usually filled with small glass beads to protect the fluid entry tube. The dosage form is kept at the place by a holder. The cell is immersed into a heated water bath.

All parts of the used device which are in contact with the preparation or the dissolution liquid are chemically inert, and thus do not adsorb
the evaluated substance(s), do not react and do not interfere with the tested sample.

7.1.2 CONDITIONS AND PROCEDURE OF THE DISSOLUTION TESTING

Conditions for the dissolution test and its performance should be set as follows:

- The apparatus to be used.
- The composition, the volume and the temperature of the dissolution medium.
- The rotation speed (usually 50–100, for the basket and the paddle method with the accuracy of ± 4 %) or flow rate of the dissolution medium (for flow-through cell method).
- The time, the method and the amount for sampling of the test solution or the conditions for continuous monitoring.
- The method of analysis.
- The quantity of active ingredients required to dissolve within a prescribed time.

7.1.2.1 Dissolution medium

The volume of the dissolution medium usually ranges from 500 to 1000 ml (when a device with paddle or basket is used). The temperature of the dissolution medium for testing dosage forms for oral administration is always 37 °C. Water is recommended as a dissolution medium if it is proven that different pH values do not influence dissolution characteristics. Solutions characterized by the pH and the ionic strength are usually used. According to the Ph. Eur., it is possible to use as a dissolution medium for instance a solution of hydrochloric acid (pH 1.0), a solution of hydrochloric acid and sodium chloride (pH 1.2; 1.5), phosphate buffer (pH 4.5; 5.5; 5.8; 6.8; 7.2; 7.5), acetate buffer (pH 4.5; 5.5; 5.8; 6.8) and others.

It is impossible to achieve satisfactory simulation of in vivo conditions by using a single dissolution medium for testing of modified-release dosage forms. Due to this reason, it is suitable to test them in more dissolution media or to perform a dissolution test during which a dissolution medium pH and composition are gradually changed. It is necessary to consider the residence time of dosage forms in individual parts of the gastrointestinal tract (GIT).
Therefore, it is not suitable to perform the dissolution test of prolonged-release tablets in an acidic pH for 12 hours because this time is much longer if compared with real stomach residence time. Most used pH values of the dissolution media and residence time in individual parts of GIT are summarized in Table 7.1.

Table 7.1 Simulation of GIT conditions in vitro; most commonly used pH values of the dissolution media and residence times

<table>
<thead>
<tr>
<th>GIT part</th>
<th>pH</th>
<th>Residence time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1–2 fasted state</td>
<td>1–2</td>
</tr>
<tr>
<td></td>
<td>3–5 fed state</td>
<td></td>
</tr>
<tr>
<td>Proximal small intestine</td>
<td>6.1–6.8</td>
<td>3*, 4**</td>
</tr>
<tr>
<td>Distal small intestine—ileum</td>
<td>6.7–7.5</td>
<td>1–2</td>
</tr>
<tr>
<td>Colon</td>
<td>6.8</td>
<td>4–6***</td>
</tr>
</tbody>
</table>

* For multiple dosage forms; ** For single dosage forms; *** To simulate conditions in patients with inflammatory bowel diseases

Various naturally occurring enzymes can be added to dissolution media with a corresponding pH to simulate GIT physiological conditions. A group of used enzymes includes pepsin for stomach simulation, pancreatin for simulation of the small intestine or a variety of enzymes occurring in the colon area. An addition of colonic enzymes, such as β-glucosidase, galactomanase, amylase or pectinase, have been recently used for dissolution testing of colon drug delivery dosage forms based on polymers biodegradable by enzymes produced by colonic microbiota. For such systems, numerous alternative dissolution tests including methods using the intestinal content of some animals (such as rats, rabbits or pigs), methods performing in a fermenter containing conventional intestinal bacteria in an anaerobic environment or in the Simulated Human Intestinal Microbial Ecosystem (SHIME) device representing the human intestinal microbial ecosystem in vitro were used.

In the case of poorly soluble drugs, so called biorelevant dissolution media are available (see chapter 7.2.3). The biphasic dissolution method (dissolution medium consists of the upper organic layer and the lower aqueous layer 0.1M HCl) can be used as a suitable and discriminatory dissolution method for oral dosage forms containing substances in the form of weak acid salts with poor solubility.
There is also a biorelevant dissolution apparatus available. This is a dynamic multi-compartmental dissolution instrument which simulates transit and biorelevant conditions in the different parts of GIT. The apparatus design allows a simulation of physiological conditions with the possibility of adjusting all vital parameters such as pH, volumes, transit times, temperature, agitation/peristaltic rate, and enzyme secretion during the testing run.

7.1.2.2 Performance of dissolution test

A dissolution test using the basket or paddle method is performed as follows (Fig. 7.2): The prescribed quantity of the selected dissolution liquid is measured in a vessel, the device is assembled and the dissolution liquid is heated to 37 ± 0.5 °C. In the device with a paddle, the unit of the tested preparation is placed on the bottom of the vessel before starting the device; preparations which float in the dissolution liquid are kept at the bottom by means of a wire or a glass spiral. In the device with a basket, the unit of the tested preparation is inserted into the dry basket before starting the device and the basket is fixed on the driving shaft.

A dissolution test using a device with the reciprocating cylinder is performed as follows: The prescribed quantity of the selected dissolution liquid is measured in the vessel, the device is assembled and the dissolution liquid is heated to 37 ± 0.5 °C. A unit of the tested preparation is inserted in every reciprocating cylinder. The reciprocating cylinder moves up and down.
A dissolution test using a device with the flow-through cell is performed as follows: Glass beads of suitable dimension (best with the diameter of 1 ± 0.1 mm) are placed into the cell. A unit of the tested preparation is placed to the cell or the layer of beads and it is loaded with a holder. The dissolution liquid is heated to the temperature of 37 ± 0.5 °C and is left flowing through the bottom of the cell at a prescribed constant flow (± 5 %) to achieve an equal flow through the closed or opened circuit. The unit of the tested preparation is placed to chamber A. The cell is assembled and closed at the upper part with the filter. The heated dissolution liquid flows through the bottom of the cell using a suitable pump at the prescribed flow rate to achieve an equal flow through the closed or opened circuit. The dissolution liquid then overflows to chamber B, the air is vented through a capillary tube and samples are taken at regular intervals.

7.1.2.3 **Sampling and determination of drug released amount**

The prescribed amount of a sample for evaluation is withdrawn from the dissolution vessel at prescribed time or time intervals. The sample is filtered through an inert filter of suitable mesh size and the content of the drug is determined. In the basket and paddle method, samples are taken from the place in the middle between the level of the dissolution medium and the upper edge of the basket or paddle. In the reciprocating cylinder method, the sample is withdrawn after taking out the reciprocating cylinder from the place in the middle between the level of the dissolution medium and the bottom of the vessel. The taken-away medium is replaced with the same volume of the drug-free dissolution medium or it is not replaced. In this case it is necessary to take this into account later during the calculation. A sample from the automatic dissolution line is returned back to the dissolution vessel after the analysis. To analyze the samples, absorption spectrophotometry in the UV/Vis area is usually used due to the simplicity and fastness of the measurement and the possibility to measure in flow-through cuvettes (an *on-line* dissolution line). In cases of insufficient sensitivity (e.g. a low molar absorption coefficient of API) or selectivity (another component of the dosage form absorbs at similar wavelengths as API), the most frequently used method is high performance liquid chromatography (HPLC).
The HPLC enables a use of a number of stationary phases based on various principles of separation (e.g. adsorption, ion exchange, size-exclusion); however, chemically bound phases (mainly reverse-phases, RP-HPLC) are of the highest significance for the pharmaceutical applications. The reverse-phases enable the separation of analytes with a wide range of polarity and enough modifications of reverse phases with required properties is at disposal. Moreover, relatively cheap and accessible solvents can be used as mobile phases. The most common carrier for chemically bound phases (including reverse ones) is silica gel. Its structure contains free OH groups (Si–OH) which can be replaced by various functional groups of various properties. The most common chemically bound phase is silica gel with bound octadecyl chains (C18) or shorter alkyls such as octyl (C8). A disadvantage of silica gel is its stability limited by a certain pH range (usually 2–8). The second important disadvantage is the presence of non-reacted OH groups of silica gel resulting in undesirable adsorption. However, end-capped stationary phases in which a part of free OH groups is deactivated are at disposal.

The sensitive detectors enabling continuous monitoring of the substances at the column output are used. Spectrophotometric detectors belong to the most common detectors used in HPLC due to a relatively low price, reliability, a possible detection of a large group of substances (with most substances exhibiting an absorption in the region of 200–800 nm) and compatibility with the gradient elution. These detectors can measure absorbance at one or more selected wavelengths or to record the entire spectrum in the UV/Vis region. Recording of the entire spectrum by so-called diode array detector (DAD) provides much more information that can be used for the identification of substances (by comparison of the spectrum with the standard) or for the determination of peak purity (by a change of the spectrum in various parts of the peak suggests a mixture of analytes). In cases when the spectrophotometric detector does not provide a sufficient response (e.g. analyte does not absorb in the UV/Vis region), some of the universal detectors can be used (e.g. refractometric detectors, evaporative light scattering detectors). In cases of insufficient sensitivity or selectivity, some selective detectors can be chosen (e.g. fluorometric, electrochemical or mass detectors).
7 DISSOLUTION TESTING

7.1.2.4 Evaluation and specification

The result of the dissolution test is a dissolution profile of the drug. A graph of dependency showing the amount of the released drug (%) in certain time periods (hours or minutes) is usually created.

Six units are usually tested in dosage forms with a conventional drug release. According to Ph. Eur., more than 80% of the drug should be released from every unit within 45 minutes.

The number of units tested in prolonged-release dosage forms is not specified in Ph. Eur.; however, twelve units are usually tested. On the dissolution profile of prolonged-release dosage forms, three or more specification points are found. The first point is determined to prevent undesirably fast release (burst effect) of the active substance (overdose); this first point corresponds to 20–30% of the released amount of API. The second point of the specification defines the time interval when approximately 50% of the API is released. The last point of the specification is intended for ensuring almost complete drug release from the dosage form, which is defined as the release of more than 80% of the API.

Six units are usually tested in case of delayed-release dosage forms. Testing is usually accompanied by a change in the pH of the dissolution medium. Points of specification must be defined individually. The number of the specification points in the gradual dissolution test corresponds to the number of used dissolution media with a different composition or pH for delayed-release dosage forms. The first point of the specification is usually after one or two hours in an acidic medium at gastro-resistant preparations (it specifies the dosage form behaviour in an acid environment). The quantity of the released drug should not exceed 10%. At the second specification point at pH 6.8, the total released amount of API should not be lower than 80% of API for each tested unit.

7.2 APPLICATION OF DISSOLUTION TESTING IN PHARMACEUTICAL TECHNOLOGY

The dissolution test is widely used in pharmaceutical technology. It is used in the field of pharmaceutical research, pharmaceutical industry as well as in education. The drug release rate (immediate, controlled), the type of controlled release (prolonged, delayed, pulsatile), the kinetics (zero order, first order), mechanism of drug release (e.g. diffusion, matrix
swelling) and other aspects can be clearly determined from the obtained dissolution data.

Drug dissolution testing is used both in the early and the late stages of drug development for many dosage forms. In the early drug development stage, the dissolution test helps to find the optimal formulation with the desired drug release profile. Later, the dissolution profiles can be used to establish an in vitro/in vivo correlation (IVIVC) which may reduce the need for costly bioequivalence studies. In the final stage, the dissolution testing is used for quality control of dosage forms.

7.2.1 FORMULATION OPTIMIZATION

A dissolution test is an essential tool in the development of single and multiple dosage forms. The final dosage form should be fully specified in terms of quality as well as quantity of pharmaceutical components. Based on the dissolution data, it is possible to select suitable pharmaceutical excipients, to investigate their effect on the drug release behaviour and consequently to find optimal composition of matrix as well as a reservoir (coated) dosage form achieving the desired dissolution profile.

For coated preparations, the dissolution test results are used for the selection of a suitable coating material and its thickness. It is of high importance for so-called functional coating when the film significantly influences the API release from the dosage form. Such an example is the case of polymeric coatings with pH-dependent solubility that are used for drug delivery to distal parts of the intestine. Methacrylic polymer Eudragit® L is soluble at a pH of 5.5–6.0 (depending on the type). A different thickness of this film can provide different drug release. A very thin coating does not in fact affect the release and it can be used for an isolation layer during the sugar-coating process. Dosage forms coated with a thicker coating layer of Eudragit® L can be used for the preparation of gastro-resistant films. They dissolve quickly after reaching the pH above their declared solubility. However, a significantly thicker coating can ensure the drug release more distally in the GIT (ileum or colon area). The selection of an optimal coating thickness can be determined by means of the dissolution test.

Figure 7.3 shows the dissolution profiles of HPMC hard capsules coated with increasing thickness of Eudragit® FS film for colon drug
delivery. The anionic methacrylic copolymer Eudragit® FS also exhibits pH-dependent solubility and starts to dissolve when pH reaches the value 7.0. Dissolution test was performed in the dissolution medium with rising pH value to mimic stomach conditions (2 hours, pH 1.2), small intestine (4 hours, pH 6.8) and colon (2 hours, pH 7.5). From the obtained dissolution data, it can be concluded that the minimal coating level of 15% ensures the drug release in colonic area (6 hours lag time is required). Moreover, it is apparent that the 30% coating level needs one hour more at pH 7.5 to be completely dissolved.

![Dissolution profiles of HPMC hard capsules coated by increasing thickness of Eudragit® FS film for colon drug delivery.](image)

**Fig. 7.3** Dissolution profiles of HPMC hard capsules coated by increasing thickness of Eudragit® FS film for colon drug delivery.18

### 7.2.2 Drug Release Rate and Mechanism

The release patterns comprise of zero and first order rate. In addition, there are those that provide an initial rapid dose, followed by zero or first order release of the sustained component. Obtained dissolution data can be used to evaluate the kinetics and mechanism of drug release from a dosage form. For this purpose, several mathematical equations that describe the dependence of release in function by time is available.

APIs are most frequently released from the matrix systems by diffusion, swelling or combination of these processes.

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Under the simplified procedure, the mathematical model that best fits the release data is selected for example determination coefficient $R^2$. Examples of frequently used kinetic models are given in Table 7.2.

**Table 7.2 Examples of mathematical models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zero order kinetics</strong></td>
<td>$\frac{M_t}{M_\infty} = K_0 \times t$</td>
<td>The ideal drug release independent on its concentration</td>
</tr>
<tr>
<td><strong>First order kinetics</strong></td>
<td>$\frac{M_t}{M_\infty} = 1 - e^{-K_1 \times t}$</td>
<td>The drug release rate depends on its concentration</td>
</tr>
<tr>
<td><strong>Weibull model</strong></td>
<td>$\frac{M_t}{M_\infty} = 1 - e^{-K_w \times t^b}$</td>
<td>This equation can be successfully applied to almost all kinds of dissolution curves; it is an empirical model, with no kinetic fundament</td>
</tr>
<tr>
<td><strong>Higuchi model</strong></td>
<td>$\frac{M_t}{M_\infty} = K_H \times \sqrt{t}$</td>
<td>Describes kinetics of drug release from the matrix by diffusion according to Fick’s Law</td>
</tr>
<tr>
<td><strong>Korsmeyer-Peppas model</strong></td>
<td>$\frac{M_t}{M_\infty} = K_{KP} \times t^n$</td>
<td>Analysis of release whose mechanism is not exactly known or is a combination of more types of drug release</td>
</tr>
<tr>
<td><strong>Hixson-Crowell model</strong></td>
<td>$\sqrt[3]{M_\infty - \sqrt[3]{M_\infty - M_t}} = K_{HC} \times t$</td>
<td>Describes drug release from systems in which a change of surface area occurs, a change of particle size or tablet diameter occurs</td>
</tr>
<tr>
<td><strong>Baker-Lonsdale model</strong></td>
<td>$\frac{3}{2} \left[ 1 - \left( \frac{M_t}{M_\infty} \right) ^\frac{3}{2} \right] \frac{M_t}{M_\infty} = K_{BL} \times t$</td>
<td>Describes drug release from systems when drug dissolution and its diffusion via channels filled with water are present</td>
</tr>
</tbody>
</table>

*M is an amount of the drug released in time t; $M_\infty$ is a total drug amount in the matrix; $K_0$, $K_1$, $K_w$, $K_H$, $K_{KP}$, $K_{HC}$ and $K_{BL}$ are respective release constants, $n$ is an exponent which characterizes the drug release mechanism and $b$ is a parameter that characterizes the shape of curve

Korsmeyer-Peppas model is an example of a mathematical model enabling the determination of drug release mechanism. To estimate the mechanism of drug release from the matrix system, the release exponent $n$ can be used. To determine the exponent $n$, it is recommended to use the portion of release curve until the point where $M_t/M_\infty < 0.6$. For API release from the tablet, the value of $n = 0.45$ corresponds to the drug release by diffusion according to Fick's Law. At $n = 0.89$, the drug is released by the matrix swelling or a polymer chain relaxation.
At exponent values of $n$ in the interval from 0.45 to 0.89, the drug release is considered anomalous, i.e., combination of these processes.

### 7.2.3 Prediction of *in vivo* Performance of the Dosage Forms

To gain knowledge of the behavior of a drug—especially a poorly-soluble one—during GIT transit, the use of biorelevant dissolution media simulating the actual *in vivo* situation is important. Biorelevant media simulating the fasted or fed stomach and upper small intestine have been described. They are characterized by pH, osmolality and surfactant type and level. They exhibit lower surface tension compared to water and therefore an increasing solubilizing capacity of poorly soluble drugs. *The fasted state simulated gastric fluid* (pH 1.6) contains pepsin and a low level of taurocholate and phospholipids. To simulate fed conditions in the stomach, milk (3.5%) or nutritional drinks are used. *The fasted and fed state simulated intestinal fluids* contain surfactants (sodium taurocholate, lecithin) and differ in their ionic strength and pH—6.5 and 5.0, respectively.

The mathematical relation between dissolution data (*in vitro*) and *in vivo* performance of the dosage form (e.g., concentration-time profile of drug) with the aim to accurately predict the *in vivo* plasma concentration-time profiles is called *in vivo/in vitro* correlation (IVIVC). Characteristic values monitoring *in vivo* during pharmacokinetics studies are, as a rule, plasma concentrations of the drug over time, expressed as the area under the curve (AUC) or maximal drug plasma concentration ($C_{\text{max}}$). Using IVIVC, it is possible to determine, based on *in vitro* dissolution, whether a change in the manufacturing of the dosage form has no effect on its *in vivo* behavior.

### 7.2.4 Evaluation of Bio-Equivalence of Generic Drugs

Original medicinal products are subject to clinical studies before their introduction to clinical practice. These studies prove safety and efficiency of the preparation on a large group of volunteers. For generic medicinal products, which may appear after the expiration of the patent protection of the original preparation, their similarity with the original product is confirmed based on a so-called bio-equivalence study. Bioequivalence means that API in two or more dosage forms reaches systemic circulation...
at the same rate and to the same relative extent. According to the FDA, bioequivalence is defined as: “the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study”.

Most frequently used “in vivo bio-equivalence” studies include a monitoring of various pharmacokinetic and pharmacodynamic parameters after the administration of tested preparations. A conventional pharmacokinetic study is usually carried out in human and animal subjects by measuring the rate and extent of drug absorption in the blood stream ($AUC$, $C_{\text{max}}$). The goal of a bio-equivalence study is to prove the similarity between the tested and compared preparations. This is considered demonstrated if the 90% confidence intervals of the ratios for $AUC$ and $C_{\text{max}}$ between the compared preparations lie within the range of 80–125%.

A basic presumption for achieving a successful in vivo bio-equivalence study is that the similarity between dissolution profiles (in vitro) of original and generic preparations is as high as possible.

Dissolution tests can only be used instead of in vivo studies in some special cases (so-called “in vitro bio-equivalence studies”), e.g. for a marketing authorization of biowaivers, lower strengths of the same product, an already marketed product newly produced by a slightly modified method, etc. In vitro studies reduce the cost and number of trials.

The dissolution profile of a generic preparation is compared with the dissolution profile of a reference (original product). Where more than 85% of the drug is dissolved (which means the mean percent of drug released for both reference ($R$) and test ($T$) samples) within 15 minutes, the dissolution profiles may be accepted as similar without further mathematical evaluation. If the previous requirement is not met, the difference factor $f_1$ (Eq. 7.2) and the similarity factor $f_2$ (Eq. 7.3) are, due to its simplicity, the metric of choice by both the EMA and FDA guidelines:

$$f_1 = \frac{\sum_{i=1}^{n} |R_i - T_i|}{\sum_{i=1}^{n} R_i} \times 100 \quad (7.2)$$
\[ f_2 = 50 \times \log \left( 1 + \frac{1}{n} \sum_{i=1}^{n} (R_i - T_i)^2 \right)^{-0.5} \times 100 \] (7.3)

where \( n \) is a number of time points, \( R_i \) and \( T_i \) are the drug release amount of the reference (\( R \)) and test (\( T \)) sample in the given time (12 units each). This method is the most suitable for the dissolution profiles comparison when three to four or more dissolution time points are available. If the difference factor \( f_1 \) takes the value between 0 and 15, the dissolution profiles are considered similar. The similarity factor \( f_2 \) reaches the values from 0 to 100. The value of 100 indicates identical dissolution profiles, the value of 50–100 indicates similar dissolution profiles and a value in the interval between 0 and 50 indicates different dissolution profiles. The dissolution measurements of the test and reference batches should be performed under the identical conditions in the presence of at least 12 individual dosage units of test and reference products. Only one measurement should be considered after 85% dissolution of both the products. To allow the use of mean data, the percent coefficient of variation at the earlier time points should not be more than 20 %, and at other time points should not be more than 10 %.

7.2.5 STABILITY EVALUATION

A dissolution test is also used for testing the stability of medical preparations. It is ranked among physical tests. The same group also includes e.g. tests of mechanical properties of tablets (friability and hardness tests), a test of the humidity content or a disintegration test.

Stability testing is necessary to be performed prior to the industrial production of a new medicinal product (original or generic), in case of various changes in already existing medicinal product (changes of a package, composition or manufacturing process) or for the regular stability monitoring of a preparation already available on the market. According to storage conditions, stability tests can be divided into stress, accelerated and long-term tests. However, other types are also known. A test after the first opening should prove the stability of an opened package and determines the shelf life after opening.

The main goal of stability tests is to have a stable medical preparation which exhibits the required characteristics for its shelf life. These characteristics of dosage forms with immediate and controlled drug
release include maintaining the drug release profile in this interval. Due to this reason, a dissolution test of the monitored preparation is regularly performed under pre-set conditions. Obtained dissolution profiles are compared (e.g. on the basis of the specification limit of dissolution or the similarity factor $f_2$) with the reference dissolution profile. Two or three batches of the final preparation (always at least 12 samples) are usually tested.

Figure 7.4 shows the slightly different dissolution profiles of drug-loaded matrix tablets at starting time point (0) and after 6 months of the storage under different external conditions. The similarity factor $f_2$ calculated between dissolution data at time 0 and 6 months, respectively, reaches the value above 50 (78.5, 57.4) and reveals the similarity of dissolution profiles and therefore stability of tested medicinal product.

![Graph showing dissolution profiles](image)

**Fig. 7.4** Stability dissolution testing (paddle method, pH 6.8) of matrix tablets stored under different external conditions at time 6 months (RV—relative humidity).

### 7.2.6 QUALITY CONTROL

The selection of a cheap, simple and discriminating dissolution method is crucial for quality control of medicinal products in relation to an optimal selection of raw materials, product specification, batch control and storage conditions as well. Such a method can reveal even small changes in the quality of the tested dosage form.

Typically, a lot of drugs may change their solid phase during manufacturing or stability testing. This change may have an adverse effect on the drug pharmacokinetics and/or pharmacodynamics in the human/
animal body. In order to capture this instability, it is necessary to use a dissolution method which provides different dissolution profiles for different solid phases of the drug in the same pharmaceutical form. The search for such a discriminating method is based mostly on changes in an agitation rate, pH, temperature or a composition of the dissolution medium, and subsequently on the comparison of the obtained dissolution curves by the similarity and difference factors. Another possibility of dissolution testing in QC is for example a confirmation of input excipients quality. As an example, Fig. 7.5 displays the influence of a change in particle size of excipient sucrose (filler) on the dissolution profile of diclofenac sodium (DS) from the povidon/cetyl alcohol matrix tablets. The obtained dissolution data were used for performing of the difference factor $f_1$ and the similarity factor $f_2$ analysis. The obtained values (Table 7.3) clearly show that the change in size of sucrose particles (Fig. 7.6) is important variable for modifying the DS release profile from such a tablet and a decrease in the sucrose particle size from 500 µm to the level smaller than 125 µm significantly accelerates the drug liberation (difference factor $f_1$ higher than 15, similarity factor $f_2$ smaller than 50). On the other hand, matrix tablets containing sucrose with particles in interval 500–250 µm and 250–125 µm exhibit a similarity in the dissolution characteristics. Based on that, the used dissolution method is discriminatory for assessment of the excipient particle size and can be used as a part of QC.
Fig. 7.5 Dissolution profiles of matrix tablets with diclofenac sodium based on a combination povidone/cetyl alcohol with sucrose of different particle size fraction in a role of filler.\textsuperscript{19}

Table 7.3 The difference factor $f_1$ and the similarity factor $f_2$ analysis for comparison of the dissolution profiles displayed in Fig. 7.5\textsuperscript{19}

<table>
<thead>
<tr>
<th>Sucrose particle size in matrix tablets</th>
<th>500–250 µm</th>
<th>250–125 µm</th>
<th>125–80 µm</th>
<th>&lt; 80 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference factor $f_1$ Reference sample</td>
<td>7.10</td>
<td>16.35</td>
<td>25.00</td>
<td></td>
</tr>
<tr>
<td>Similarity factor $f_2$ Reference sample</td>
<td>63.47</td>
<td>43.73</td>
<td>35.53</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7.6 The real difference in sucrose particle size: the lines at the bottom right of the images, 500 µm.\textsuperscript{19}

7.3 REFERENCES


8 EVALUATION OF SIZE, SHAPE AND SURFACE OF PARTICLES

8.1 SIZE AND SHAPE OF PARTICLES

The size and shape of particles significantly influences physicochemical, mechanical and biopharmaceutical characteristics of medical preparations. The API particle size has significant effect on the surface area, the rate of dissolution and its bioavailability. It influences the flow properties and during fluidization it affects their compressibility, homogeneity and filling to capsules or matrices of pressing machines. The size of powders or microforms (especially microparticles) intended for inhalation basically pre-determines the place of expected release and effect of contained active ingredients. Inspecting the particle size in a completed batch is a typical method for evaluating the reproducibility of a production process.

The shape and surface of particles are also very important parameters. The more the particles approximate to a sphere in their shape, the smaller surface they have at the given diameter (i.e. a smaller ratio between the surface and the volume). A regular spherical shape and a smooth surface without significant unevenness is optimal with regard to flow properties, coating and further technological processing. The particle shape influences their flow properties and compressibility as well as the rate of dissolution and drug release. Despite this fact, the problems of particle shape are paid much less attention than their size.

Several methods for measuring the particle size which differ in their principle, technique and sensitivity. They include sieving and sieve analysis, microscopic methods (described in detail in chapter 9) and image analysis, laser diffraction methods, sedimentation analysis using the Stokes' sedimentation law and Coulter counter. The selection of a suitable measuring method depends especially on the presumed size range of particles and on physicochemical properties of the measured material, such as particle shape, morphology of their surface, solubility and wettability (the principle of some methods requires liquid environment for measuring particle size in which the measured sample
is dispersed) and on the desired accuracy and measuring rate. The shape is evaluated mainly by microscopic techniques, such as light and electron microscopy (see chapter 9) usually in combination of these methods with image analysis.

8.1.1 SIEVING AND SIEVE ANALYSIS

The simplest and historically oldest method for particle size analysis and distribution is sieve analysis suitable for sieving of powders, granulated powders, pellets and microparticles in dry condition with the particle size exceeding 75 µm.

The equipment used for the sieving method (Sieve shaker) consists of a set of sieves with metal (or other material) mesh and a device platform ensuring their defined movement: shaking or vibrations. The sieve set is assembled in opening size decreasing order. Therefore, the top sieve has the largest openings and the lowest sieve has the lowest opening size. A weighed sample (usually 25 g, 50 g or 100 g) is put on the upper sieve and the assembly is closed with a lid. By shaking, the sample is divided into two parts at every sieve: one part remains on the sieve and it is called the fraction or residue, the second part falls through the sieve onto the sieve below. The fraction from a particular sieve is not formed by particles with the same uniform dimension, but by particles whose dimensions lie in the interval of upper and lower sieve mesh size. A weight of each fraction is determined by weighing. Results are usually expressed in per cents, partial or cumulative. The maximum number of fractions obtained equals the number of sieves used increased by one, as the collection bowl is placed under the lowest sieve to collect particle dust fraction.

The sieve analysis has its limitations. It needs a higher quantity of a sample (at least 25 g). It is also difficult to sieve oily or cohesive powders because they tend to adhere to the walls of sieves and clog their mesh openings. Obtained values must be understood as two-dimensional estimates of particle dimensions, as the passage through the sieve openings depends on their maximum width and thickness, not on their length.

A mean diameter $d$ can be determined from the results of the sieve analysis by using following equation (Eq. 8.1):
where \( d \) is a mean diameter (mm), \( d_i \) is a size class diameter defined by mesh dimensions of the upper and the lower sieve and \( w_i \) is a weight of the fraction caught by the given sieve (e.g. if 100 g of a sample is used for sieving, then \( \sum w_i = 100 \)).

As a simple example of the sieve analysis utilization with an added value, an evaluation part of an experiment with pellets prepared from the microcrystalline cellulose can be mentioned. Pellets were intended as enzyme carriers for naked-eye detection of specific chemical warfare agents. Prior to the detection test pellets were filled into narrow glass tubes. For these reasons, pellets had to be big enough for the naked-eye detection but small enough for the glass tube filling. However, the preparation process usually yields wider size distribution. Sieve analysis was used to collect the particles with suitable size (sieves 1.0 and 0.8 mm) and to discard the rest. Fig. 8.1 shows that the preparation process was well set as the desired fractions form a majority of the prepared sample.

![Sieve analysis size distribution](image)

**Fig. 8.1** Sieve analysis size distribution. Bars show percentage of individual fractions on a given sieve, Black line shows cumulative percentage increase. Only fractions on sieves with opening size of 1.0 and 0.8 mm were suitable for further work.

### 8.1.2 Laser Diffraction

Laser diffraction (LD) is currently the most used method for measuring the particle size of pharmaceutical powders. Traditionally, by means of LD it is possible to measure particles in a relatively wide size range of 0.1–2000 μm, however due to the recent advances in optics and construction these range limits can be nowadays exceeded in some
instruments. The method is based on the ability of particles to diffract light. Diffraction of light is defined as the bending of light around the corners of an obstacle or aperture into the region of geometrical shadow of the obstacle and a diffraction pattern is created on a screen formed by light or dark strips of different width and intensity. When light is diffracted, beams are deviated from directions determined by straight-line light propagation. Radiation diffracts at any time when the wave front reaches the edge of any barrier, or if it passes through a small hole in the barrier. It is important that dimensions of the barrier/hole are smaller or comparable to the wavelength of radiation. A barrier can be formed either by a geometrically shaped hole in an object impermeable for light, or in contrast, a non-homogeneity impermeable for light, which is sharply bordered in the environment in which light is propagated (i.e. an object preventing the passage of a part of light radiation, e.g. a measured particle).

A laser beam passes through a cuvette with a sample placed in a laser diffraction device. Measured particles are suspended in the transparent liquid or gaseous environment. When the beam passes through the cuvette, beams are diffracted under the influence of present particles under an angle which is inversely proportional to their size (Fig. 8.2). The diffraction angle logarithmically increases with the decreasing size of a particle. Scattered radiation is directed by a Fourier lens and then it is detected by photodetectors (detectors producing electrical current, if radiated) situated under various angles. The measured course of laser beam diffraction is then used for the calculation of the distribution of particle size. An important condition for measuring is to maintain optimal sample concentration in the cuvette. High concentration can cause undesired levels of scattering, resulting in opacity, meanwhile too low concentration will produce weak signal. Wet dispersion type (liquid environment) of LD devices also includes an ultrasound probe, a mechanical mixer to prevent aggregation of particles and a pump which enables sample re-circulation. Dry dispersion type (air environment) of LD devices prevents aggregation of particles by air flow which may be less effective than the procedures used for measuring in a liquid environment. On the other hand, it is not necessary to consider the solubility of the measured particles in the used medium and the measurement is faster than using the wet dispersion type of LD devices.
A basic problem in measuring particle size by LD is the ability to interpret diffraction patterns created by diffraction on a mixture of particles of various sizes. Obtained mixed patterns are mathematically evaluated and the size of present particles or their shape is determined. Fraunhofer and Mie theories are the most often used for calculations depending on the size of measured particles. Fraunhofer theory can be applied to spherical non-transparent particles with the size higher than 50 µm. Fraunhofer approximation does not consider the existence of other optical phenomena, such as absorption of radiation, scattering or refraction. Another important prerequisite is that measured particles should be sufficiently distant from each other to prevent overlapping of particles and multiple scattering. Mie theory considers both diffraction and scattering of radiation and it is used for analysing a diffraction pattern created by transparent particles of sub-microscopic dimensions. To use the Mie theory, it is necessary to know the refraction index of the sample and of the dispersion environment. It is also very important to know the imaginary part of the measured particle index (value which characterize light absorption by the material) to obtain correct measurement. The measured particle size is based on the prerequisite of a spherical particle shape, which means the calculated size corresponds to the size of a theoretical sphere (a sphere of the same volume as the analysed particle). The basic distribution type obtained through the laser diffraction is so-called volume distribution. This type gives percentage of total volume of measured particles assigned to the appropriate particle size. However, coupled software allows recalculation to classic number distribution or surface area.

LD does not require lengthy calibration and determines the complete distribution of particle size in a sample. A large set of particles dispersed in various dispersion media is very quickly measured (measuring a single sample takes approximately 1–5 minutes). Devices for LD are easy.
to operate and provide results with high reproducibility (within measuring on a single device). LD is suitable for size measuring of powders, granulates, microparticles, suspensions, emulsions and aerodispersions.

Laser diffraction also has its disadvantages and limitations. Expensive instrumental equipment is necessary for its implementation. A sub-micron analysis is relatively difficult and LD shows high inter-instrumental variability. Very serious setback is poor implementation of the Mie theory by common users when measuring small micron and sub-micron sizes, as the personnel often forgets the need to reflect the optical properties of the material. It was estimated that the majority of scientific papers using this technique could have incorrect data because of this problem. Also, laser diffraction is much more suitable for spherical particles. Irregular particles with sharp edges can cause inaccuracies due to large angles. This can even generate a “false particle” if the angle is too large. Nevertheless, it is usually recommended to couple the method with other technique like optical image analysis to verify the results.

![Graph showing laser diffraction analysis results of PLGA microparticles. Sample A dispersed in 0.1% polysorbate 80 water solution, Sample B dispersed in water. For both samples, size distribution chart and cumulative curve are available. The q on the left y axis describes amount of given size by volume in %.](image)

Choice of dispersive environment is also very important as it is often needed to add a surfactant for proper redispersion of solid particles, or eventually to reflect solubility of the analysed sample in various dispersion liquids. Fig 8.3 shows a laser diffraction analysis of PLGA microparticles in plain water and in 0.1% water solution of polysorbate 80. It is evident that analysis performed with the surfactant polysorbate 80 yielded lower size values and narrower size distribution. The redispersion could be therefore concluded more thorough in comparison with pure water, where the particles remained probably partially agglomerated.
8.1.3 Dynamic Light Scattering

Dynamic light scattering (DLS) enables measuring particles in a size range of approximately from 0.5 nm to 6 μm, i.e. it is usable mainly for characterization of nanoparticles. The DLS principle is based on diffraction of light beams (laser) which are passing through the analysed sample (suspension, emulsion). A thermal movement of molecules of the medium causes position changes of observed particles (Brownian movement), which affects diffraction pattern. Thanks to this, the intensity of an observed point of a diffraction pattern changes in time. A change of intensity depending on time is observed by a correlation function (Fig. 8.4). The smaller the size of measured particles, the faster they move in the solution and the fluctuation of scattered radiation increases directly proportionately to the size of particles. It is possible to calculate the distribution of particle sizes according to the Stokes-Einstein equation (Eq. 8.2) from the Brownian movement, i.e. the fluctuation of scattered radiation:

\[ d_h = \frac{k_B \times T}{6\pi \times \eta \times D} \]  

(8.2)

where \( D \) is a diffusion coefficient, \( k_B \) is a Boltzmann constant, \( T \) is an absolute temperature, \( \eta \) is viscosity of the medium and \( d_h \) is a hydrodynamic diameter of a spherical particle.

![Intensity vs. Time](image1)

![Correlation Coefficient vs. Time](image2)

Fig. 8.4 Changes of radiation intensity and resulting correlation function. The time point at which the start of correlation function decrease, corresponds to the size of particles and the slope of correlation function decrease corresponds to polydispersion of the sample; (—) small particles, (--) large particles.\(^{20}\)

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\(^{20}\) Adapted from: Olivier, S., 2011. Malvern Instruments Ltd, seminar: Characterisation of proteins and biomolecules using light scattering techniques and size exclusion chromatography.
In DLS, a laser beam passes through a temperature-controlled cuvette with a sample. The intensity of scattered light is converted to an electrical signal in the detector, which is further led to a digital correlator which calculates the correlation function and the particle size distribution is consequently calculated from it. A suitable concentration of particles for the determination of their size is 0.01–0.1%. The range of measured particle size is limited by the fact that very small particles (< 5 nm) scatter light poorly and particles above 0.5–1 μm can sediment during the measurement and their Brown movement is very slow (long measuring times are necessary). A disadvantage is that particles measured in this way are interpreted as spherical, which may distort results in case of non-spherical particles.

The temperature and viscosity of the medium in which the measured particles are dispersed must be known for measuring. A diffusion coefficient can be determined from the correlation function and a hydrodynamic particle diameter $d_h$ can be calculated by its incorporation into equation (Eq. 8.2). A hydrodynamic diameter is a diameter of a particle and its surrounding which moves in the same velocity as the measured particle (it is influenced by surrounding molecules which form a particle solvation shell). For poly-dispersion samples, the correlation function is a sum of contributions of all particles of various sizes and the interpretation of $D$ is less straightforward. Computer programs supplied by a manufacturer together with a DLS device serve for its calculation.

A result of particle size distribution measuring in samples by DLS can be interpreted in several ways. A primary result is a particle size distribution according to the intensity of the scattered light. This evaluation is highly sensitive to the presence of larger particles because larger particles scatter radiation more efficiently than smaller ones. A disadvantage is that the presence of several larger particles (impurities, clusters etc.) significantly influences the resulting particle size distribution in a sample. Due to this reason, the evaluation of particle size distribution is used according to their volume (using the Mie theory). The resulting particle size distribution is equivalent to the weight or distribution of weights of measured particles. Another possibility for the evaluation of the results is the particle size distribution according to the number (using the Mie theory) which is equivalent to the number of particles in the sample (Fig. 8.5). Figure 8.6. shows one of many uses
of DLS in pharmaceutical area. In this specific example particle size obtained via DLS also gives the information about successful preparation process.

Fig. 8.5 The evaluation of particle size distribution of the same sample. Particle size distribution according to intensity (a), Particle size distribution according to volume (b), Particle size distribution according to number (c).\(^{21}\)

Fig. 8.6 Picture of the size distribution of cationic liposomes and cationic liposomes complexed with the DNA used for transfection of cells. Successful complexation can be monitored using DLS method. Two curves show the size distribution of cationic liposomes before and after the complexation. The slight change in the size can be observed (the difference is approximately 8 nm).

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\(^{21}\) Adapted from: Olivier, S., 2011. Malvern Instruments Ltd, seminar: Characterisation of proteins and biomolecules using light scattering techniques and size exclusion chromatography.
8.1.4 IMAGE ANALYSIS

An image analysis can be defined as a process of preparation and processing of an image for purposes of measuring dimensions of an object displayed in the image. Input data can be in the form of an image taken by a camera, a digital camera, a video camera etc., either in the analogue form (i.e. the display having a permanent continuous sequence of values), or the digital form (i.e. the display created by discontinuous line of separate point). In this way, a real spatial (3D) object is first displayed as a flat one (2D). A 2D image is converted to a digital one by means of suitable software (the process of image digitalization). Digital image consists of image points: pixels (abbreviation for picture element), the smallest units of digital scanning (bitmap) graphics. Pixels bear information about colour (a colour value) and brightness (a brightness value). Points are arranged horizontally and vertically and they form a square net in which every pixel can be clearly identified according to coordinates.

For the image to be able to be processed in a computer (i.e. measured), it has to be in the form of a binary code. The smallest unit of this code is a bit which can only take two values: truth or untruth, recorded in logical expressions of 1 and 0. It is only defined for these expressions whether the point is lit, which corresponds to the binary logics of bits (truth—lit, untruth—not lit). The conversion of a digital image to a binary one differs according to the fact whether the input image is black-and-white, in shades of grey, or colourful RGB one (R—Red, G—Green, B—Blue) or CMYK (C—Cyan, M—Magenta, Y—Yellow, K—blackK). The process of conversion to the binary form requires an operator's intervention because he has to decide during the process of thresholding which colour values will be assigned to the displayed object and which will be excluded from the analysis by their marking on the background. The conversion of colourful images to a black-and-white binary image is relatively complicated, therefore the colour value of the pixel is usually neglected and only its brightness value is considered.

The system of pattern analysis consists of several parts. Firstly, an optical or electron microscope (microscopic methods are described in chapter 9), which nowadays contains micro-photographic equipment enabling observing microscopic specimen at the magnification up to 1000 times. A macro system consists of a stand and a lighting system. On the
stand there can be a colour or black-and-white CCD camcorder which enables creating documentary images. Images from the microscope or macro optics can be archived, modified by software, measured and further mathematically processed.

Important parameters which can be a source of variability of results in case of pattern analysis systems based on photomicroscopy or videomicroscopy are, among others, resolution, the light source location and light intensity, the size of a sample and the number of measurements of a single particle. According to the number of pixels per unit of area, an optical resolution ability is evaluated, i.e. the ability to draw details. The image resolution is measured in units dots per inch (DPI), more correctly in pixels per inch (PPI). The higher the resolution (the higher PPI) is, the more realistic display of the object can be observed and the values are measured more precisely. A general recommendation for measuring a pellet with the diameter of 1.2 mm is that the side length of 1 pixel should not cover more than 30–35 μm.

A sample size, i.e. the number of measured particles, is another variable influencing the precision and accuracy of results. Data from technical literature are highly variable; it is usually necessary to measure at least 200 particles of mono-dispersion and 500 particles of poly-dispersion systems. By measuring the sample of 625 particles (and more), the error of measurement reduces to 2 % only. To calculate a sample size (i.e. the number of particles to be measured), several means were proposed, including ISO 13322-1 (Eq. 8.3):

\[ \log n = -2 \log \delta + K \]  

(8.3)

where \( n \) is the number of analyzed particles, \( \delta \) is a relative error and \( K \) is determined numerically based on the population standard deviation, probability and constants.

Image analysis combined with microscopic techniques is suitable for analysing particles of suspensions, powders, granulates, sprays, micro forms etc., and offers numerous advantages: it is suitable for measuring particles with the dimension from 100 nm to several mm, it requires a small quantity of a sample, it is non-destructive, highly reproducible, precise, and it can be automated to a larger extent. Possibility to analyse the shape of particles is another advantage. However, several inconveniences exist. It is relatively time-consuming (the analysis of a single sample usually takes more than 15 minutes), it operates
with 2D images of spatial objects, it is sensitive to the system of sampling and it is also sensitive to setting, which means a trained operator is necessary.

8.1.4.1 Parameters of particle size

The size is easily defined only for regular spherical particles, about which it can be said that their size corresponds to the diameter or radius. For irregular particles, a variety of definitions of particle size exist (Fig. 8.7). In general, for irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several diameter types exist.

Surface equivalent diameter is a parameter used for characterizing dispersion particles of irregular shape. It equals the diameter of a circle with the same surface area as the area of observed particle projection (Eq. 8.4):

$$D_p = \sqrt{\frac{4 \times S}{\pi}}$$  \hspace{1cm} (8.4)

where $D_p$ is a surface-equivalent diameter and $S$ is a surface of the measured particle.

Another diameter type is Feret's equivalent diameter. It equals the distance of points where two parallel tangents touch the perimeter of the projection of the particle (it is determined on an image from an optical or electron microscope). The direction in which tangents are led must be identical for all measured particles (alternatively it can be randomly oriented, but only if all particles have the same size and shape).

Last but not least Martin's diameter can be measured for irregular particles. It equals the length of a line which divides the area of projection of a dispersion particle to halves (it is determined on an image from an optical or electron microscope). The direction in which the dividing line is led is arbitrary but it has to be identical for all measured particles.
Fig. 8.7 Most frequently used parameters in measuring of particle size.\(^{22}\)

### 8.1.4.2 Parameters of particle shape

A shape can be assessed on the basis of calculated shape parameters values, such as the sphericity factor, 2D shape factor or aspect ratio. It needs to be pointed out that the technical literature is not fully unified in terminology and it uses many other designations and synonyms for the same mathematical expressions.

The sphericity factor (SF) is calculated according to equation (Eq. 8.5):

\[
SF = \frac{4\pi \times A}{P^2} = \frac{4 \times A}{\pi \times d^2} \tag{8.5}
\]

where \( A \) is a projected object area, \( P \) is the perimeter and \( d \) is the diameter of the particle. In an optimum case, i.e. when the particle is perfectly spherical, \( SF \) takes the value of 1.0 (in case of optical analysis, where measuring error occurs due to the conversion of the image to pixels, the \( SF \) value of an ideally spherical particle is about 0.96). An acceptable value (for pellets) is the value of \( \geq 0.80 \).

Meanwhile sphericity describes the overall shape and its closeness to sphere, roundness (\( R \))—sometimes called circularity—indicates rounding of particle edges. It can be calculated according to equation (Eq. 8.6):

---

where $A$ is a projected object area and $d$ is the diameter of the particle.

An aspect ratio ($AR$) is defined as a ratio of the longest of measured Feret's diameters $d_L$ of the particle width perpendicular to this diameter $d_B$ according to equation (Eq. 8.7):

$$AR = \frac{\text{max } d_L}{\text{min } d_B} \quad (8.7)$$

In case of a round particle, the value of $AR$ equals one. A critical value of $AR$, which would still express that the tested particle is round differs in literature again. The value of $\leq 1.2$ can be generally considered acceptable for pellets intended for being filled into capsules.

Shape factor in general is a shape of an object expressed by number. Several expressions for various shape factors exist. Formula for shape factor ($e_R$) defined to measure oblong granules with a certain roughness is stated in equations (Eq. 8.8 and 8.9):

$$e_R = \frac{2\pi \times r_c}{P} \times f - \sqrt{1 - \left(\frac{d_B}{d_L}\right)^2} \quad (8.8)$$

$$f = 1.008 - 0.231 \left(1 - \frac{b}{l}\right) \quad (8.9)$$

where $r_c$ is an average radius derived from an average distance between the centre of gravity of the object and perimeter, $P$ is a measured perimeter of the particle, $l$ is the longest of measured Feret's diameters, $b$ is the longest width perpendicular to $l$ and $f$ is a correction factor. The values of factor $e_r$ can range from 0 to 1, where the value equal to one indicates a particle the perimeter of which creates a perfect circle. In practice, factor $e_r$ is highly sensitive to the presence of any surface unevenness, so values of $\geq 0.6$ can be considered as the values indicating a round shape.

8.1.5 Sedimentation methods

Sedimentation methods represent the most traditional methods of the particle analysis. They can be divided to those which are performed in the gravitation field, in the centrifuge field and in the forcefield—sedimentation field-flow fractionation (SFFF). The methods are used for indirect determination of the particle size from the sedimentation rate.
8.1.5.1 Sedimentation in gravitation field

Sedimentation in the gravitation field is the simplest and historically oldest variant, where sedimentation happens under natural conditions. Sedimentation of spherical particles in liquid can be used for the determination of particle size as shown in equation of Stokes' law (Eq. 8.10):

$$v = \frac{h}{t} = \frac{d^2 \times (\rho_1 - \rho_2) \times g}{18 \times \eta}$$

(8.10)

where $v$ is a rate of sedimentation, $d$ is a diameter of the particle, $\eta$ is viscosity of the liquid, $\rho_1$ is a density of the solid substance, $\rho_2$ is a density of the liquid, $h$ is a trajectory of the particle fall, $t$ is time of sedimentation and $g$ is a gravitation acceleration.

Advantages of sedimentation methods using the gravitation field include a clear measuring principle, an easy performance not requiring complicated equipment and physically unambiguous interpretation of results. Disadvantages, as time exigency (from 15 minutes up to 8 hours), a relatively narrow range of measurement and the dependence of results on sample preparation, in particular the sensitivity of results to an optimum degree of de-agglomeration, can hinder result reproduction. Particles too large cause turbulent flow, while too small particles are influenced by the Brown movement. Sedimentation methods are therefore suitable for analysing powders and suspensions with the particle size in the range of 0.3–200 µm. The most used devices utilizing gravity sedimentation is so-called Andreasen pipette and sedimentation balance.

Andreasen pipette device

The principle of the method lies in sorting the analysed particles to fractions due to different sedimentation rate of differently sized particles.

The device consists of a 500 ml cylinder and a pipette with a three-way tap, which ends 20 cm under 500 ml mark (height is 20 cm) (Fig. 8.8). An analysed sample (suspension) is left standing and the concentration of particles at a certain place, i.e. at a certain height of the sedimentation column, is monitored in pre-set time intervals. Concentrations are determined by taking away a small quantity (10 ml) of the suspension with a pipette. Fractions are dried and the dry matter
weight is determined. With regards to the optimum measurement, time intervals between individual samplings should grow in geometrical series; in case of particles at the size of approximately 1 μm and smaller, the complete measuring by this technique can last even for several days. Particle diameters corresponding to various time intervals can be then calculated from Stokes' law.

Measuring conditions include an identical density of analysed particles and constant temperature (due to constant viscosity and density of the liquid). The concentration of particles should be lower than 0.2 % (v/v) to prevent interaction of the particles.

**Fig. 8.8** Andreasen pipette.\(^{23}\)

*Sedimentation balance*

It is a device which serves for measuring of a sedimentation curve. A balance pan is submerged in a suspension dispersion. Particles sediment on the pan and the increase of weight is recorded continuously. Increase corresponds to the settled size fractions. There are two types of sedimentation balances:

- Double-arm balance with continuous automatic weighing and registration of the weight of sediment.
- Single-arm balance, e.g. Figurovsky balance (Fig. 8.9), where the balance pan is hung on a quartz or glass fibre which bends under the growing load; the bend of the fibre is observed e.g. by a cathetometer.

Conditions to be met for achieving reliable results are the absence of interactions between particles (diluted suspensions are suitable) and laminar flow, therefore before measurement too large particles must be removed, usually by sieving through a sieve with the mesh size of 63 μm; the sieve fraction above 63 μm can be evaluated by sieve analysis and the results from both methods can be then combined to obtain full particle size distribution. This is possible because the sedimentation balance method and the sieve analysis both provide weight-balanced distribution.

### 8.1.5.2 Sedimentation in centrifugal field

The method uses the determination of particle size in an ultra-centrifuge. Rotation velocities of the centrifuge range from 500 to 24000 rpm; higher rotation velocities are used for particles smaller than 0.1 μm. To detect particles, an optical system using most often white or monochromatic UV/Vis radiation or X-ray radiation is used. The weakening of radiation intensity after the passage through the sample is measured in both cases (absorption of X-ray radiation or scattering of UV/Vis radiation). The particle diameter can be calculated on basis of weakening of radiation intensity (it is proportional to the number of particles) and their density. A basic requirement is that all particles of the sample have the same density. Advantages include the speed of analysis (usually 10–20 minutes) and measuring of small particles at the range of 0.02–10 μm.

### 8.1.5.3 Sedimentation in force field

Sedimentation field-flow fractionation (SFFF) is a flow method which uses a force field of the centrifuge for separating particles according to their size. This method can analyse particles smaller than 1 μm.
The principle of the method is the action of a laminar flow of the carrying liquid inside a separation channel and an external physical field (acting perpendicularly on the flow direction) on analysed particles. Mutually perpendicular orientation of the field action direction and the flow direction differ SFFF from other classical methods based on the action of fields (e.g. centrifugation). Particles of various sizes have different velocity of passage through the separation channel and they are detected at the output by means of an UV extinction detector.

8.1.6 COULTER PARTICLE COUNTER

The Coulter principle was revealed by Wallace H. Coulter in 1948 but it was not patented until 1953. This invention uses the measurement of electrical resistance for the determination of the number and size of microscopic particles (originally of blood elements). Particles suspended in a conductive liquid (electrolyte) act as an insulator. The suspension of measured particles slowly flows through a glass tube with a single small hole which connects the liquid inside and outside the tube. The passage of every single particle through the hole increases the electrical resistance between the two submerged electrodes situated on both sides of this hole at the given moment. This decreases electric current briefly, inducing an electrical impulse which can be registered, and its volume can be measured. While the number of impulses indicates the number of particles in the measured sample, the pulse amplitude is proportional to the volume of a particle (Eq. 8.11):

$$U \propto \frac{V \times \sigma \times I}{R^4}$$  \hspace{1cm} (8.11)

where $U$ is the voltage pulse amplitude, $\sigma$ is a resistivity of the liquid, $V$ is particle volume, $I$ is the aperture current and $R$ is the aperture radius. Coulter particle counters can count and determine the size of particles with dimensions of range from 0.5 $\mu$m to 1200 $\mu$m.

8.2 PARTICLE SURFACE STRUCTURE

One of the parameters which define quality of solid pharmaceutical microforms is surface. Surface and surface unevenness are proportional to the porosity of particles, selected technological procedure and properties of powder mixtures and used raw materials. The surface of uncoated dosage forms influences the rate of API release, abrasion
and the adhesion of potential sugar or polymeric coat. The surface of coated forms is closely related to the dissolution rate, permeability and gloss.

Methods used in the study of surface structure provide information (physical, chemical or physicochemical) about characteristics of the studied material. A result of measuring physical properties can be a volume or area of the solid phase and the size of pores (chapter 8.2.1. and 8.2.2.) or the 3D profile height or width (chapter 8.2.3.). To evaluate physicochemical properties of the surface, e.g. the inversion gas chromatography is used (chapter 8.2.4). Modern microscopic methods are very often used in the surface analysis, such as laser confocal microscopy (chapter 9.1.5), methods of scanning probe microscopy (chapter 9.3) or scanning electron microscopy (chapter 9.2.4). The analysis of particle surface structure can also include its chemical composition. Methods such as chemical mapping by means of infrared or Raman spectroscopy (chapter 3.2.2) or scanning electron microscopy complemented with an X-ray analysis with energy dispersion (EDAX; chapter 9.2.1.5) are suitable for these purposes.

**8.2.1 SPECIFIC SURFACE AREA DETERMINATION**

Specific surface area is an important property of solid particles defined as the total surface area of a material per unit of mass. The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbed gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbed gas molecules and the adsorbent surface of the tested powder. One of the most used concept is Brunauer, Emmett and Teller (BET) surface area determination. The method expands the Langmuir adsorption model by assumption that the gas adsorption can happen in multilayers. The determination is usually carried out at the boiling point of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure. BET is useful in evaluation of product performance and manufacturing consistency (e.g. the specific surface area is proportional to the dissolution rate). For example, the ISO 9277 standard for SSA solids calculations is based on the BET method.
8.2.2 MERCURY POROSIMETRY

Porosity is a property of solid or semi-solid materials. These materials contain a number of small more or less evenly distributed cavities and openings of various shape and origin. Porosity lowers the weight of the material and can affect many other abilities, such as water absorption. For porosity measurement, non-wetting liquid substances are used, most often mercury.

Mercury porosimetry is based on the existence of a surface tension and on the phenomenon of capillary depression. Mercury does not wet porous substances, but only envelopes their surface. The volume of solid phase is determined on the basis of the volume of mercury displaced by the sample. The volume measured in this way corresponds to the tapped volume.

Mercury can also penetrate to pores but in such case it is necessary to use an external pressure on the system. By increasing the pressure, mercury penetrates even into the finest pores and the values of intra-particular porosity and the information about pore size can be obtained in this way. The calculation of pore size is derived from an inverse ratio between the radius of the filled-in pore and the value of the used pressure.

However mercury porosimetry has numerous disadvantages: mercury is toxic and special procedures must be adhered to when handling it, a tested sample must be inert to mercury, a sample is contaminated after measurement and therefore it must be disposed of in the respective way.

8.2.3 PROFILOMETRY

8.2.3.1 Parameters of surface unevenness

The rate of surface unevenness can be quantitatively expressed by means of several parameters. An arithmetic average roundness \( R_a \) is most often determined, which can be defined as an arithmetical average of deviation of the surface profile above and below the central reference line referred to the measured length. \( R_a \) of the linear scan is calculated according to the following equation (Eq. 8.12):

\[
R_a = \frac{1}{N} \sum_{n=1}^{N} |Z_n - Z^-|
\]  

(8.12)
where \( N \) is a number of measured points, \( Z_n \) is an individual height of the \( n \)th point and \( Z^- \) is an average value of all the measured heights.

Parameter \( R_t \) expresses the maximum distance between maxima and minima of the measured profile (peak to valley height). In case of a linear scan, the value of \( R_{tm} \) is an arithmetical average of \( R_t \) values obtained from five sampling sections and it is determined according to the following equation (Eq. 8.13):

\[
R_{tm} = \frac{1}{5} \sum_{n=1}^{5} |R_t|
\]

8.2.3.2 Optical profile microscopy

Profile microscopy enables contactless non-destructive measuring of the thickness of transparent coats and analysing the surface unevenness of solid dosage forms, e.g. tablets, pellets or individual grains of a granulate.

Optical analysis of the surface and measuring of film thickness require special equipment, so-called profile microscope. Light radiation of variable intensity comes from a source (a lamp) and passes through a narrow slot which narrows the beam and directs it to an object lens. The directed beam impacts on the surface of the substrate under the angle of 45°. A part of the light is reflected from the film surface (if present) and a part is reflected from the core surface. A profile line of the core and the coat is displayed at the same time in the eyepiece of the microscope situated in the opposite angle of 45°. A squared net in the eyepiece enables deducting the distance at the accuracy of a tenth of micrometre. To measure the coat thickness, the method is limited with its transparency. Parameters of peak to valley height roughness \( R_t \) and mean of the maximum peak to valley heights \( R_{tm} \) can be determined by means of a profile microscope. The value of arithmetic average roundness \( R_a \) can only be determined from a photographic record.

8.2.3.3 Surface profilometry

Equipment for measuring surface unevenness (profilometer) examines surface of a sample by means of a special tip which directly touches the sample. Vertical movement is converted to an electrical signal in a piezoelectric cell where it is amplified and processed by software, thus directly providing values of parameter \( R_a \). A typical surface analysis
is performed in 0.8 mm sampling sections along the total length of 5 mm. During measuring the tip and its support must not destroy or damage the surface of the measured sample.

8.2.3.4 Contactless laser profilometry

Contactless laser profilometry uses infrared radiation from a semiconductor laser focused on the examined surface by means of an object lens. Radiation reflected by the sample surface is directed by a prism to a beam distributor and is displayed as a pair of points (with the diameter of 1 µm) on photodiodes. If the object lens is situated exactly at the focal distance from the tested sample, diodes are evenly radiated. If the distance between the sample surface and the object lens is disturbed due to surface unevenness, the measured beam is diffracted and diodes are unevenly radiated. This change causes an error signal which results in the check and adjustment of the position of the moving object lens hung in the probe so that points of impact of the focused beam always remained identical. In this way, changes in lighting are converted to amplitude parameters ($R_a$, $R_t$ and $R_{tm}$), which express the rate of surface unevenness. Instrumentation enables to obtain output in form of an image with surface profile diagram (Fig. 8.10).

![Fig. 8.10](image)

**Fig. 8.10** The use of contactless laser profilometry for characterisation of the surface topography of solid dosage forms. The picture shows the topography of the coating layer made from ethyl cellulose. 3D Optical Surface Metrology System Leica DCM8 was used. The profile of selected part (white line on the surface picture) is shown in the bottom part of the picture.
8.2.4 Inverse gas chromatography

Inverse gas chromatography (IGC) is a method suitable for the determination of surface energy and an overall description of particle surfaces. It is used for measuring the surface properties of a wide spectrum of materials including pharmaceutical powders. An advantage of this technique is in testing the material without any special pre-measuring adjustment.

IGC has inverse principle compared to the classical gas chromatography. This means the chromatographic column is filled with an unknown (measured) sample and molecules of known composition in gaseous phase (e.g. ethanol) are carried through the column by a suitable carrier (inert) gas. These molecules interact with the analysed sample and on the basis of this interaction they are washed out from the column in different times. It is possible to calculate a distribution coefficient for the gas-solid phase interaction from the retention time or the volume. Numerous physical-chemical properties of solid materials can be determined from these basic data, such as surface energy, acid-base surface properties, or solubility parameters. Surface energy has been established as a key property of a particulate or powdered material. The quantification of attractive intermolecular forces within a solid provides its surface energy, an analogous value to a liquid's surface tension. These intermolecular forces are the cause of the attraction among solids such as powder particles and liquids, occurring through short range chemical forces (polar forces) and long-range van der Waals forces. Hence, surface energy is related to several key properties of solids such as surface chemistry, adsorption capacity, static charge, adhesion, process-induced disorder, agglomeration, powder flowability, dispersability, and wetting.

8.3 References


8 Evaluation of Size, Shape and Surface of Particles


9 MODERN MICROSCOPIC METHODS

Microscopic techniques and their applications have an irreplaceable use in many scientific fields and can significantly contribute to important discoveries in pharmaceutical technology.

To assess the shape and surface of solid particles in powders, granulates, aero dispersions and suspensions, to observe the appearance of drug microforms (pellets, microparticles, nanoparticles), to evaluate the structure of uncoated and coated tablets and films or to measure the size of drops in emulsions and creams, visualization is necessary. For this purpose, optical or electron microscopes are usually used. System evaluation by microscopic methods does not only provide highly valuable information on the shape, but also on the size, microstructure, surface topography, porosity and often also on the physical condition and distribution of substances in the final product. This information can be also used as a clue what measurement will be needed with respect to the particle characteristics.

There are currently numerous techniques available. Microscopic methods can be divided according to various criteria, e.g.:

- According to the principle of observing and display of the object: light, electron, probe microscopy.
- According to viewing the object: displaying all points at one time (e.g. trans-illuminating electron microscopy) or gradually by scanning the surface (e.g. scanning electron microscopy).
- According to the illumination of an object: microscopy in transmitted light (lighting up techniques) or microscopy in incident light etc.

Every microscopic method has its advantages and disadvantages. They differ in their principle, resolution, magnification and demands on the sample preparation.

9.1 OPTICAL (LIGHT) MICROSCOPY

Although the importance of optical microscopy has decreased a bit with the development of other instrumental analytical methods
(especially of electron microscopy analysis), it is still often used in practice due to its relative instrumental and financial availability. In pharmaceutical technology it is used especially for observing morphological properties (particle size, shape, coat thickness, etc.). Methods of optical microscopy are based on observing the interaction of Vis light with a sample. It enables up to thousands of times magnification with standard preparation of sample and without any special modification to the microscope. It is possible to observe objects in natural conditions including humidity and at lower or higher temperature after minor modifications.

Optical microscopy represents a very fast and simple method. Combined with a digital camera or a camcorder, it enables collecting and saving a lot of data which can be further mathematically processed by means of specialized computer software (chapter 8.1.4).

9.1.1 PRINCIPLE OF DISPLAY AND MAGNIFICATION BY MICROSCOPE

Optical devises use lenses made usually of glass or plastics (e.g. polycarbonate), which can be of two basic types: converging and diverging, both further distinguishable to several subtypes according to the surface shapes (Fig. 9.1).

![Fig. 9.1 Basic types of lenses. Converging lenses: biconvex (1), plano-convex (2), concave-convex (3); diverging lenses: biconcave (4), plano-concave (5), convex-concave (6).](image)

Every lens has two focal points, one in object plane and one in image plane. The distance of the focal point from the lens principal plane (virtual plane going through the centre of the lens perpendicularly to the optical axis) is called the focal length \((f, f')\) (Fig. 9.2).
Fig. 9.2 Lens: $C$ and $C'$—centres of lens curvature, $F$—object focal point, $F'$—second focal point, $O$—optical centre of lens, $f$—object focal length, $f'$—image focal length.

When light passes through the lens (Fig. 9.3), light is refracted on the interface of the lens and environment, and a small part of light is reflected. Refraction is subject to the Snell's Law of Refraction. The following rules apply to light refraction through a converging lens:

- Ray falling in parallel with the optical axis is refracted to the second focal point $F'$ (Fig. 9.3, ray $a$).
- Ray passing through the centre of curvature is not refracted (Fig. 9.3, ray $b$).
- Ray passing through an object focal point $F$ is refracted in parallel with the optical axis (Fig. 9.3, ray $c$).

Fig. 9.3 Passage of light rays through a converging and diverging optical lens; $a$, $b$, $c$—impacting light rays; $a'$, $b'$, $c'$—light rays after passage through the lens.

The principle of sharp image creation is the fact that light rays spreading from a certain point of object in various directions and falling on the lens to converge again, really or virtually, in a single point, compile a sharp image of the object. The image appearance in converging lenses imaging depends on the distance of the object from the lens (Table 9.1).
If an object is far more than double of the focal length, a real, reduced and inverted image of the object is created. If an object is between the double of focal length and the focal point, the created image is real, magnified and inverted. If an object is between the focal point and lens, the created image is virtual, magnified and upright (Fig. 9.4).

The following two equations apply to the observation by a lens: the display equation (Eq. 9.1) and the magnification equation (Eq. 9.2):

\[
\frac{1}{a} + \frac{1}{a'} = \frac{1}{f} \quad (9.1)
\]

\[
Z = \frac{1}{y} = -\frac{a'}{a} \quad (9.2)
\]

where \(a\) is a distance of the object, \(a'\) is a distance of the image, \(f\) is a focal length, \(Z\) is magnification, \(y\) is a height of the object and \(y'\) is a height of its image.

**Table 9.1 Rules for displaying through the lens**

| Lens           | \(a\)  | \(a'\) | \(Z\) | \(|Z|\) | Image properties                  |
|----------------|-------|--------|-------|-------|-----------------------------------|
| Condensing     |       |        |       |       | Magnified, upright, virtual       |
| lens \(a < f\) | \(a' < 0\) | \(Z > 0\) | \(|Z| > 1\) |       | The image is in infinity           |
|                | \(a = f\) | \(a' \rightarrow \infty\) |       |       | Magnified, inverted, real         |
|                | \(f < a < 2f\) | \(a' > 0\) | \(Z < 0\) | \(|Z| > 1\) | Equally large, inverted, real     |
|                | \(a = 2f\) | \(a' > 0\) | \(Z < 0\) | \(|Z| = 1\) | Reduced, inverted, real           |
|                | \(a > 2f\) | \(a' > 0\) | \(Z < 0\) | \(|Z| < 1\) | Reduced, upright, virtual         |

* Example of an optical device: magnifier, microscope eyepiece; ** Example of an optical device: microscope eyepiece; *** Example of an optical device: camera
The operation of a basic optical microscope can be explained on a layout of two sets with converging lenses, as shown in Fig. 9.5. Near the sample there is a system of converging lenses called an objective. It is characterized by a very short focal length. An observed object is placed between the focal point and the twice of the focal length of the objective ($2f > a > f$). Objective creates a real, inverted and magnified image of the object (Fig 9.5; image 1), which is projected between the focal point of the eyepiece and the eyepiece lens ($a < f$). Consequently, this image is observed through the eyepiece as it would be under a magnifier. A final virtual, magnified and inverted image of the sample is created in the eyepiece (Fig 9.5; image 2).
9.1.2 DESIGN OF MICROSCOPE

A classic optical microscope is relatively simple device which consists of several mechanical and optical parts (Fig. 9.6).

The mechanical part of a microscope includes:

- Base and arm ensure stability of the device. It has a leg in the lower part to which an arm bearing a body tube is connected.
- Body tube is a basic part of a microscope. One (monocular microscope) or two eyepieces (binocular microscope) are inserted to its upper part. There can be another optical output (trinocular microscope). A revolving objective holder is attached on the opposite end of the body tube. An optical and mechanical length of the body tube belongs among basic parameters of a microscope.
- Stage with clips serves for placing an observed object to an optical axis of the microscope. Sample is usually placed on standard microscope slide (76 × 26 × 1 mm). It is covered with a cover slip with the standard thickness of 0.17 mm. Two metal clips enable fixing the sample. A cross horizontal movement of the stage is ensured by two edge-notched screws which control the movement of a sample guide.
- Screws ensure micrometric and macrometric vertical shift of the stage, i.e. the shift in the direction of the optical axis.
- Revolving nosepiece enables fast exchange of objectives. A revolving nosepiece can take up to 5–6 objectives which are fixed to it by a thread.

The optical part of a microscope consists of three sets of lenses:

- Eyepiece magnifies the image created by the objective. It consists of two or more lenses. The magnification is printed on the shell (usually 5–20 times).
- Objective ensures magnification and resolution of a microscope. Its magnification and numerical aperture are noted on the shell (e.g. 100/1.4).
- The third set is called a condenser. The condenser ensures the maximum illumination of the objective. It is a set of two or three lenses with a short focal distance. Its task is to concentrate the light rays from the light source to a sample. Important part
is an iris diaphragm which enables selecting width of the passing beam. A condenser is usually equipped with a bracket in which colour filters can be inserted. In general, when weaker objectives are used, the condenser is lowered and the iris diaphragm is closed.

The optical part is further supplemented with a light source built in into the stand of the microscope. It consists of a power transformer to 220 V/50 Hz with the input voltage control (6 or 12 V) which supplies power to a halogen lamp with the output of 20–100 W, which controls the intensity of illumination. The light is directed to the condenser of the microscope by a collector and a collector diaphragm.

**Fig. 9.6** The design of an optical microscope.

### 9.1.2.1 Resolution and magnification of microscope

Microscope resolution (and in a broader sense optical resolution) is a distance between two points which can be still distinguished as two separate entities (resolving ability). Thanks to the diffraction influences, no objective displays a point as a point again, but as a system of concentric circles of various intensity. These circles are also called diffraction, bending or dispersion rings. The central, and the brightest ring is called Airy disc (according to Sir George Airy). Airy disc is surrounded by a system of diffraction rings, whose brightness quickly decreases
with the distance from Airy central disc. When two near points are displayed, respective diffraction rings can overlap and at certain minimum distance they become unresolvable (Fig. 9.7).

Fig. 9.7 Microscope resolution and Airy ring (y is a distance of centres of two displayed points, δ is a resolution limit, the two points on the right image merge and the image will remain unresolved)

A microscope resolution can be thus understood as a measure of image clarity. It is limited by the wavelength of Vis light as can be deduced from the formula (Eq. 9.3) describing relation between microscope resolution and the wavelength of the used radiation:

$$\delta = \frac{\lambda}{n} \times \sin \alpha$$  \hspace{1cm} (9.3)

where δ is a resolving power (or resolution), λ is a wavelength of radiation, n is a refractive index of the environment between the objective and the observed object, α is an opening angle, i.e. a half of the angle formed by rays going from the object which are captured by the objective (Fig. 9.8). If a yellow-green light with the wavelength of ~ 550 nm is used for display, the achieved resolution is about 0.6 µm, shorter wavelength, e.g. a blue light with the wavelength ~ 400 nm, will decrease the resolving power value even further.

The expression $n \times \sin \alpha$ is called a numerical aperture (NA) of an objective which expresses the acceptance cone of the objective. The numerical aperture of the highest quality objectives reaches the values about 1.3–1.5.

Fig. 9.8 Opening angle of an objective.
Most objectives are dry objectives with air between the objective lens and the sample. The refractive index \( n \) of air is 1; the numerical aperture thus cannot exceed the approx. value of 1. From the aforementioned equation it is evident that by replacing air environment with a so-called immersion medium with a higher refractive index (water, paraffin, glycerol, cedar oil) it is possible to increase numerical aperture. In practice the immersion medium is dropped straight on the microscope slide with the sample, immersing both, the sample and the objective. Maximum achievable resolution is approximately 0.17 µm if a short-wavelength blue light is used. Achievable resolution of various microscopic technologies is shown in Table 9.2.

### Table 9.2 Resolution of microscopic technologies

<table>
<thead>
<tr>
<th>Microscopic technology</th>
<th>Achievable resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked eye</td>
<td>~ 0.1 mm</td>
</tr>
<tr>
<td>Light microscopy</td>
<td>~ 0.2 µm</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>&gt; 2–3 nm</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>&gt; 0.1 nm</td>
</tr>
<tr>
<td>Scanning Probe Microscopy</td>
<td>~ 0.1 nm</td>
</tr>
</tbody>
</table>

Another microscope characteristic is magnification which is increase in apparent size of the displayed objects. A total microscope magnification \( Z_m \) is given by a product of objective magnification and eyepiece magnification and the following equation (Eq. 9.4) applies to its calculation:

\[
Z_m = Z_{objective} \times Z_{eyepiece}
\]  

(9.4)

There are few limits to the amount of total magnification in order to prevent the so-called empty magnification. Empty magnification occurs when the image continues to be enlarged but no additional detail is further resolved. This usually occurs when eyepieces with higher magnification are employed. To avoid the empty magnification, it is necessary to take the numerical aperture into account. Simple tool to keep the magnification in useful values to resolve the image is to have its value between \( 500 \times NA \) (minimum value magnification) and \( 1000 \times NA \) (maximum value magnification). For example: microscope with objective magnification \( 100\times \), eyepiece magnification \( 15\times \) and \( NA \) 1.25 will result in empty magnification \( (1500 > 1250) \). However, if the eyepiece
magnification is decreased to 10×, the empty magnification will be removed (1000 < 1250).

A depth of objective focus is a difference between the distance of the nearest and the furthest place of the displayed object, which are still displayed with acceptable sharp definition. This means the point lying in this plane is seen sharply and the points in other planes are seen defocused. A very low depth of focus thus causes that it is impossible to focus and observe all structures at one time in a spatial object. Less magnifying objectives (4–10 times) have a deeper depth of focus compared to objectives which enable a higher magnification (40×, 60×, 100×).

9.1.3 BRIGHT FIELD MICROSCOPY

The principle and design described above represent the basic bright field microscope set up. However, several modifications and upgrades exist, which enable to observe objects at more specific condition, even allowing visualization of images which would be unobservable by standard technique. As an example, Fig 9.9 (a, b) shows use of a classic bright field transmission microscope for PLGA microparticles. Observer can see polydisperse system with particle size not exceeding 50 micrometers, in case of Fig. 9.9 (a) with almost all particles smaller than 10 micrometers. Computer software enables to measure the particle size and the method can be thus used as complimentary/verification method to laser diffraction. Moreover, classic microscope can be used to observe emulsions if dyed appropriately. For example, fig. 9.9 (b) shows lipophilic phase released from pellets formulated with liquid-solid system.

9.1.3.1 Inverted microscope

Unlike common microscopes, these microscopes have the optical system inverted, i.e. the objective is under the observed object and the light source is above the sample. The microscope is equipped with two body tubes with eyepieces for observation by both eyes, and usually a third body tube for connecting a digital camera or a camcorder. This set up eliminates the need of microscope slide and slip, allowing observation without sample preparation and in more natural conditions. Inverted microscopes are often equipped with instruments for phase-contrast imaging (deliberate phase wave change to increase contrast). Inverted
Microscopes are mostly used for observing tissue cultures in nutrient solutions and as metallographic microscopes. In pharmaceutical technology they can be for example used for measuring angle of contact of microparticle carriers to determine wetting properties.

**Fig. 9.9** Classic bright field microscopy: PLGA microparticles (a, b); An emulsion o/w formed after release of lipophilic phase from pellets, dyed with Sudan III (c). Red bar in c corresponds to 100 micrometers.

### 9.1.3.2 Polarized light microscope

Polarized light microscopy solves the fact that some substances in sample can be properly observed only thanks to their anisotropic character, which means they double-refract polarized light. However, a non-modified light beam is non-polarized, being a random mixture of waves with different characteristics. To obtain a polarized light beam, a polarizer is used. It is a type of optical filter, usually situated near condenser, which removes the randomness by letting through only the waves with defined characteristics. Such created polarized light passes through the sample and double-refracts on anisotropic structures. These two wavefronts are recombined in an analyser, which is a second
polarizer placed between the objective rear aperture and the observation tube. Double-refracting structures (anisotropic) are imaged as light or coloured (interference) structures, meanwhile isotropic structures remain darkened. In pharmaceutical technology, polarized light microscopy can be used for example for crystal detection in various excipients used for dosage form formulation.

**9.1.3.3 Stereo microscope**

Another type of optical microscopes is a stereo microscope (a stereo magnifier). A stereo microscope was named for the possibility to observe the sample by both eyes while preserving the stereoscopic spatial effect of vision, typically using reflected light from the object instead of light transmission. Unlike classical optical microscopes, it does not use one, but two independent optical systems with two objectives (or one wide objective with two separate optical pathways) and two eyepieces. This arrangement results in slight differentiation of view angles for the right and the left eye. The resulting image is created by the compilation of images in the brain of the observer. These microscopes are thus adjustable to human physiology and unlike classical microscopes, they enable three-dimensional observation of the studied object. A stereo microscope should not be confused with a common assembled microscope equipped with two eyepieces, where both eyes see the same image in such device.

Modern stereo microscopes are produced on the basis of two different concepts (Fig. 9.10). The first one is called the “Greenough concept”. Two identical objectives are arranged in such way that their optical axes form a minimum angle. Two separated images are generated there, and they are observed through two separated eyepieces. A spatial image is created by the combination of these two images in the human brain. The second concept is called the “telescope concept”. In this case, two microscopic systems, in which a light signal is directed by a system of lenses to a common objective, are arranged in parallel. The stereo effect is achieved by the combination of two axes of beams. This system is more complicated, thus more expensive, but it offers a possibility to use numerous additional modules increasing the quality and flexibility of microscopic analyses.
An advantage of stereo microscopes is a long working distance and a relatively low purchase price. They are suitable for achieving lower magnifications (compared to classic optical microscope) from 5 to 120 times. Their disadvantages are a relatively low depth of focus and the necessity to use an external light source. For solid dosage forms excipients characterization in pharmaceutical technology stereo microscope is used more often than classic bright field microscope. With appropriate software the observer can evaluate particle size and some other dimension-based characteristics along with sphericity and cheap visualization of particle shape. It can be also used for some other special observation/measurement like coat thickness measurement or observation hydrophilic matrix hydration (Fig. 9.11).

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9.1.4 Fluorescent Microscopy

Fluorescent microscopy is based on the fluorescence phenomenon, a subtype of luminescence. A substance absorbs light or another electromagnetic radiation, causing electron excitation, which is then followed by return of the electron to lower energy level and emission of light with longer wavelength. Emitted light is captured by a detector and creates an image of the observed object. Residual excitation radiation is filtered off and does not participate in image creation. The most graphic transition is in the cases when UV radiation is absorbed (invisible) and Vis light with longer wavelength is emitted. For the excitation of visible radiation in objects to be efficient, these objects must contain chemical substances called fluorochromes (photoindicators, fluophores) able to specifically absorb impacting UV radiation and emit coloured Vis radiation.

A specific light source system is necessary for fluorescence microscopy to obtain needed wavelengths. High-pressure mercury discharge lamps are often used, alternatively fluorescence lamps can be employed. These devices emit high quantity energy with peaks in broader spectrum including UV radiation. To sift desired wavelength peak for sample illumination, fluorescence filters are used.

Fluorescence microscopy is currently one of the most often used microscopic techniques suitable especially for observing biological structures (e.g. proteins, lipids or particular DNA sequences) and processes. It is also used in dosage form structure and degradation studies (Fig. 9.12). Advantages of fluorescence microscopy include a high contrast of resolution, a high sensitivity to present molecules and low concentrations of fluorescence colorants used for marking the specimen.

Fig. 9.12 Fluorescent microscopy of coumarin-6 loaded PLGA particles: classic general view (left), 3D – model (right).
9.1.5 Laser Confocal Microscopy

Laser confocal microscopy is one of the most important progresses in microscopy, thanks to bright, sharp and/or possible three-dimensional visualization of an observed object. A confocal microscope eliminates non-focused light (i.e. light coming from areas situated outside the focused plane of the sample) which can causes image “blurring” of thicker three-dimensional samples in case of conventional optical microscopy. Individual 2D plane images can then be used for computer design of a 3D model of the sample.

An observed object in confocal microscopy is lit with an intensive point illumination source, most often a laser beam. It is focused on a diaphragm (dichromatic mirror), from where it passes through the objective to the sample. It is displayed there as a point with the diameter equal to the minimum distance of two resolvable points. The same objective collects the light reflected and dispersed by the sample up to another confocal point diaphragm. It filters the light coming from areas outside the focal plane of the microscope. After the passage of this radiation through the point diaphragm the light falls on the detector (photomultiplier) which amplifies the formed image. During scanning the signal from the photomultiplier is registered by a computer together with the information about coordinates of analyzed points. At one time one point is displayed, the whole image is formed by rasterizing. This scanning is automated and controlled by a computer. The computer compiles a complete image from the gathered information. A disadvantage of the confocal microscope is its high price.

Abilities of the confocal microscope to display specimen with three-dimensional resolution is used e.g. in studying surface properties of materials (pattern and composition of surfaces, erosion of materials); it is especially suitable for newly developed applications in micro and nanotechnological field. It can be also used for characterization of particle deformation during tablet compression. Biology and medical research benefit from the fact that the confocal microscopy is a non-invasive and non-destructive method for studying the spatial structure of cells and tissues. By means of special fluorescence probes it can be used e.g. for studying intracellular concentration of physiologically important ions (e.g. K+, Ca2+), measuring a membrane potential.
or intracellular pH. Intracellular tracking of nanoparticle-based drug delivery systems can be performed using fluorescently labeled nanoparticles, e.g. liposomes or polymeric nanoparticles and microparticles. Colocalization studies with various cellular organelles, e.g. lysosomes can be performed.

![Fig. 9.13 Laser confocal microscopy in liposomes internalisation study.](image)

As a laser confocal microscopy example, the Fig. 9.13 shows fluorescently labelled drug-loaded liposomes (green) internalised by a cell. The cell nucleus was stained using DAPI (blue) and endosomes were stained in red. Colocalised signal is visualised as a yellow colour. The fate of nanoparticles in cells can be thus observed using confocal microscopy.

### 9.2 Electron Microscopy

Microscopic display techniques using a flow of electrons can be divided according to the method of image creation to two basic groups. The first group is based on a well-known microscopic technique concept in which
A specimen is observed as a whole image at once. Radiation passed or diffracted by the specimen is processed in a display system and the result is an image of the observed object. An example is the transmission electron microscopy (TEM). The second type of display equipment is represented by scanning (raster, line) microscopes. They scan a specimen point by point and the whole image is created by compiling individual signals. A typical example is the scanning electron microscopy (SEM). It uses an electron beam concentrated into a smallest area possible (a point). A series of signals arise by the interaction of the electron beam with the specimen. Signals are detected and they can be used for obtaining an image of the observed object.

Main advantages of electron microscopy include high magnification (up to 1,000,000 times), high resolution (tenth of nm in orders) and a large depth of focus (several mm). An advantage is also the fact that an electron microscope can provide information about topography of the sample as well as about its material composition. Disadvantages include especially high demands for space, a high purchase price and the necessity to place a sample into vacuum.

9.2.1 Properties of Electron Flow and Principles for Image Creation

As optical microscopes use Vis light as a signal carrier, electron microscopes use electron beams accelerated in vacuum for displaying the observed objects.

Electron radiation, similarly to light and other forms of radiation, has a dual character, i.e. it has the properties of particles as well as waves. According to Louis de Broglie, a wavelength can be assigned to a moving electron (Eq. 9.5):

$$\lambda = \frac{h}{m \times v}$$  \hspace{1cm} (9.5)

where $h$ is a Planck constant ($6.626 \cdot 10^{-34} \text{ J s}$), $m$ is a weight of the electron ($9.109 \cdot 10^{-31} \text{ kg}$) and $v$ is a velocity of the electron.

Negative charge of electrons enables their acceleration by an external electrical (so-called accelerating) voltage. The following equation (Eq. 9.6) applies to the kinetic energy $E_k$ of an electron accelerated by an external electrical field in vacuum:
\[ E_k = e \times U = \frac{1}{2} m \times v^2 \]  

(9.6)

where \( e \) is the charge of the electron \((1.602 \times 10^{-19} \text{ C})\), \( U \) is an accelerating voltage \((\text{V})\), \( m \) is a weight of the electron and \( v \) is its velocity. An equation (Eq. 9.7) for the calculation of resulting velocity of a travelling electron can be obtained by modifying this equation to the form:

\[ v = \sqrt{\frac{2e \times U}{m}} \]  

(9.7)

By combining equations (Eq. 9.5 and 9.7), equation (Eq. 9.8) can be obtained which shows that the wavelength of the flow of accelerated electrons is inversely proportional to the square root of the used accelerating voltage:

\[ \lambda = \frac{\hbar}{\sqrt{2e \times U \times m}} \]  

(9.8)

By incorporating constants, this equation is simplified to the following form (Eq. 9.9):

\[ \lambda = \frac{1.227}{\sqrt{U}} \]  

(9.9)

When accelerated electrons interact with the mass of the sample, they are dispersed and decelerated in the field of nuclei and electron shells of atoms of the sample. Signals (Fig. 9.14) which give various information about the elementary composition of the tested object arise, such as topography of sample surface, chemical composition, dispersion of electrical and magnetic fields etc.

Interactions between primary electrons and atoms of the specimen can be simply divided into two groups, namely to flexible or elastic collisions, which result in the occurrence of reflected electrons, and inflexible or non-elastic collisions, in which the energy of primary electrons is handed over to atoms of the sample and consequently secondary and Auger electrons are released, X-ray radiation is emitted and cathodoluminescence occurs. In TEM, electrons penetrate through the observed specimen, where they can be deviated from the original direction by interactions with the sample. The majority of deviated electrons is excluded from the beam by means of a diaphragm. The image
is then created by an impact of mostly non-deviated electrons in the display system.

Figure 9.14 shows an impact of an electron beam on the surface of thicker samples and the depth from which the given signal still can be observed. For example, if primary electrons have energy $E$ which enables them to penetrate into the substance into a defined depth, it can be presumed than reflected electrons are able to get back to the surface of the substance from a half of this maximum distance.

![Fig. 9.14 Signals induced by the interaction of an accelerated electron beam with the material of the sample and volume amount from which signals are emitted.](image)

**9.2.1.1 Primary electrons**

Primary electrons have energy $10^3–10^5$ eV and they represent signal detected in TEM (the resolution of 0.5 nm). A part of primary electrons is absorbed by the sample (absorbed electrons). The quantity of absorbed electrons depends on the amount of electron flow passing through the sample and on its chemical composition (the higher the proton number of atoms in the sample, the more electrons it absorbs). An image is created in the basis of different “permeability” of electrons through materials with different chemical composition. If a sample is sufficiently thin, a part of electrons passes through it and elastic scattering of the primary beam can or need not occur, i.e. the deviation of flying electrons from their original direction.
9.2.1.2 Secondary electrons

Secondary electrons (SE) are electrons released after the impact of the primary beam but they have much lower energy (lower than 50 eV) and are the usual detected signal at SEM. Secondary electrons are released from a thin surface layer (5–15 nm) and bring information about surface topography (spatial image with a large depth of focus). An image is created on the basis of different emission of secondary (resp. reflected) electrons depending on the chemical composition of individual phases in the sample.

9.2.1.3 Backscattered electrons

Backscattered electrons (BSE) have energy comparable with the energy of the primary electron beam (the resolution of 50–200 nm) and are also detected at SEM. They emerge from a deeper depth (tens of micrometers) and thus bring information about local changes of the material (material composition of the specimen: morphological and chemical composition). This is called a material contrast.

9.2.1.4 Auger electrons

If an electron from inner layers of the electron shell is driven out, e.g. by a primary electron beam, and an unoccupied shell (energy layer) appears in this layer, an electron from higher layers of the electron shell transfers to this unoccupied inner shell. The energy released in this way can be radiated in the form of photon (X-ray radiation, see below), but in some cases it is handed over as kinetic energy to an electron in the outer shell which thus obtains enough energy to leave the atom. Driven-out electrons are identified as Auger electrons (AE). The probability whether the released energy is radiated by photons as X-ray radiation or by Auger electrons depends on the atomic number of the element in which this process occurs. In general, Auger electrons much more significantly manifest in lighter elements. An element analysis can be performed by measuring their energies.

9.2.1.5 X-ray radiation

X-ray radiation is excited by the impact of a primary beam of electrons and bears information about the chemical composition of the sample (emission X-ray spectrum). The technique of energy dispersive X-ray analysis (EDAX) is used in connection with electron microscopes. EDAX
measures the intensity of X-ray radiation and its energy. The energy of X-ray radiation is characteristic for individual chemical elements; it is possible to identify individual elements present in the sample by comparing known characteristics. Quantitative representation of individual elements is proportional to the intensity of X-ray radiation.

9.2.1.6 Cathodoluminescence
Electron beam induces emission of photons, usually in the Vis, UV and IR spectrum, this process is referred as cathodoluminescence. It is used in specialized applications, investigation and interpretation of the composition and structure of minerals and materials.

9.2.2 Design of Electron Microscope
The source of electrons in electron microscopes is an emission cathode, called emission gun. The oldest type of emission gun, which is not used presently, is a secondary emission cathode. In this case the material of the cathode is bombarded by accelerated ions which drive out electrons from the cathode. The second type of emission gun is represented by thermionic emission cathodes. If any material is heated to a high temperature, electrons are supplied with sufficient energy to overcome a natural energy barrier which prevents them from escaping. The escape energy of the electron is called “work function”. Heating up and consequent thermionic emission can occur at the passage of an electric power through a filament and the probability of an escape of electrons can also be increased by its shaping to a V-shape, when the damage of the ultra-structure at the place of bending facilitates the release of electrons.

A cathode is usually made of tungsten because it has a low output energy of valence electrons ($E_v = 4.5$ eV) and a high melting point ($T_t = 3653$ K) and because it does not require a high vacuum value for its operation. The service life of cathode filament is inversely proportional to the temperature to which the filament is usually heated up. A filament of a tungsten cathode has the operating temperature of approximately 2600 °C. Its service life is then about 40 hours. Cathode made of LaB$_6$ (lanthanum hexaboride) is used in modern types of high-performance electron microscopes instead of a tungsten cathode. This type of cathode has about ten-time higher emission of electrons than a tungsten cathode, but it requires a higher value of vacuum
(10^{-5} \text{ Pa}). This cathode has an operating temperature of about 1600 °C and the service life about 250 hours.

A more efficient source of electrons with unlimited service life is an auto-emission nozzle when a cold tungsten filament removed to a tip with the radius of 60–200 nm emits electrons. An electrode with the positive voltage of 5 kV is placed in the opposite of the tip. An electrical field is formed around the tip which is able to tear out a large quantity of electrons from the surface of the tungsten tip. A disadvantage of the auto-emission nozzle is a high value of vacuum (10^{-6}–10^{-8} \text{ Pa}).

A coherent beam of electrons is required from the electron source, which means that electrons should come from a point source, they should have an identical energy and their accompanying wave should even be at the same phase. Due to these reasons, designing an electron nozzle is more complicated. A nozzle consists of a cathode emitting electrons and an anode with a round hole in its centre which attracts the electrons and gives them sufficient acceleration for travelling through the microscope body tube (Fig. 9.15). A cathode filament is centred to the hole of a Wehnelt column which has negative bias voltage. The Wehnelt column forms an electrical field around the cathode filament which causes that the beam of electrons emitted from the cathode is narrowed so that it forms a cross-over point, i.e. the narrowest place of the beam, immediately in front of the hole in the anode. This place can then be considered a point source of accelerated electrons. The velocity of accelerated electrons from the source is approximately \( v = 600 \times U^{\frac{1}{2}} \).

The trajectory, velocity and width of the electron beam is then adjusted by a system of diaphragms and lenses which create, together with the electron nozzle, together creating an assembly analogous to a lighting system of the light microscope. In this simple method, when the Wehnelt column acts in fact as the first electrostatic lens of a microscope, sufficient stock of electrons with approximately identical initial energy is ensured so that the electron beam has the aforementioned properties.
A trajectory of the flying electron can be influenced by the action of a static homogeneous magnetic field. Magnetic force $F$ than acts on the charge of an electron, the amount and direction of which can be determined according to the following equation (Eq. 9.10):

$$F = e \times \mathbf{v} \times \mathbf{B} \times \sin \alpha \quad (9.10)$$

where $\mathbf{v}$ is an electron velocity vector, $\mathbf{B}$ is a magnetic induction vector and $\alpha$ is an angle formed by these two vectors. In case this angle is not null (i.e. both vectors are not parallel), a force which curves the trajectory of the electron acts on the travelling electron. Electron moves along the circle in homogeneous magnetic field (Fig. 9.16), so the angle $\alpha$ is 90° and the magnetic force equals (Eq. 9.11):

$$F = e \times \mathbf{v} \times \mathbf{B} \quad (9.11)$$

The action of the magnetic field on the trajectory of a travelling electron can be used for constructing an electromagnetic lens which performs an analogical task as a glass lens of an optical microscope. The simplest electromagnetic lens is a solenoid (a round coil). Electromagnetic lenses show so-called aberrations as the result of non-homogeneity and asymmetry of the magnetic field. They include spherical and chromatic aberrations and axis astigmatism.
An electron microscope needs vacuum for its operation due to several reasons:

- Air absorbs electrons and thus shortens the range of an electron beam to the maximum of 1 m.
- Air is not a good insulator and therefore there is a risk of air ionization due to the flow of electrons and consequent occurrence of an electrical discharge between the anode and the cathode.
- Air contains molecules of oxygen, carbon dioxide and hydrocarbons which contaminate walls of the tube as well as the surface of the sample.

Vacuum is not identical in all areas of the microscope; it ranges from $\approx 10^{-3}$ Pa in the screen area to $\approx 10^{-5}$ Pa in the cathode area (for LaB$_6$). A vacuum system of an electron microscope consists of a system of exhauster pumps (ion, diffusion, rotary pumps) and valves which is automatically controlled and checked by vacuum gauges.

### 9.2.3 TRANSMISSION ELECTRON MICROSCOPY

By means of transmission electron microscopy (TEM) it is possible to distinguish structures with the diameter lower than 0.2 nm and reach the magnification of 500–1 000 000×.

A TEM display system consists of a specimen holder, an objective, intermediate lenses, projectives and a fluorescence screen. Nets on which specimens are situated are placed to the holder. Thanks to the holder which fits into a goniometric stage, the specimen can be slightly moved in all directions by means of micrometric screws and it can be even tilted. In modern TEM microscopes, stage movement is controlled by means of small engines and user controls it with a joystick through a computer.
An objective is the most efficient lens of the microscope and it is often called the heart of the whole system. It is able to ensure the highest magnification and also it has the shortest focal distance. To achieve the required performance, the coil of the objective has a large number of threads through which high electric current flows. To prevent its overheating, it is usually cooled with water. The area around the focus point of the objective is often a critical part of the microscope because a lot of parts are situated there. Also, in majority of microscopes, an anti-contamination device is also placed. It enables freezing the space around the specimen and thus reducing its contamination. An objective screen which reduces a spherical aberration of the objective and significantly increases contrast of the resulting image must be placed immediately below the specimen. An image produced by the objective lens is further magnified to the required size by means of projective and intermediate lenses. A specimen at the magnification about 100 times can be observed in the image plane of the objective. A part of this image is projected to an auxiliary projective which is able to change the focal point depending on a change of flow and therefore the magnification, e.g. at the range from 0.45 times to 24.5 times. Another lens, which is involved in image magnification, is a main projective, usually with a constant magnification of 100 times. Nowadays, a second auxiliary projective is commonly incorporated in this system so that the resulting maximum magnification of the whole display system, which equals the product of magnification of all lenses, reaches the value of 1 000 000.

To see electrons which passed through the specimen and the display system, the information they bear must be transferred to the area of Vis light. For this purpose, a screen covered usually with zinc sulphate, which is able to emit light with the wavelength of 450 nm depending on the energy and the quantity of impacting electrons, is placed on the bottom of the body tube. Due to impurities the emission is shifted closer to 550 nm, i.e. the green light.

TEM can be used in many scientific fields for studying the inner structure of materials at the atomic levels (Fig. 9.17). Samples for TEM must be sufficiently small (the diameter of 3 mm) and sufficiently thin (less than 0.5 μm), so the electrons can pass through them. Every research field uses its own specific methods of sample preparation. All fields however use an object screen for comfortable handling of the sample. To prevent the sample falling through the meshes of the screen, the screen
must be “coated” with a film. Formvar (polyvinyl formaldehyde which has the ability to create thin film on surfaces after drying) or carbon films (used at higher accelerating voltages) are most often used for this purpose. When measuring e.g. a nano-powder material, the sample must be dispersed in ethanol. The formed solution is dropped on the object screen which is coated with a steamed carbon film. The sample is then left dry at the room temperature and then it is ready for analysis.

Fig 9.17 TEM picture of monodisperse 100 nm PLGA-PEG nanoparticles. Phosphomolybdic acid negative staining was used.

9.2.4 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is a very popular scanning technique in pharmaceutical technology. The resolution of SEM fluctuates according to the accelerating voltage used, useful magnification of the conventional SEM microscope is 10–30000×. It does not enable resolution and magnification of TEM but it provides valuable information on surface topography of a sample, and/or on its chemical composition, because in addition to morphological images of observed objects obtained by the detection of secondary electrons, SEM can be used for detecting other signals (X-ray radiation, reflected electrons, Auger electrons), which provide information on material composition. An example of using SEM in pharmaceutical technology can be the observation of surface morphology of dosage forms (tablets, pellets, capsules), the observation of undesirable re-crystallizing of a drug or an excipient, the thickness analysis of individual layers (e.g. polymeric coat or a drug layer applied on an inactive core) etc. (Fig. 9.18).
Fig. 9.18 Observation of the surface of poly(D,L-lactide-co-glycolide) (PLGA) microparticles by SEM; analysis shows successful preparation of particles with porous structure.

At first sight, scanning electron microscope shows significant differences in design of the device compared to a transmission electron microscope (Fig. 9.19). The different length of the body tube, which is half as large, is caused by the fact that a scanning electron microscope detects signals released by the primary beam of electrons above the surface of the specimen and there is no need for the system of lenses which form the display system in the lower part of the tube of TEM. Instead, SEM is equipped with detectors of secondary and reflected electrons and the electronics for amplifying and processing of the signal and image creation. A beam of primary electrons concentrated by electromagnetic lenses is moved by deflecting coils before the impact on the surface of specimen so that it covers a small area with lines (scans). Obtaining an image on the scanning electron microscope is based on the interaction of the primary beam with the surface of the viewed object. Every product of such interaction gives information about physical and chemical properties of the tested object. In the lower part of the body tube there is a chamber of specimen which is bigger compared to TEM. There is a goniometric stage in it, on which samples are fixed in a specimen holder; samples often have up to several centimetres. The stage enables moving, turning and tilting the specimen. A big advantage of this layout is e.g. the possibility...
to simply record the movement on the specimen and to return to individual viewed places. Near the specimen there are detectors of individual signals, e.g. of secondary and reflected electrons or X-ray radiation.

![Diagram of the principle of Scanning (a) and Transmission (b) electron microscope.](image)

**Fig. 9.19** A diagram of the principle of Scanning (a) and Transmission (b) electron microscope.  

### 9.2.4.1 SEM types

There are several types of scanning electron microscopes on the market nowadays:

**High vacuum SEM**

A source of electrons is a thermionic emission cathode; the inner spaces reach the vacuum of values from $10^{-4}$ to $10^{-5}$ Pa. The microscope provides the maximum magnification up to 100 thousand times depending on the type of specimen. Samples for this type of microscope must be dry and their surface must be coated with a layer of electrically conductive metal (most often with gold or platinum).

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High resolution SEM

A source of primary electrons is an auto-emission nozzle which is able, unlike the previous type, to provide a beam of primary electrons with the diameter in the order of nm. It requires a higher value of vacuum \((10^{-9} \text{ Pa})\) but the microscope is able to provide magnification up to five hundred thousand times on a suitable specimen. As in previous case, samples must be dry, deaerated and their surface coated with metal (Fig. 9.20).

![Image of liposomes observed using high resolution microscope Hitachi SU8020. Sample of liposomes was dried under room temperature and sputter coated with Pt/Pd before observation. The resolution up to nanometers can be achieved using this technique.](image)

Environmental SEM

Environmental SEM enables observing specimens with water content up to 90 %. It can be equipped with a thermionic emission or auto-emission nozzle. The inner space of the microscope is divided to chambers with the increasing pressure value. The pressure of 2 kPa can be achieved in the specimen chamber (atmospheric pressure is about 100 kPa), which enables observing samples containing water without its turbulent release. To detect secondary electrons, the microscope is equipped with an ionization detector: the signal is led to the detector by means of gas or air molecule ionization by SE. Moreover, electrically non-conductive specimens can be observed without metal coating.
**Low vacuum SEM**

Low vacuum SEM enables observing specimens with water content up to 70% at the pressure in the specimen chamber of 0.2 kPa. To create an image, it uses the detection of back-scattered electrons which unfortunately provide a smaller resolution. Metal coating of the sample increases the quantity of back-scattered electrons and eliminates charging.

### 9.2.4.2 Preparation of a sample for SEM

Samples for an electron microscope analysis must be always specifically prepared due to several reasons:

- A sample must not contain water for a high vacuum SEM.
- A sample must be stable under the electron beam.
- A biological material must be fixed not to undergo post-mortem changes.
- A sample should be electrically conductive so it will not be charged with negatively charged electrons.

With regards to the fact that most observed objects are thermally and electrically non-conductive, the scanned surface is charged during the SEM analysis. This results in the deviation of the primary electron beam, which clogs the detector of SE. Depending on the charging intensity, disrupting screen pulsation can be observed, resulting in white lines on the whole image. As objects in high vacuum SEM, as well as in TEM, must be observed dried, when they are not electrically conductive, they have to be coated with a thin layer of metal before observation (gold, silver, platinum, carbon or a palladium and platinum alloy with a good electrical and thermal conductivity. The thickness of layers ranges in tens nm. A sputter coater serves for metal-coating of a sample. The layer of metal ensures the take-away of the negative charge and heat in which the majority of energy of accelerated primary electrons is changed. The heat locally released by primary electrons in the sample is high and manifests e.g. in the contamination of the scanned area, the movement of the specimen under the beam or directly by its damage, e.g. cracking.

### 9.2.5 Cryo-Electron Microscopy Imaging

Cryo-electron microscopy (cryo-EM) is a modern imaging method enabling the study of primarily biological samples in their native frozen-
hydrated state with minimal distortions in near-atomic resolution (3.8–4.5 Å). In 2017, the Nobel Prize in Chemistry was awarded jointly to Jacques Dubochet, Joachim Frank and Richard Henderson for the development of the cryo-EM.

In classical EM of biological materials the damage of specimen surface induced by electron bombardment is the major problem. Moreover, in the case of samples in solution, the noisy images are commonly occurring due to scattering of electrons for sample and for solvent in a similar way. Therefore, the transforming liquid samples into a solid can be an asset in terms of preservation of the natural state and structure of the material at extremely high resolution.

Cryo-EM can be performed for the sample in aqueous solvents or in various organic and ionic solvents after cryofixation. The sample preparation is based on freezing below approximately −185 °C (or −196 °C, which is the nitrogen boiling point). The cooling rate must be extremely high (about 105 °C s⁻¹) to prevent crystallisation of water, which is present in the sample solution. Instead, the formation of ice in a vitreous state (vitrification) is achieved to preserve hydrated state of the sample. In cases of freezing water more slowly or at higher temperatures, the formed ice structure expands during the cooling process, which results in damage of biological material structure and occurrence of drying artifacts in the image. The process of cryofixation also stops all motion and metabolic activity in the sample. After embedding in vitreous ice, the sample is placed to the electron microscope vacuum chamber and observed at about −185 °C, at high voltage in the range from 200 to 300 kV. The single particles in the images can be detected based on the difference in sample and vitreous ice densities. Moreover, specimen surface is not metal coated as in the case of classical EM, so it is possible to avoid any distortion of the surface image.

The cryo-EM usually operates in transmission electron microscopy mode (cryo-TEM), where the specimen is embedded in a thin, vitreous layer of ice. Low temperatures during the whole experiment are achieved using liquid nitrogen or liquid helium. At first, about 2–3 ml of hydrated sample are applied to special EM grid (Cu, Au, Mo, Si₃N₄, SiO₂) covered by a perforated support film (C, Au, silicon derivative). Then the excess amount of solution is removed with filter paper, the grid is plunged rapidly into a cryogen and the EM imaging can be carried out. Cryo-SEM
uses a cold stage in a cryogenic chamber of scanning electron microscope, mostly cooled with liquid nitrogen.

Cryo-EM can be preferably used for the study of macromolecular complexes, especially for samples, which are difficult to crystallize or which are inappropriate for X-ray crystallography or NMR analysis for another reason. Combined with 3D reconstruction techniques, cryo-EM can be utilized to visualize 3D models of intricate biological structures or macromolecular interactions. Other typical samples for cryo-EM are plant or animal tissue, microorganisms or suspensions of viruses or viral capsids, cosmetics, pharmaceuticals or food products. Therefore, in pharmaceutical technology, the ability to achieve near-atomic resolution can play an essential role in the development of new dosage forms. Cryo-EM also enables monitoring of time-related changes in samples via taking a series of images at the defined time intervals and it is also possible to make a movie recording.

The good example of cryo-SEM use can be demonstrated on analysis of the gel layer structure. Gel layer is a typical formation with high content of water. The water cannot be removed without serious changes in the gel structure. Cryo-SEM thus represent suitable method for the gel structure observation (Fig. 9.21). Firstly, the sample was frozen in liquid nitrogen first, the water was let to sublimate from the thin surface layer of the sample at −90 °C, sample was sputter coated with Pt/Pd mixture and observed at −130 °C. 3D structure of the gel is visualised in the picture.

**Fig. 9.21** Cryo-SEM image of a gel structure.
Using so-called freeze-fracture technique it is also possible to observe the inner structure. The frozen sample is fractured by a force (knife, released spring load, etc.), usually breaking along the lines of least resistance. Fig. 9.22 shows an example of freeze-fracture use. Coated pellet was frozen using liquid nitrogen and broken to reveal the inner structure. The image clearly shows the core of the pelet with the coating.

As opposed to X-ray crystallography, where sample purity is essential, rather structural homogeneity or integrity is the limiting factor toward atomic resolution for cryo-EM, even in the case of a chemically pure sample.

There are two main problems, which poses an obstacle when monitoring a sample at high resolution: low signal-to-noise ratio and movement of the samples induced by electron beam during exposure. The first mentioned causes poor contrast of captured images and limits the size of the observed macromolecules, the latter can result in image quality deterioration. Solution of these two problematic situations is possible by reducing electron dose, which, however, leads to necessity of special recording device. Conventional media as CCD cameras are not suitable for this purpose, because recording takes a few seconds, during which the sample movement degrades the image resolution.

Further development and improvements are expected in the near future, particularly in the following areas: The development of direct detection cameras with thinner sensor chips enabling capture of high-resolution images with high signal-to-noise ratio, improvement
of the motion-compensation methods for images at higher quality and also the progress in the process of sample preparation, for instance the methods to facilitate visualization of very small biomolecules.

9.3 SCANNING PROBE MICROSCOPY

Scanning probe microscopy (SPM) is another representative of scanning (raster, line) microscopy. They scan a specimen point by point and the whole image is created by compiling individual signals. Scanning probe microscopy is a summary name designating a group of methods based on the same or similar general principle, the most important of which are the scanning tunnelling microscopy (STM) and the atomic force microscopy (AFM).

SPM methods have numerous significant advantages. They have high resolution ability which ranges in tens pm to tens Å. They are non-destructive, highly precise, enable to examine sample in its natural environment (liquid, air, vacuum) and thus are an invaluable tool for particle surface analysis.

9.3.1 DESIGN OF SCANNING PROBE MICROSCOPE

Every SPM microscope consists of a mechanical part—a stage for fixing a sample, a positioning device, which enables movement in three axes, a probe (a tip on a cantilever) and an electrical part—supply, feedback, signal collection and movement control. An important external part of the microscope is a device for absorbing vibrations. A microscope can further be equipped with other auxiliary devices such as a vacuum chamber, a cryostat etc.

The essential part of the whole device is the probe, or nanosensor, which moves along the surface along axes \( x \) and \( y \) according to pre-defined patterns called “raster”. A very fine and precise movement of the probe is ensured by the positioning device which must ensure both precise positioning of tips, and stability of the position. Two positioning functions must be ensured from the technical point of view: (1) macroscopic movement of the sample towards the tip after it is placed into the device, which can be performed piezoelectrically, mechanically by transfer levers or a screw, or electrostatically; (2) nanoscopic movement performing the approximation of the sample to the tip to the distance which makes it possible to measure and to select
an area of examination. This part is performed exclusively piezoelectrically and several designs of the positioning device (scanner) are used as a standard which differ in their maximum range, linearity and other parameters. A piezoelectric element converts electric impulses to mechanical ones (a converted piezoelectric effect: electric current induces mechanical changes of crystal with piezoelectric properties; after the end of electric current presence the crystal obtains its original form). Any changes in nanosensor position along axis z caused by surface unevenness are detected and converted to realistic 3D models of the examined surface by means of computer software.

There can be numerous interactions between the tip of the probe and the surface of the observed specimen. An example of such interactions are a quantum tunneling, used in STM; van der Waals forces, used in AFM; electrostatic interactions, used in AFM; magnetic forces, used in magnetic force microscopy (MFM); optical interactions below the diffraction threshold, used in scanning near-field optical microscopy (SNOM); elastic and non-elastic interactions, used in friction force microscopy (FFM); thermal exchange, used in scanning near-field thermal microscopy (SNTM); ion transport, used in scanning electrochemical microscopy (SECM) or capillary phenomena.

9.3.2 SCANNING TUNNELLING MICROSCOPY

This method is directly based on the phenomena called quantum tunnelling. Normally, electron is not able to travel through the vacuum environment, where the sample is placed. However, if the conducting tip is brought close enough to the sample surface, bias between the sample and the tip enables electrons to travel through the vacuum barrier, thinned thanks to tip close position. Now flowing electrons create so called tunnelling current. Tunnelling current depends on tip position, voltage used and so-called local density of states. Monitoring of the current along with the tip position gives the signal and this information can be displayed in image form by appropriate software (Fig. 9.23).
There can be two types of the measured signal depending on the mode of measurement. In the constant height mode, the tip moves in the horizontal plane above the sample and the tunnelling current changes depending on the surface topography and local surface electrical properties of the sample. The tunnelling current measured in every point of sample surface provides a set of values of which the resulting image is created.

In the constant current mode, feedback is used to maintain constant value of the tunnelling current (it is evaluated in every point of the sample surface depending on the height of the tip above the surface). In this case the system detects an increase in the tunnelling current, corresponding voltage is brought into the piezoelectric system, which ensures putting the tip further away from the surface. In the constant current mode, the movement and position of the scanner tip are decisive for data creation. If the detection system registers a constant tunnelling current (with the error of several per cent), the height of the probe above the surface will also be constant (with the deviation in the order of $10^{-12}$ m).

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Both modes have its advantages and disadvantages. The constant height mode is faster because the system does not need to ensure the scanner movement up and down, but it is less precise because current can go below the well measurable level at high distances of the tip. It therefore gives useful information especially for relatively smooth surfaces. The constant current mode is more precise for rugged surfaces but measuring takes more time. A disadvantage can be potential damage to the surface if the tip goes over the area with significantly different electrical properties (e.g. oxidized places); to maintain the set current, the tip lowers deeply to the sample, sometimes it even touches it, and consequently the surface of the sample is damaged.

STM has some limitations and its main disadvantage is that a sample must be situated in vacuum and must be conductive. This is addressed by applying a conductive layer on the sample which is impossible in case of studying biological phenomena. It also requires elimination of external environment vibrations and stable working place.

9.3.3 Atomic Force Microscopy

This method uses the force action of atomic origin which arises between atoms of the tip and the sample at small distances (max. several tens of nm). Especially the repulsive and attractive forces of short range are in play (van der Waals forces, electrostatic forces, Pauli repulsive forces etc.). These forces act on any sample, independently on conductivity, therefore the tip and the sample surface does not need to be conductive, which is a big advantage of this method compared to STM. Adhesion, binding, abrasion, deformation and capillary forces can also act, the most important of which is the last one which manifests in special ways and often makes measuring more difficult. It always acts at the presence of a condensate, but it only manifests at the passage through the liquid-air interface. During measurement, the action of a single force is always monitored only; all the other forces interfere.

The method principle is as simple as STM; a very sharp tip moves above the sample or is in contact with it and is repulsed or attracted by the sample. The tip sensing the interaction is perpendicularly fixed on a thin flexible arm (a cantilever). Cantilevers usually have the shape of A or a rectangle and they are integrated in a carrying plate. The tip is conical or pyramidal and it is usually made of quartz or silicon nitride
as the cantilever. Very important parameters are the radius of curvature of the tip end and the vertex angle of the tip. Cantilevers with the tip are additionally covered with thin layers of selected elements or compounds for special purposes of AFM (e.g. to increase electrical conductivity).

The design of an AFM microscope is in fact identical with the design of a STM microscope. They only differ in the manner of detecting the deviation of the cantilever. While a tunnelling current is measured in STM, most atomic microscopes use optical methods for detecting the deviation of the cantilever. A laser beam focuses on the end of the cantilever and it is reflected to a quadrant photodetector from there. Depending on the change of laser trace position, a change of cantilever position is detected (Fig. 9.24). On the basis of this scanning a computer creates an overall image of the surface of a sample (Fig. 9.25).

![Fig. 9.24 A diagram of AFM instrumentation.](image)

According to the type of interaction (attractive or repulsive forces), several operating modes used for scanning the surface by the AFM microscope exist.

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9.3.3.1 Contact mode

The tip is brought to proximity of the sample where prevailing repulsive force acts on it. The tip is in contact with the sample and the total force acting between the tip and the sample ranges in the order from $10^{-6}$ to $10^{-8}$ N, which is relatively high. As the tip is too close to the surface, it is suitable to use a cantilever with low rigidity to prevent the tip damaging the sample. In the contact mode of AFM there are two display modes which are parallel to the STM modes.

A contact force mode: the feedback loop controls the position of the sample towards the tip in this mode so that a constant bend of the cantilever is maintained. A real height of the scanned surface is then proportional to the voltage on the piezo-manipulator necessary to maintain the constant force.

A constant height mode: the feedback loop is disconnected during scanning at the constant height mode. Resulting topographic data are generated by the photodetector detecting a cantilever deviation from the balanced position which is caused by variable force conditions between the tip and the sample due to a change of sample height.

9.3.3.2 Contactless mode

The tip is guided closely to the sample in the contactless mode so that an attractive force starts acting on it. The total force acting between the tip and the sample ranges in the order of $10^{-12}$ N but it is almost impossible to detect it by cantilever bending. For this reason, sensitive detection methods based on the principles of resonance frequency of an oscillating cantilever with the tip were developed. The cantilever starts oscillating at the frequency close to the resonance frequency outside the area of attractive forces and approximates to the sample. Van der Waals forces start acting on the tip at certain distance from the sample which shifts the effective resonance frequency down and it changes the amplitude of oscillation and the phase shift towards the excitation signal. On the basis of observing these changes a computer creates an image of topography of the sample surface. Measuring is therefore more complicated than in the contact mode. A lower resolution is achieved in the contactless AFM mode than in the contact mode. Despite this fact, the contactless mode is more suitable for studying soft and elastic samples because the surface of the sample is not damaged or contaminated during the analysis.
9.3.3.3 **Tapping Mode**

This is a transition mode between the contact and the contactless method. A cantilever with the tip oscillates at its resonance frequency at the amplitude of approximately 100 nm, the angle of oscillation of the cantilever is thus higher than in the contact mode. At every swing closer, the tip comes into contact (and then outside of it) with the surface of the sample. The obtained image consists of the contribution of topography and elastic properties of the given sample. This mode is more advantageous than the contact one if there is a danger of damaging the sample. It is also more advantageous than the contactless mode in case of a scan surface which has a higher span in axis $z$.

![AFM image](image.png)

**Fig. 9.25** AFM image of the silicone surface of *in vitro* model of artery used in pharmaceutical research. Micrometer-sized surface imperfections can be observed in the image as 3D objects coloured in shades of grey.

9.3.4 **Other Techniques**

9.3.4.1 **Lateral Force Microscopy**

Lateral Force Microscopy (LFM) operates in the contact mode and uses atomic forces acting on the tip. During scanning it does not scan the bending of the cantilever but its torsion due to forces parallel with the plane of the surface of the sample. Images obtained using this method present a change in the friction force between the tip and the surface. Its amount depends on material properties of the surface and its micro-roughness. Unfortunately, the total topography of the surface can often be reflected in data obtained in this way.

9.3.4.2 **Magnetic Force Microscopy**

Magnetic Force Microscopy (MFM) displays spatial layout of magnetic fields on the surface of the sample. The microscope operates
in the contactless mode where the shift of the resonance frequency is caused by magnetic force (the distance between ferromagnetically coated and magnetized tip and the sample surface must be of such value to ensure that the atomic forces acting on the tip are lower than the acting magnetic forces). A resulting image is a combination of information about topography of the sample and its magnetic properties.

9.3.4.3 Electrostatic Force Microscopy

Electrostatic Force Microscopy (EFM) studies the spatial layout of charges on the surface of a sample. In this method, voltage is inserted between the tip and the sample and it is scanned in the contactless mode but without oscillation of the cantilever. Due to the presence of an electrostatic force on the sample surface the cantilever with the tip changes its deviation which is proportional to the density amount of the charge.

9.3.5 SPM Application

All SPM methods are used especially for studying topography and properties of various surfaces and for studying surface processes (e.g. chemical reactions). They are also used in pre-formulation and formulation studies of medical preparation development. Pre-formulation studies include the determination of a crystalline condition and distinguishing polymorphs of API, size and shape of particles, drug stability, interactions between API and excipients. Formulation studies evaluate e.g. the stability of solid/liquid dosage forms, the drug release from a dosage form etc. Another application can be the search for molecules of APIs which have the same structure as active places of proteins.

A very interesting application of SPM techniques in pharmaceutics is nanotechnology. The possibility to display atoms and precise positioning of the tip and the sample can be used for handling of atoms. In this way it is possible to create structures at the atomic level. A sample having a high-quality surface is necessary for handling the atoms and high vacuum must be maintained around the sample.

Besides the physics and chemistry of surfaces, the method can also be used in other fields. The biggest contribution of this method for biology is the possibility of display in any surrounding environment, which enables displaying live organisms (e.g. bacteria, DNA etc.).
By means of AFM it is also possible to detect the presence of biologically active materials (toxins, bacteria, viruses etc.) in a studied sample. SPM can be also used in metrology (for precise dimensional measurements, surface roughness determination etc.).

The price of the equipment is less favourable compared to the price of standard scanning electron microscopes. Unlike other methods (TEM, SEM), it does not require difficult sample preparation. Its disadvantage is that it does not provide immediate image, but scanning is performed gradually.

9.4 References


Validation is a confirmation by examination and providing of the objective evidence that the particular requirement for a specific intended use can be consistently fulfilled. The principle is to establish that the process and its manufacturing, controlling, monitoring and measuring equipment operate within the desired parameters. Validation performs replicate cycles representing the required operational range of the equipment to demonstrate that the processes have been operated within the prescribed parameters (limits) and so the product meets the predetermined quality. The validated process is monitored during a routine operation. A requalification and recertification of the equipment is performed as needed. The following description is generally not based only on a specific directive but attempts to describe the basic principles of validation in a clear way.

Contemporary there is a wide variety of procedures, processes, and activities that need to be validated. The field of validation is divided into number of subsections including the following. According to the different areas of pharmaceutical technology four most important types are distinguished:

- Analytical method validation (analytical method).
- Process validation (production of dosage forms).
- Cleaning validation (method of cleaning).
- Computer system validation (electronic equipment and computers).

This text deals only with the validation of analytical methods and the process validation. However, it is important to know that all the validation types are involved in all above-mentioned types of instrumental methods.

The Validation Master Plan (VMP), a part of Good Manufacturing Practices (GMPs) for pharmaceutical, biotech and medical device companies, is a document that outlines and defines the processes and equipment that are to be validated and the priority and order in which this should be done. It also lists who should be responsible for the validation process. The VMP describes the strategy of preparation and planning of various steps in the process and the overview
of the validation operation. All validation activities related to critical technical operations, relevant to product and process controls are involved. The format and content should include: the validation policy, scope, location and schedule; organizational structure: personnel responsibilities; process/product description; specific process considerations that are critical and those requiring extra attention; a list of products/processes/systems to be validated; pre- and postmarked validation activities, current status and future planning; key acceptance criteria; references to the required standard operating procedure (SOP) and time plans of each validation project and sub-project.

Each process of the product validation must be monitored and documented in a written form (validation protocol). It is a written plan including personal responsibilities, test parameters, product characteristics, production and packaging equipment, and acceptable test results of critical steps. It also contains the product composition, process flow chart, described manufacturing process; a review of equipment/utilities; a review of raw materials and packing materials; a review of analytical and batch manufacturing records; a review of process parameters; the validation procedure; sampling location; acceptance criteria; a summary and conclusion.

10.1 **Analytical Method Validation**

Method validation is the process of verification that the analytical procedures are suitable for their intended use and that they support the identity, strength, quality, purity and potency of the drug substances and medicinal products. The development of a validated method should be carried out using an instrument that has validated software and is regularly tested to confirm its performance. The instrument installation should conform with the manufacturer's Installation Qualification (IQ). The recommended manufacturer's Operational Qualification (OQ) should also be carried out, with the instrument performance being tested using secondary standards on a routine basis between each OQ visit (for more details see chapter 10.2). The validation process shall be performed, and the results reported, according to the documented procedure (validation protocol). The validation of the analytical methods is addressed in the ICH Q series guidelines. The first is the ICH Q2(R1) “Validation of Analytical Procedures: Text
and Methodology”. The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests.
- Quantitative tests of impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of a drug substance or a product or other selected component(s) in the medicinal product.

Typical validation characteristics which should be considered according to ICH Q2(R1) are listed in the Table 10.1.

**Table 10.1** Typical validation characteristics according to the type of analytical procedure

<table>
<thead>
<tr>
<th>Type of analytical procedure</th>
<th>Identification</th>
<th>Testing for impurities</th>
<th>Assay: dissolution, content/potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quantitative</td>
<td>Limit</td>
</tr>
<tr>
<td>Accuracy</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Precision (Repeatability)</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Specificity</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Detection limit</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Linearity</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Range</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

These validation characteristics should be considered for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. Robustness is not listed in the table but should be considered at an appropriate stage of the development of the analytical procedure. The robustness of an analytical method is an indication of its ability to remain unaffected by small variations in the test parameters and thus to provide assurance of its reliability during routine usage.

There are many other analytical procedures which have not been addressed in the initial text on validation of analytical procedures.
Validation of these additional measuring procedures is equally important to those listed herein. For example, when validating a method for particle characterization (e.g. laser diffraction, microscopic method), the following main variables must be considered during the method development: sampling (whether the sample selected for analysis is representative of the bulk material); sample preparation (e.g. primary particle or agglomerate state is important); the instrument range (whether the instrument covers the size range of the tested sample); the concentration range (for the particle characterization in the dispersion); selection of an appropriate model for data assessment (e.g. Fraunhofer or the Mie theory, image analysis parameters).

An integral part of many analytical procedures is the system suitability testing. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. The system suitability test parameters to be established for a particular procedure depend on the type of the procedure being validated.

The pharmacopoeia methods are validated for their purpose and do not have to be validated for routine analysis. When using the pharmacopoeia methods, it is usually sufficient to perform the System suitability test. If the pharmacopoeia methods for stability assessment are used, they must be validated for this purpose, e.g. it has to be demonstrated that this is a stability indicating method.

The current ICH Q2(R1) “Guideline on Validation of Analytical Procedures: Text and Methodology” does not cover the more recent application of analytical procedures, (e.g. NIR spectroscopy or Raman spectroscopy). Spectroscopy tools are commonly used in process controlling and real time testing of pharmaceutical products using multivariate analysis methods. Taking into consideration the difference between the multivariate and traditional methods, the current approach of Q2(R1) is not sufficient to establish the suitability of the multivariate methods. It was proposed to develop a new quality guideline on the Analytical Procedure Development and to revise the ICH Q2(R1). The scope of the revision will include the validation principles that cover the analytical use of spectroscopic or spectrometry data (e.g. NIR, Raman, NMR or MS), which often require the multivariate statistical analyses.


### 10.2 PROCESS VALIDATION

The aim of the process validation is the objective evidence that the process consistently produces a product meeting its predetermined specifications. Before the product is validated, the device must be checked in the form of the following qualifications:

- **User requirements specification (URS)**, which is the definition of a specification for the equipment, premises, media or systems. It outlines the basic elements of quality and mitigates the GMP risks to an acceptable level. This is a reference point throughout the validation life cycle.

- **Design qualification (DQ)**, which ensures the equipment, space, media, or systems where it is necessary to demonstrate and document the compliance of the GMP design. When creating the design, the demands for the specification of the user requirements are verified.

- **Factory acceptance testing (FAT)** is a set of tests performed at the vendor to ensure that the device meets the customer's requirements. These tests can replace the IQ/OQ if it can be ensured that the transport and installation does not affect the functionality.

- **Installation qualification (IQ)** is the correctness verification of the installation of the instrumentation according to the drawings and specifications according to the established criteria. It also concerns checking the supplier's operational and working instructions, calibration of the instrumentation and verification of structural materials.

- **Operation qualification (OQ)** follows the IQ. These are examinations developed based on knowledge of the processes, systems and devices, ensuring the functionality of the system according to the design. It includes tests confirming the upper and lower operating limits or conditions of the so-called “the worst-case” which documents the evidence that the process equipment operates within the established limits.

- **Process performance qualification (PQ)** follows the IQ/OQ. These are usually placebo tests under normal operating conditions and “the worst-case” scenario. The sampling frequency applied
for the verification of the control process should be justified in order to confirm the required range.

10.2.1 Types of Process Validation

Process validation generally means a complex of sub-types that are related to the production of a medicinal product.

10.2.1.1 Traditional process validation

In the traditional approach, a lot of batches of finished products is produced under normal conditions, thus confirming the reproducibility. The number of batches produced and the number of samples taken should be based on the principles of quality risk management, allowing the determination of the normal range of variations and trends and providing sufficient data for the evaluation. Each manufacturer must specify and justify the number of batches required to prove a high level to ensure that the process is able to provide a quality product on a sustainable basis. It is generally accepted that the validation process may consist of at least three consecutive batches produced under normal manufacturing conditions.

10.2.1.2 Continuous process verification

For products developed based on the “quality by design” (QbD), where it has been scientifically demonstrated during the development that the established control strategy provides a high degree of product quality assurance, the continuous process validation may be used as an alternative. It is necessary to determine a suitable validation method. There must be scientifically verified control strategies for the required attributes for materials (excipients), critical attributes of quality and critical process parameters to confirm the quality of the product. This should include a regular evaluation of the control strategy. Process analytical technology and process control using multivariate statistics can be used as tools. Each manufacturer must determine and justify the number of batches required to demonstrate a high level of assurance that the process is capable of consistently providing a high-quality product.
10.2.1.3 **Hybrid approach**

In the cases, where the extensive product and process knowledge and understanding of the experience gained from the production and historical batch data exists, a combination of the traditional approach and ongoing process verification may be used. This approach can be applied to any validation activities after the changes or during the interim process verification, even though the product was originally validated by the traditional procedure.

10.2.1.4 **Continuous verification of the process during the life cycle**

Continuous verification of the process is used throughout the life cycle of the preparation to support the validated state of the product. It must be documented in the Product Quality Assessment Quality Review. Furthermore, changes over time must be taken into account and any further steps, such as expanded sampling, considered. This concept also includes the principles of the former concept of postmark “revalidation”.

10.2.2 **Representative cases**

This chapter briefly summarizes the issues of validation in pharmaceutical technology, including the basic statistical tools. The examples are based on the validation of the content uniformity of tablets as a model dosage form. During the validation, the product should meet the defined requirements and specifications on a statistically significant confidence level. The validation also covers the stage of the development, i.e. the choice of excipients, procedure, and process parameters, including the manufacturing process control, control of intermediates, the final product and sampling. Furthermore, the subjects of the validation are common physical parameters, such as mass uniformity, dissolution test, friability, etc. The content uniformity is one of the most important qualitative parameters, described in number of scientific and industrial guidelines.

Statistical methods offer several possibilities to evaluate the significance of the process (e.g. ANOVA). Nevertheless, the experience suggests that complex equations demand professional statisticians. In practice, simpler procedures such as the process capability index ($C_{pk}$) calculation or Bergum methods are used to evaluate
the statistical significance. The use of $C_{pk}$ enables to assess the accuracy and precision of the process, based on simple parameters demanded by pharmacopoeia: arithmetical average, maximum, minimum, and standard deviation. The $C_{pk}$ can be calculated according to the equation (Eq. 10.1):

$$C_{pk} = \min \left( \frac{USL - x_i}{3s}, \frac{x_i - LSL}{3s} \right)$$  \hspace{1cm} (10.1)

where $USL$ is the upper specified limit, $LSL$ is the lower specified limit, $x_i$ is the arithmetical average and $s$ is the standard deviation.

The limit $C_{pk} \geq 1.0$ is suitable for the content uniformity validation. Since in practice only the estimation of $C_{pk}$ and its confidence interval (CI) is known, it is preferable to use the lower limit of CI for $C_{pk}$. The lower limit of CI for $C_{pk} \geq 1.0$ guarantees, usually with the 90.0% or 95.0% assurance (according to the statistical significance level for CI) that at least 99.73% of samples produced by this process will pass the used acceptability criterion. The Bergum method is adapted to the USP monograph “Uniformity of Dosage Units” <905> and its harmonized European counterpart (Ph. Eur. 2.9.40). The Bergum method is based on the tabulated RSD value which guarantees, with 90.0% or 95.0% assurance, that at least 95.0% of the samples tested for content uniformity will pass the <905> USP test.

**10.2.2.1 Content uniformity validation: traditional validation**

The subject of validation was the process of preparing tablets containing 2% warfarin sodium. Based on the development, the basic process parameters were collected and verified by producing three consecutive batches (Table 10.2). Optimum conditions such as mean API particle size, time and mixing rate were found. The 10 tablets were collected during compression of each batch. For the analysis of API contents the HPLC method was used in this case.
### Table 10.2 Content uniformity of tablets

<table>
<thead>
<tr>
<th>Batch</th>
<th>$x_i$ (%)</th>
<th>RSD (%)</th>
<th>Ph. Eur. (2.9.6)**</th>
<th>Ph. Eur. (2.9.40)**</th>
<th>$C_{pk}$ (2.9.6)**</th>
<th>Bergum method**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>102.3</td>
<td>2.52</td>
<td>+</td>
<td>+</td>
<td>1.64</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>99.9</td>
<td>1.67</td>
<td>+</td>
<td>+</td>
<td>2.96</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>102.2</td>
<td>2.54</td>
<td>+</td>
<td>+</td>
<td>1.65</td>
<td>+</td>
</tr>
</tbody>
</table>

* Average content of 10 tablets from the particular batch; ** (+) passed, (–) failed; *** $C_{pk}$ values for Ph. Eur. 2.9.6 limits calculated according to the Equation 10.1.

### 10.2.2.2 Content uniformity validation: hybrid validation

Based on the results of the production batches that have been produced under different conditions, it is possible to select adequate process parameters through multivariate statistical methods. The process of preparing tablets containing 2% of warfarin sodium was validated. The Table 10.3 lists the manufactured batches, including the production conditions.

The impact of the formulation and process parameters (particle size distribution of API and filler, the content of API, duration of mixing after the addition of a lubricant) on the content uniformity of the blend and the tablets were evaluated. A data pack of 32 samples with variable RSD and $C_{pk}$ of tableting blends or tablets was evaluated using the factor analysis (FA) in order to find the optimal technological parameters. Supposedly, the content uniformity is improved with the decreasing RSD values and increasing $C_{pk}$ values. In FA loading plot (Fig. 10.1), the first factor is explained by the blend content uniformity and the second factor is explained by the content uniformity of tablets. There are correlations between the particle size of the used filler and blend content uniformity represented by $C_{pk}$ and between the API particle size and blend content uniformity represented by the RSD. These results show that a better blend content uniformity was achieved when combining a filler with a larger particle size ($D_{50} = 152 \mu m$) and the API with a smaller particle size ($D_{50} = 10 \mu m$).

---

Table 10.3 Content uniformity of blends and tablets containing a particular amount of warfarin sodium, manufactured under specific formulation and process parameters

<table>
<thead>
<tr>
<th>Batch</th>
<th>API</th>
<th>Filler</th>
<th>Blending</th>
<th>Blend</th>
<th>Tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c (%)</td>
<td>$D_{50}$ (μm)</td>
<td>$D_{50}$ (μm)</td>
<td>t (min)</td>
<td>RSD (%) $^*$</td>
</tr>
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<td>10.3</td>
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<td>15</td>
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<td>2.73</td>
<td>152.3</td>
<td>10.3</td>
<td>15</td>
<td>3.44</td>
</tr>
</tbody>
</table>

* Relative standard deviation of 10 samples from a particular batch; **$C_{pk}$ values for Ph. Eur. 2.9.6 limits
The FA was also used to evaluate the mixing duration after the addition of a lubricant ("blending" variable). The optimal duration of mixing with the lubricant is obvious in the FA scores plot (Fig. 10.2). The best results were achieved with mixing for 5 minutes. On the other hand, the blends mixed for a shorter time (2 min) or a longer time (10 or 15 min) had deteriorated content uniformity of both the blend and tablets. The Fig. 10.2 (a) suggests that the API concentration of 0.5 % correlates significantly with the content uniformity RSD in tablets, with a negative impact on the content uniformity. The API concentration in the range of 2–5.5 % does not have any significant impact on the content uniformity of the blend or tablets. The best results (Fig. 10.2 (b)) were achieved with the API concentration of 2, 2.7, or 5 %, with the duration of mixing after the addition of the lubricant for 5 minutes, and when using the API of a small particle size ($D_{50} = 10 \mu m$) and a filler of a large particle size ($D_{50} = 152 \mu m$). Based on the FA, raw materials of this particle size distribution and mixing duration of 5 minutes were chosen for further experiments. The API concentration of 2 % was chosen, as at this concentration both manufactured batches had suitable content uniformity. This concentration also allows the manufacturing of tablets.
with reasonable dimensions and weight for all therapeutic strengths (1–10 mg) by a common blend technology.

**Fig. 10.2** FA scores plot (a); The part of FA scores plot showing the best results (b); Batch label: x %_Wy_Dz_mixing time with magnesium stearate in min; x—warfarin sodium content in %; y—D_{50} of warfarin sodium; z—D_{50} of filler (Di-Cafo); D_{50}—50 % of measured particles are smaller than this size (µm).
On the basis of the selected formulation and process parameters, tablets with 8 concentrations of warfarin sodium were produced (2−10 mg). Three batches (A, B, C) were produced of each concentration. Batch B corresponded to 100 % of warfarin sodium target concentration. The other two corresponded to 96 % (batch A) or 104 % (batch C) of target concentration, respectively. This means “the worst-case scenario”. The target concentration of warfarin sodium in “the worst-case scenario” batches approaching the pharmacopoeial limits by “Warfarin Sodium Tablets USP” that require a content within 95−105 %. All tablet blends (batches A, B and C) met the Ph. Eur. 2.9.6 and the FDA requirements. $C_{pk} \geq 1$ was confirmed for Ph. Eur. 2.9.6 limits (85 % and 115 %).

Tablets containing 2, 2.5, 3, 4, 5, 6, 7.5, and 10 mg of warfarin sodium were manufactured from the blends (batches A, B and C), with the amount of API in the tablet set by the weight of the tablet. The results of the evaluation of the content uniformity of the tablets are listed in Table 10.4. Tablets of all strengths produced from common blends (batches A, B, and C) met the pharmacopoeial requirements defined by Ph. Eur. 2.9.6 and Ph. Eur. 2.9.40 (harmonized with USP). The $C_{pk}$ values for Ph. Eur. 2.9.6 were not lower than 1.0. Meeting of the Bergum criteria ensures with 90% assurance, that at least 95.0 % of the subsequently manufactured batches should meet the Ph. Eur. 2.9.40 limits.

Based on these results, it is possible to say that tablets of all concentrations (including the “worst-case” scenario batches) meet the pharmacopoeia limits according to the current GMP regulations and can be considered as valid.
Table 10.4 Content uniformity of tablets

<table>
<thead>
<tr>
<th>Batch*</th>
<th>$x_1$ (%)**</th>
<th>RSD (%)**</th>
<th>Ph. Eur. (2.9.6)***</th>
<th>Ph. Eur. (2.9.40)***</th>
<th>$C_{pk}$ (2.9.6)***</th>
<th>Bergum method***</th>
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<td>2_A</td>
<td>98.24</td>
<td>2.37</td>
<td>+</td>
<td>+</td>
<td>1.89</td>
<td>+</td>
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<tr>
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<td>101.26</td>
<td>1.67</td>
<td>+</td>
<td>+</td>
<td>2.71</td>
<td>+</td>
</tr>
<tr>
<td>2_C</td>
<td>102.50</td>
<td>2.31</td>
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<td>+</td>
<td>1.76</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>1.89</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
<td>1.96</td>
<td>+</td>
</tr>
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<td>1.97</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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</table>

* Batch label: API content_batch; ** Average content of 20 (Batch A and C) or 10 tablets (Batch B); *** (+) passed, (–) failed

10.3 REFERENCES


10 Validation at a Glance


11 INTELLECTUAL PROPERTY
AND PATENT PROTECTION
OF PHARMACEUTICALS

Instrumental analytical methods used in pharmaceutical technology play an important role in the intellectual property protection of pharmaceuticals. The subject of intellectual property could be the pharmaceutical forms themselves, their composition and applications, and the procedures that lead to their production. On the border between pharmaceutical technology and chemical synthesis are different API modifications in the form of derivatives, salts or polymorphs. Their formation may occur either during the synthesis or the formulation of the dosage form. Other components of the dosage form may also be involved in this process.

In the application of intellectual protection, the physical-chemical properties of pharmaceuticals, including API and possibly other components, as well as the manufacturing processes, including the individual final and interposed parameters, need to be monitored. In all these situations, it must be clearly demonstrated on specific examples that the result is a product or manufacturing process, which has advantages over the present state of the art. This is practically impossible without the use of modern instrumental analytical methods.

11.1 REASONS FOR INTELLECTUAL PROPERTY PROTECTION

For R&D of new (original) and generic pharmaceuticals, companies and other research facilities spend a considerable amount of money. The protection of intellectual property is used in order to recover the costs of research. For this reason, patents that regulate competition are used. This occurs in the form of intellectual protection of the solution of the invention, which prevents competitors from using it. This protection is usually limited to 20 years.
11.2 Forms of Intellectual Property Protection

The patent is the basic and most common form that provides the most powerful protection. Next to it, there exist also an industrial design and utility model, a geographical indication and a designation of origin. Patent protection starts when a patent application has been registered with the relevant national or international authority, thus creating the so-called “date of priority” and it lasts usually 20 years. Granting of patents usually takes 2−5 years, but sometimes even longer. The patent guarantees the owner the exclusive right to industrial use of the invention. He may then use the subject of protection or grant a license to other persons or subjects. The patent may not be granted for inventions, the use of which would be contrary to public order or good morals. The plant varieties and animal breeds, biological methods of plant breeding or animal husbandry cannot be patented. Intellectual property rights are territorial, which means that each country reserves the right to define this concept in accordance with local law.

11.3 Conditions of Intellectual Property Protection

In general, the invention must include a reasonable amount of human intelligence, ingenuity, creativity and inventiveness to improve the state of the art. The invention, which is recognized as a patent, must meet the following criteria: 1. it must be new in the world that means that subject of solution is not part of the prior art; 2. it must be the result of an inventive activity, and it is not possible for a person skilled in the art to derive a solution in a clear manner from the state of the art; 3. it must be industrially utilizable, and its object may be manufactured or otherwise utilized in industry, agriculture or other areas of the economy. The requirement of industrial applicability also means that the invention must be useful and usable for practical purposes and repeated use. In addition, the solution must be reproducible regardless of place and time, which means it cannot be tied to unrepeatable natural conditions.

11.4 Structure of Patent Application

For correct handle with the data of his measurements, the must have at least a general idea of the structure and philosophy of the patent
application. He should know in what form the data should be processed, how to interpret the results and what should not be forgotten or emphasized. Each application contents: 1. *The data on the originator* (data and information about inventor(s) and applicant). 2. *Description of the prior art* (relating to the evaluation method; in this case it must be clear, what instrumental methods were used, their sensitivity and limits of detection should be known). 3. *Description of the invention* (the description of progress must employ a minimally comparable analytical method as used in comparison with the state of the art). 4. At least one *example* of embodiment (an analytical method comparable to the state of the art as well as to the description of the invention must be used here) 5. *Patent claims* (if they contain specific limits based on analytical measurements, they must be declared on the basis of an adequate method). 6. *Drawings* (not obligatory, it describes the solution), and 7. *Annotation* (briefly characterizing the essence of the invention). Each patent application in the field of pharmaceutical technology protects either a specific *formulation* or a pharmaceutical form, or a *process* of preparation. The claims must be chronologically based on the description of the invention and must be based on a comparison with the state of the art. The claims should be an expression that summarizes and gradually develop the essence of the protection of the given solution. Each individual claim must consist of only one sentence.

11.5 **GENERAL PATENT FILLING PROCEDURE**

Each patent is usually the intersection of the work of the formulator or technologist, analyst, and person skillful in the patent law. Many disagreements and complications occur when professionals do not respect legal aspects and, on the contrary, officials and legislators do not fully understand the technical aspects. Therefore, the companies address this issue to patent departments where professionals are familiar with patent legislative and can process the results in a proper form. There is a national, European (EP) and International (PCT) way of filling the patent application. The national patent application is valid only in the territory, where the patent was registered. Within 18 months after the publication of the national application is there possibility of filling a foreign application. The EP protects the subject matter of the invention in all EU
member states and follows uniform rules. The Patent Cooperation Treaty (PCT) application covers more than 140 members of the International Patent Cooperation Union, which cannot deny the patent application. This is followed by the patent application procedure in the countries, where the patent is applied for. These countries should not provide for any other formal adjustment and content requirements.

### 11.6 Subject of Intellectual Property Protection in the Pharmaceutical Technology

As mentioned above, the subject of patent protection may be an API, whether as a product of synthesis or formulation of a pharmaceutical dosage form; the pharmaceutical dosage forms itself or its composition and properties or the process of preparation.

In the case of API, with the exception of new molecules, the novelty is found in particular polymorphism, particle size distribution, specific surface, crystalline form, electrostatic charge, etc. These variables must always relate to some progress compared to the state of the art. Most often, it is increasing the dissolution rate and bioavailability, facilitating and accelerating the technology preparation, or improving the physicochemical properties like stability of the dosage forms. Appropriate changes to the API may occur during the preparation of the dosage form. For example, the API may be converted to a more soluble amorphate or salt. Amorphate is constituted in the form of a solid dispersion after drying the solution or a liquid dispersion with a suitable excipient; and the salt may be created by neutralization during the preparation of solutions intended for granulation or for direct use in final pharmaceuticals such as oral or parenteral drugs. This is called “in situ” modification of the API.

Another common form of the subject of protection is the composition or the resulting properties of the pharmaceutical dosage form. The composition is the qualitative or quantitative presentation of individual components, including the use of unusual substances. The composition must exhibit advances in the art and is associated with the pharmaceutical formulation or its preparation. The resulting properties of the dosage form are, for example, new or more preferred dissolution profiles associated, with a more advantageous dose, optionally
with a novel indication; increasing bioavailability or, on the contrary, achieving controlled release; improvement of the physical parameters of the pharmacopoeia or non-physical properties and, last but not least, the improvement of stability, resulting in prolongation of the expiration time, reducing the packaging material or increasing the product safety by reducing toxic degradation product and impurities.

The last area is the technology (procedure) of drug production. This field is so extensive that only principles can be mentioned. Today, biotechnology, gene engineering or the 2D and 3D printing can be mentioned. Generally, protected may be the new procedures and process parameters that improve, facilitate, shorten or reduce the production process or enhance the product. The technology may not be simplified, shortened or discounted if it leads to the advantageous properties or the price of the dosage form compared to state of the art.

11.7 REPRESENTATIVE CASES

This chapter gives examples of patent protection applied in pharmaceutical technology areas. In all mentioned examples, instrumental methods are used, and the examples are illustrative for API polymorph protection, pharmaceutical dosage forms, their properties and the technology of preparation in relation to this subject.

Patent protection is mainly used in the case of new drugs (originals), but also in generics. There is no rule that procedures for the preparation of new drugs are more original or more sophisticated. Conversely, in the preparation of generics, the researcher must demonstrate considerable ingenuity to overcome the current state, but also to overcome the current protection of the same pharmaceuticals. While patents of new drugs and procedures are aimed to protect against competition, patent protection for generics is used to prevent the producer from suspecting that his practices are plagiarism. The patent itself is a guarantee of originality and nonconflictability of the chosen solution, and the court takes it into account.

In this chapter, five inventions are demonstrated, which were created with the support of instrumental analytical methods. They document the form of API, the preparation process, the composition and the properties of the resulting pharmaceutical dosage forms.
11.7.1 Polymorphism

Methods of instrumental analysis have an irreplaceable position in identifying individual polymorphs of existing or new API molecules. Producers of generics, thanks to the polymorphism, try to bypass the protection of specific forms of API so far. At the same time, originators are trying to prepare different polymorphs to prevent this bypass. The principle applied here says that if the substance is monomorphic, the reason is its polymorphs have not yet been discovered. In addition to common polymorphs, there is also an effort to protect “pseudo polymorphs”, which are, for example, differently hydrated forms of API. The items of protection of “pseudo polymorph” raise discussion among experts. For each polymorph, it is usual to demonstrate what progress has been made in the state of the art.

A typical example is the Czech original anti-tumor drug designated LA-12, which chemically corresponds to \{\((OC-6-43)\)-bis (acetato) (1-Tricyclo [3,3,1,13,7] decylamine)\} amminedichloroplatinum (Fig. 11.1). It is a tetravalent platinum complex that allows (unlike bivalent platinum) oral administration.

Overall, four solid forms with different physical properties were found, one of which is amorphous. Polymorphism was confirmed by differential scanning calorimetry, X-ray powder diffraction and infrared spectroscopy. The Table 11.1 lists the differences that have been confirmed by the different responses of the given methods.

![Fig. 11.1 Molecule of LA-12.](image_url)
**Table 11.1 Characteristics of LA-12 polymorphs**

<table>
<thead>
<tr>
<th>Solid form</th>
<th>DSC (melting temperature °C ± 10 / fusion enthalpy J g⁻¹ ± 15)</th>
<th>XRPD 2Theta angle peaks (degrees ± 0.1 / spacing-d Å ± 0.1)</th>
<th>IR Representative peaks (cm⁻¹ ± 2)</th>
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<td>Amorphous</td>
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<td>No peak</td>
<td>2909; 1655; 1635; 1361; 1288; 1080; 701</td>
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<tr>
<td>Crystalline (I)</td>
<td>120 / 81</td>
<td>7.5; 9.4; 15.1; 17.8 / 11.7; 9.4; 5.9; 9.0</td>
<td>2909; 1665; 1596; 1339; 1278; 1081; 697</td>
</tr>
<tr>
<td>Crystalline (II)</td>
<td>150 / 77</td>
<td>8.4; 15.4; 15.7 / 10.5; 5.7; 5.6</td>
<td>2909; 1662; 1629; 1593; 1373; 1268; 1087; 704</td>
</tr>
<tr>
<td>Crystalline (III)</td>
<td>197 / 72</td>
<td>5.0; 8.6; 9.9; 14.5; 17.8 / 17.8; 10.3; 8.9; 6; 5.0</td>
<td>1661; 1602; 1544; 1365; 1306; 1283; 1085; 707</td>
</tr>
</tbody>
</table>

11.7.2 DISSOLUTION

Specific dissolution profiles related to new or improved pharmaceutical dosage forms are another frequent subject of patent protection. For these dosage forms, conventional dissolution devices such as USP II (Paddle Method) and USP I (Basket Method) are sometimes not sufficient. Therefore, other sophisticated devices are often used. A certain compromise is the use of existing methods, for example with a continuous pH gradient, to monitor the imitation of the release of the API throughout the passage through the digestive tract from the stomach to the colon.

One example is a new pharmaceutical form of the so-called “colonial suppositories”, where an anti-tumor drug under the designation LA-12 is used again. This invention relates to an oral pharmaceutical composition for targeted transport of LA-12 into colon wherein the pharmaceutical composition is characterized in that it is composed of a core consisting of pharmaceutically acceptable material, which liquefies at body temperature or is dispersible or soluble in the colon juice (for example refined plants or animal fats). API is dispersed in the core material, the core is disposed within a capsule enabling release of the active substance in the colon. The capsule is coated with a colonic coating material Eudragit® FS enabling release of the active substance in the colon only.
The passage of the digestive tract takes about 2–4 hours in the stomach environment at pH 1–5, then 3–5 hours with a small intestine at pH 5.5–7 and finally with a large intestine at a pH of 6–7.5. A limit for dissolution profile was selected to simulate colon when the amount of the drug was not more than 5% during 120 min; 50–65% after 6 hours and 85–100% after 12 hours. For this purpose, a paddle method was developed at 50 rpm paddle rotation in 900 ml of 0.1M HCl at pH 1.2 and changing to pH 6.8 by adding 20.7 g of Na₃PO₄·12 H₂O after 120 min. Achieving this dissolution profile with the use of the “colonial suppositories” formulation is an object of the present invention.

11.7.3 CONTENT UNIFORMITY

Obtaining improvements over the prior art is, for example, achieving more strict parameters than pharmacopoeia generally requires. In the example, there is no new or innovative form of medicine. The required parameter is the technological process leading to a pharmaceutical dosage form that complies with a stricter pharmacopoeia parameter, which must be properly explained and must progress towards the state of the art. An example of this is the technology of preparing tablets containing warfarin sodium.

Warfarin has low therapeutic index. When titrating the dose, it is typically increased or decreased by only 5–15% of the daily dose. Therefore, content uniformity is an important parameter of warfarin tablets. The content uniformity hence represents a critical parameter for drug forms particularly in the case of active substances with only narrow interval between their active and toxic doses (warfarin sodium salt). Keeping the content uniformity in individual dosage units of the pharmaceutical dosage form guarantees that the patient receives only the effective dose of the API and not a toxic one. The content uniformity is checked by the appropriate analytical method (e.g. HPLC) in a defined number of dosage units (10–150). The obtained data are calculated as an average with the RSD value, which is compared with the labeled average content with acceptance RSD limits for used number of samples (Bergum method). Meeting limits guarantees with 90% assurance that at least 95% of all samples tested for content uniformity will pass the pharmacopeial test (USP/EP) for content.
uniformity. Bergum's limits for RSD are listed for content uniformity test of tablets and it is valid for harmonized USP/EP monographs. Bergum method is also accepted by FDA for process validation according to GMP requirements.

11.7.3.1 Solution A (suitable technology)

The invention relates to a method of producing tablets with warfarin sodium salt in an amount of 1 to 10 mg and having high degree of content uniformity satisfying the Bergum method. In the manufacturing process, the aqueous solution of warfarin sodium is sprayed in a high-shear mixer on a mixture of lactose monohydrate (Pharmatose® DC-11), microcrystalline cellulose (Avicel® PH101), modified starch (Starch® 1500), and colloidal silicon dioxide (Aerosil® 200). Particle size distribution of this mixture was measured as 10 % of particles < 40 μm, 90 % < 200 μm and 99 % < 300 μm. Having an active surface area of 150 m² g⁻¹, colloidal silicon dioxide acts as a sorbent, preventing over-wetting and changing of particle structure. When the spraying of the solution was finished, the blend was transported to a fluid-bed dryer and dried. Dried particles were then mixed with disintegrant, cross-carmellose sodium salt (Ac-Di-Sol®) and lubricant (magnesium stearate) and compressed into tablets. Obtained particles were free of the powder and both the intermediate blend and tablets met Bergum limits (Table 11.2).

<table>
<thead>
<tr>
<th>Strength (mg)</th>
<th>Average Content (%)</th>
<th>RSD (%)</th>
<th>Bergum limit (%)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>102.0</td>
<td>2.09</td>
<td>2.70</td>
</tr>
<tr>
<td>2</td>
<td>98.1</td>
<td>1.00</td>
<td>2.83</td>
</tr>
<tr>
<td>2.5</td>
<td>95.8</td>
<td>1.27</td>
<td>2.39</td>
</tr>
<tr>
<td>3</td>
<td>97.3</td>
<td>0.88</td>
<td>2.68</td>
</tr>
<tr>
<td>4</td>
<td>98.7</td>
<td>1.17</td>
<td>2.94</td>
</tr>
<tr>
<td>5</td>
<td>98.0</td>
<td>0.67</td>
<td>2.81</td>
</tr>
<tr>
<td>6</td>
<td>99.5</td>
<td>1.11</td>
<td>3.09</td>
</tr>
<tr>
<td>7.5</td>
<td>99.8</td>
<td>1.25</td>
<td>3.14</td>
</tr>
<tr>
<td>10</td>
<td>99.4</td>
<td>1.27</td>
<td>3.07</td>
</tr>
</tbody>
</table>
11.7.3.2 Solution B (suitable particle size)

Another way to achieve consistent content uniformity is to define the particle size distribution of the API in relation to the other components of the tablet blend. This method is mainly used in the field of direct compression. Particle size is commonly evaluated by laser diffraction methods, both on the “dry way” in aerosdispersion or in the “wet way” in suspension. D-values ($D_{10}$, $D_{50}$ and $D_{90}$) are used to describe the representation of particles with diameter (usually in μm) smaller than 10 %, 50 % or 90 % of the cumulative mass. In this case, tablets containing crystalline sodium warfarin ($D_{50} = 10.3$ μm) with 2 percent API concentration (by weight) in one tablet mass. As the main filler dibasic calcium hydrogenphosphate (Di-Cafos 92–12 resp. 92–14) was used with two particle sizes ($D_{50} = 61$ μm resp. $D_{50} = 152$ μm). Other components were microcrystalline cellulose (Avicel® PH101), cross-carmellose sodium salt (Ac-Di-Sol®) and lubricant (magnesium stearate). All constituents were mixed for 15 minutes (procedure A) or all constituents, without magnesium stearate, were mixed for 10 minutes, then magnesium stearate was added and another 5 minutes of mixing followed (procedure B). Two batches (1 and 2) were produced for every procedure/composition combination. An additional third batch was produced using the composition and procedure that showed the best results.

Table 11.3 Content uniformity of tablets prepared by direct compression

<table>
<thead>
<tr>
<th>Batch*</th>
<th>Average content (%)</th>
<th>RSD (%)</th>
<th>Bergum method**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1_A_D61</td>
<td>101.4</td>
<td>3.57</td>
<td>−</td>
</tr>
<tr>
<td>2_A_D61</td>
<td>102.0</td>
<td>5.06</td>
<td>−</td>
</tr>
<tr>
<td>1_A_D152</td>
<td>101.7</td>
<td>2.58</td>
<td>+</td>
</tr>
<tr>
<td>2_A_D152</td>
<td>101.0</td>
<td>2.62</td>
<td>+</td>
</tr>
<tr>
<td>1_B_D61</td>
<td>100.1</td>
<td>3.20</td>
<td>−</td>
</tr>
<tr>
<td>2_B_D61</td>
<td>103.2</td>
<td>1.62</td>
<td>+</td>
</tr>
<tr>
<td>1_B_D152</td>
<td>102.3</td>
<td>2.52</td>
<td>+</td>
</tr>
<tr>
<td>2_B_D152</td>
<td>99.9</td>
<td>1.67</td>
<td>+</td>
</tr>
<tr>
<td>3_B_D152</td>
<td>102.2</td>
<td>2.54</td>
<td>+</td>
</tr>
</tbody>
</table>

* Batch label: batch number_procedure_filler particle size (50 % of particles smaller either than 61 or 152 μm); ** (+) passed; (−) failed.
The above Table 11.3 shows that the particle size along with process parameters has a significant effect on content uniformity. Its proper choice leads to the production of tablets with a content uniformity meeting the Bergum method. Procedure B together with a suitable particle size distribution of API ($D_{50} = 10.3 \mu m$) and particle size of filler ($D_{50} = 152 \mu m$) has become the subject of successful patent protection. The patent claimed that tablets comprise at the most 5 percent by weight of sodium warfarin and 50 to 80 percent by weight, preferably 70 percent by weight of calcium hydrogen phosphate. The particle size distribution ratio of the API particle size to the particle size of the calcium hydrogen phosphate expressed by the $D_{50}$ parameter was in the range from about 14 to about 20. Expressed by the $D_{90}$ parameter it was found to be in the range from about 3 to about 5.

### 11.8 References


### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>Auger electrons</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredients</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflection</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BSE</td>
<td>backscattered electrons</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer, Emmett and Teller</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CP</td>
<td>cross-polarization</td>
</tr>
<tr>
<td>$C_{pk}$</td>
<td>process capability index</td>
</tr>
<tr>
<td>CPX</td>
<td>ciclopirox olamine</td>
</tr>
<tr>
<td>cryo-EM</td>
<td>cryo-electron microscopy</td>
</tr>
<tr>
<td>CSA</td>
<td>chemical shift anisotropy</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>DCP</td>
<td>dual-circular polarization</td>
</tr>
<tr>
<td>DD</td>
<td>dipolar decoupling</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DPI</td>
<td>dots per inch</td>
</tr>
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<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
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<td>DTA</td>
<td>differential thermal analysis</td>
</tr>
<tr>
<td>DQ</td>
<td>design qualification</td>
</tr>
<tr>
<td>EDAX</td>
<td>X-ray analysis with energy dispersion</td>
</tr>
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<td>EFM</td>
<td>electrostatic force microscopy</td>
</tr>
<tr>
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<td>electron microscopy</td>
</tr>
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<td>EMA</td>
<td>European Medicinal Agency</td>
</tr>
<tr>
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<td>far infrared</td>
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<td>GIT</td>
<td>gastrointestinal tract</td>
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<td>GMPs</td>
<td>good manufacturing practices</td>
</tr>
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<td>HPHD</td>
<td>high power heteronuclear decoupling</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICH</td>
<td>The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>ICP</td>
<td>incident circularity polarization</td>
</tr>
<tr>
<td>IGC</td>
<td>inverse gas chromatography</td>
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<td>IMC</td>
<td>isothermal microcalorimetry</td>
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<td>IQ</td>
<td>installation qualification</td>
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<tr>
<td>IR</td>
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<tr>
<td>IVIVC</td>
<td>in vitro/in vivo correlation</td>
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<td>lateral force microscopy</td>
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<td>MAS</td>
<td>magic angle spinning</td>
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<tr>
<td>MFM</td>
<td>magnetic force microscopy</td>
</tr>
<tr>
<td>MIR</td>
<td>mid infrared</td>
</tr>
<tr>
<td>MLR</td>
<td>multiple linear regression</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OQ</td>
<td>operation qualification</td>
</tr>
<tr>
<td>PAT</td>
<td>process analytical technology</td>
</tr>
<tr>
<td>PCR</td>
<td>principal component regression</td>
</tr>
<tr>
<td>PEO</td>
<td>polyether oxide</td>
</tr>
<tr>
<td>Ph. Eur.</td>
<td>European Pharmacopoeia</td>
</tr>
<tr>
<td>pHHPMA</td>
<td>poly(N-(2-hydroxypropyl)methacrylamide)</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares</td>
</tr>
<tr>
<td>PPI</td>
<td>pixels per inch</td>
</tr>
<tr>
<td>PQ</td>
<td>process performance qualification</td>
</tr>
<tr>
<td>QA/QC</td>
<td>quality assurance and quality control</td>
</tr>
<tr>
<td>QbD</td>
<td>quality by design</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>research and development</td>
</tr>
<tr>
<td>ROA</td>
<td>Raman optical activity</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
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<tr>
<td>SCP</td>
<td>scattered circular polarization</td>
</tr>
<tr>
<td>SE</td>
<td>secondary electrons</td>
</tr>
<tr>
<td>SECM</td>
<td>scanning electrochemical microscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SF</td>
<td>sphericity factor</td>
</tr>
<tr>
<td>SFFF</td>
<td>sedimentation field-flow fractionation</td>
</tr>
<tr>
<td>SHIME</td>
<td>Simulated Human Intestinal Microbial Ecosystem</td>
</tr>
<tr>
<td>SNOM</td>
<td>scanning near-field optical microscopy</td>
</tr>
<tr>
<td>SNTM</td>
<td>scanning near-field thermal microscopy</td>
</tr>
<tr>
<td>SOP</td>
<td>standard operating procedure</td>
</tr>
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<td>SORS</td>
<td>spatially offset Raman spectroscopy</td>
</tr>
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<td>SPM</td>
<td>scanning probe microscopy</td>
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<tr>
<td>ssNMR</td>
<td>solid-state nuclear magnetic resonance spectroscopy</td>
</tr>
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<td>STM</td>
<td>scanning tunnelling microscopy</td>
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<td>transmission electron microscopy</td>
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<td>thermogravimetry</td>
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<td>transmission Raman spectroscopy</td>
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<td>user requirements specification</td>
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<td>The United States Pharmacopeia</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible</td>
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<td>VPM</td>
<td>validation master plan</td>
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<td>WAI</td>
<td>wide area illumination</td>
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<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>XRPD</td>
<td>X-ray powder diffraction</td>
</tr>
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IN SOLID DOSAGE FORM ANALYSIS

Edited by: Jan Muselík, Jakub Vysloužil, Kateřina Kubová

Text by: Eva Bartoníčková, Jiří Brus, Jan Gajdziok, Aleš Franc, Kateřina Hickey, Martina Kejdušová, Josef Mašek, Sylvie Pavloková, Ivana Šeděnková, Martina Urbanová, David Vetchý

Design and Typesetting by Sylvie Pavloková

Proofreading by Jakub Vysloužil

Published by Masaryk University Press; Žerotínovo nám. 617/9, 601 77 Brno, Czech Republic

1st, electronic edition, 2021


ISBN 978-80-210-9723-0 (paperback)

https://doi.org/10.5817/CZ.MUNI.M210-9724-2021

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The book “Modern Instrumental Methods in Solid Dosage Form Analysis” is focused on the description of selected modern instrumental methods widely used in the evaluation of dosage forms. It explains their main principles, provides a brief description of the instrumentation, and offers numerous examples of practical applications in both the pharmaceutical research and development, as well as in the commercial pharmaceutical manufacture. For the latter, these methods are used as valuable tools for quality assurance and quality control (QA/QC) of incoming raw materials, final medicinal products, and manufacturing processes.