

Unit 5: Electrophoresis, X-Ray Crystallography, Immunological Assays, Potentiometry and Thermal Techniques

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5.1 Electrophoresis: principle

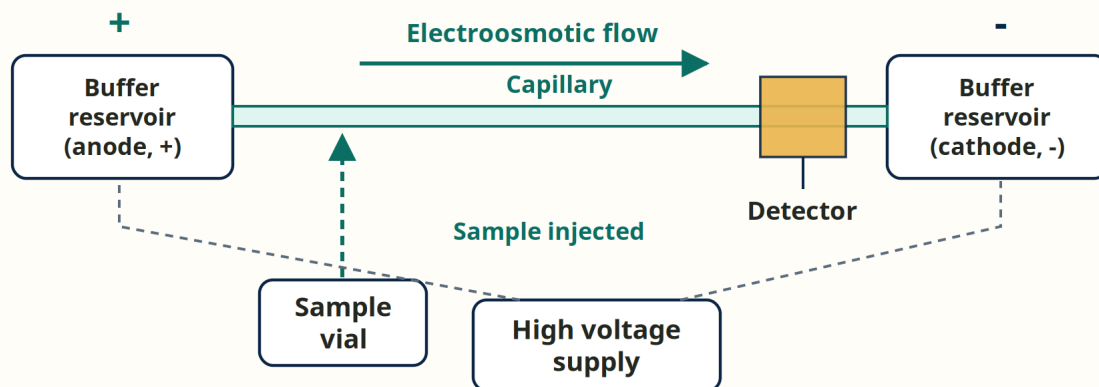
Electrophoresis is the movement of charged particles under the influence of an electric field through a medium. A molecule carrying a net positive charge moves towards the cathode (negative electrode), a molecule with a net negative charge moves towards the anode (positive electrode), and the speed depends on the charge to size ratio: highly charged, small ions move fastest. The mobility of a particle increases with its net charge and the field strength, and decreases with its size and the viscosity of the medium. Because the charge on a drug, amino acid or protein depends on pH relative to its isoelectric point (pI), the buffer pH is the master variable of the whole technique: at pH below pI the molecule is net positive, at pH above pI it is net negative, and exactly at pI its net charge and mobility are zero.

The basic apparatus is the same for most forms: a power supply giving a stabilised direct current, an electrophoresis tank or cell with two buffer reservoirs, electrodes (usually platinum), and the supporting medium that carries the sample. Factors affecting separation are: the net charge and size of the molecule, field strength (higher voltage is faster but generates more heat, which distorts bands), buffer pH, ionic strength (high ionic strength slows migration and sharpens competition for current by buffer ions), temperature, and the nature of the support (adsorption and electro osmotic flow can move even neutral compounds, a useful effect in capillary electrophoresis).

5.2 Types of electrophoresis

- **Paper electrophoresis:** the sample is applied on filter paper wetted with buffer, held between the two buffer compartments. Simple and cheap, used historically for amino acids, small peptides and serum proteins, with moderate resolution and some diffusion of bands.
- **Gel electrophoresis:** the run is carried out in a gel slab or rod, usually agarose (for large molecules such as DNA and big proteins) or polyacrylamide (for proteins and smaller fragments, giving molecular sieving because the gel pores sort by size as well as charge). In SDS-PAGE, the detergent sodium dodecyl sulphate coats proteins with a uniform negative charge per unit mass, so separation is by molecular size alone, which gives the molecular weight against standards. Gels are stained after the run (Coomassie blue or silver stain for proteins, ethidium bromide under UV for DNA).
- **Capillary electrophoresis (CE):** separation happens inside a narrow fused silica capillary (25 to 100 micrometre bore) filled with buffer, at very high voltage. The capillary dissipates heat well, so high fields give very fast, high efficiency separations from nanolitre samples, detected on column usually by UV. Electro osmotic flow sweeps everything towards the cathode, so cations, neutrals and anions can be recorded in one run. Variants include capillary zone electrophoresis, micellar electrokinetic chromatography (which also separates neutral compounds using micelles) and capillary gel electrophoresis. Pharmaceutical uses: chiral separations, purity of peptides and proteins, counter ion and small ion analysis.

Capillary Electrophoresis



High voltage drives ions through the capillary towards the cathode

Figure: Capillary electrophoresis set up, electroosmotic flow carries the sample towards the cathode.

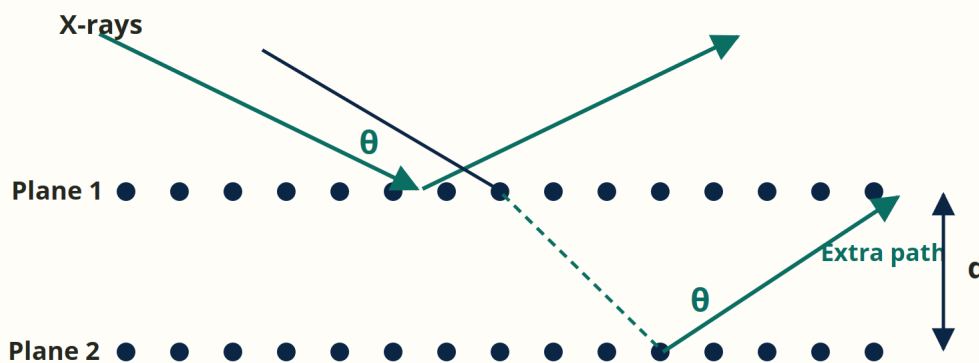
- **Zone electrophoresis:** the general family name for methods in which the sample moves as discrete zones or bands through a stabilising support such as paper, cellulose acetate, starch, agar or polyacrylamide. Cellulose acetate electrophoresis of serum proteins and haemoglobin variants is a standard clinical example.
- **Moving boundary electrophoresis:** the classical Tiselius method, done in free solution without any support, in a U shaped cell where the boundary between the sample solution and pure buffer moves under the field and is followed optically. It is the historical foundation of the technique; free solution gives true mobilities but convection limits resolution, which is why supported and capillary forms replaced it.
- **Isoelectric focusing (IEF):** the medium carries a stable pH gradient, created with carrier ampholytes. A protein migrates until it reaches the region where the pH equals its pI, where its net charge becomes zero and it stops, focused into a very sharp band. IEF gives the highest resolution of any electrophoretic form, separates proteins differing by as little as 0.01 pH unit in pI, and is used for charge variant profiling of monoclonal antibodies, haemoglobin variants and as the first dimension of two dimensional gel electrophoresis.

Applications of electrophoresis in pharmacy: purity and molecular weight of proteins and biopharmaceuticals, charge heterogeneity of antibodies, DNA and RNA analysis, haemoglobin and serum protein profiling, chiral drug separation by CE, and identity checks for peptide drugs.

5.3 X-ray crystallography

Production of X-rays: X-rays are produced when a stream of fast electrons from a heated cathode strikes a metal target anode (commonly copper or molybdenum) in an evacuated tube. Most of the energy becomes heat; a small part is emitted as a continuous spectrum (bremsstrahlung) plus sharp characteristic lines of the target metal, such as copper K alpha radiation at wavelength 1.54 angstrom, which is the radiation used for diffraction work. For single crystal work in modern laboratories, rotating anode sources and synchrotron radiation give much higher intensity.

Bragg Diffraction from Crystal Planes



$$\text{Bragg law: } n\lambda = 2d \sin \theta$$

Path difference must equal a whole number of wavelengths

Figure: Bragg diffraction, reflected X-rays reinforce when the path difference equals a whole number of wavelengths.

Bragg law: a crystal acts as a three dimensional diffraction grating for X-rays because the spacing between atomic planes is of the same order as the X-ray wavelength. When X-rays reflect from successive parallel planes of spacing d , the reflected beams reinforce only when the path difference equals a whole number of wavelengths:

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

where n is the order of reflection, λ is the wavelength, d is the interplanar spacing and θ is the angle of incidence measured from the plane. Bragg law converts the angles at which diffraction is observed into the set of plane spacings of the crystal, the raw data of the structure.

X-ray diffraction methods:

- **Rotating crystal technique:** a single crystal is rotated about one axis in a monochromatic X-ray beam. Each set of planes satisfies Bragg law at some point of the rotation and produces a spot; the pattern of spots, layer by layer, reveals the unit cell dimensions and symmetry. This is the basis of single crystal structure determination, the definitive method of knowing the exact three dimensional arrangement of atoms in a drug molecule, including absolute configuration and hydrogen bonding.
- **X-ray powder technique (powder diffraction, XRPD):** the sample is a fine powder of many tiny crystals in all orientations, so instead of spots the diffracted beams form concentric cones and rings. Every crystalline substance gives a unique powder pattern of line positions and intensities, its fingerprint. In pharmacy this is the standard tool for polymorph identification and quantification, checking crystallinity versus amorphous content in formulations, and detecting form changes during stability studies. It needs only milligrams of powder and does not require growing a single crystal.

Types of crystals: crystals are classified into seven crystal systems on the basis of their unit cell geometry: cubic, tetragonal, orthorhombic, hexagonal, trigonal (rhombohedral), monoclinic and triclinic. The unit cell is defined by three edge lengths (a , b , c) and three angles (α , β , γ); in the cubic system all edges are equal and all angles 90 degrees, while in the triclinic system nothing is equal and no angle is fixed. Fourteen Bravais lattices describe all ways of arranging lattice points in these systems (primitive, body centred, face centred and base centred variants). The same drug can crystallise in more than one arrangement; these are polymorphs, which may differ in solubility, stability and bioavailability, which is why diffraction is a regulatory quality topic and not only a research one.

Applications of X-ray diffraction: absolute structure determination of new drugs and natural products, polymorph screening and control, salt and co crystal characterisation, crystallinity testing of excipients, and identification of crystalline drugs in mixtures and in forensic samples.

5.4 Immunological assays

Immunological assays use the highly specific binding between an antigen and its antibody to measure drugs, hormones, proteins and infectious markers at very low concentrations, in complex samples such as blood, without prior separation.

RIA (radioimmunoassay): the sample antigen competes with a fixed amount of the same antigen labelled with a radioisotope (commonly iodine-125) for a limited number of antibody binding sites. After incubation, bound and free fractions are separated and the radioactivity of one fraction is counted. The more unlabelled antigen in the sample, the less labelled antigen can bind, so the signal falls as concentration rises; a standard curve converts counts into concentration. RIA is extremely sensitive (picogram level) and was the first technique to measure hormones and drugs like digoxin routinely, but it needs radioisotope facilities, shielding, waste disposal and short lived reagents, so it has largely been replaced by non isotopic labels.

ELISA (enzyme linked immunosorbent assay): the label is an enzyme (commonly horseradish peroxidase or alkaline phosphatase) instead of a radioisotope. In the widely used sandwich ELISA, a capture antibody fixed on a plastic well binds the antigen, a second enzyme labelled antibody binds another site of the same antigen, unbound material is washed away, and a substrate is added that the enzyme converts into a coloured product measured in a plate reader. In competitive ELISA, sample antigen competes with labelled antigen, and colour falls as concentration rises. ELISA keeps most of the sensitivity of RIA, is safe, has long reagent shelf life, and is easily automated in 96 well plates; uses include therapeutic drug monitoring, infectious disease markers, vaccine potency work and residue testing.

Bioluminescence assays: these use a naturally light producing enzyme system, most often firefly luciferase, which in presence of luciferin, ATP, oxygen and magnesium produces light. The light output is proportional to the limiting component, usually ATP, so the assay measures ATP as an index of living microbial cells: it is used for rapid microbial contamination and sterility related screening, hygiene monitoring of manufacturing surfaces and water, and cell viability testing. Related chemiluminescence immunoassays, using labels such as acridinium esters, are now common in automated clinical analysers because light based detection has almost no background and a very wide measurement range.

Feature	RIA	ELISA	Bioluminescence (ATP) assay
Label or signal	Radioisotope such as iodine-125	Enzyme producing a coloured product	Light from the luciferase reaction
Signal direction in competitive format	Falls as analyte rises	Falls as analyte rises	Rises with the amount of ATP present
Safety and waste	Radiation hazards, special disposal	Safe, routine laboratory waste	Safe, routine laboratory waste
Typical use	Hormones, digoxin, research assays	Drug and marker assays, screening in plates	Microbial load, hygiene and cell viability

5.5 Potentiometry

Potentiometry measures the potential (voltage) of an electrochemical cell at effectively zero current, and relates it to the concentration of a specific ion through the Nernst equation: $E = E_0 + (RT/nF) \ln a$ times the natural log of the ion activity, which at 25 degrees Celsius becomes a change of about 59.16 mV

for every ten fold change in activity of a singly charged ion. The cell has two electrodes: an indicator electrode whose potential responds to the analyte, and a reference electrode (saturated calomel or silver silver chloride) whose potential is constant.

Ion selective electrodes (ISE) are indicator electrodes whose membrane responds mainly to one ion: the glass electrode for pH and H^+ , the fluoride electrode with a lanthanum fluoride crystal membrane, crystalline and liquid membrane electrodes for ions such as calcium, nitrate, potassium and chloride, and gas sensing electrodes for ammonia and carbon dioxide. A practical assembly combines both electrodes in one combination probe with a meter reading in mV or directly in pH or concentration units; calibration with at least two standards bracketing the sample is essential, and an ionic strength adjuster keeps activity coefficients constant. Applications: pH measurement and potentiometric titrations (acid base, redox, precipitation and complexometric, where the end point is the steepest jump in potential), direct assay of fluoride and other ions in formulations and water, and monitoring of dissolution media. Its strengths are simplicity, low cost and no need for separation; its limits are interference from similar ions and the need for frequent calibration.

5.6 Thermal techniques: DSC, DTA and TGA

Thermal analysis studies how a sample changes when it is heated or cooled at a controlled rate. In pharmacy it answers questions no spectrum can: at what temperature the drug melts, whether it is polymorphic, whether it is crystalline or amorphous, whether drug and excipient interact, and how much solvent or moisture it holds.

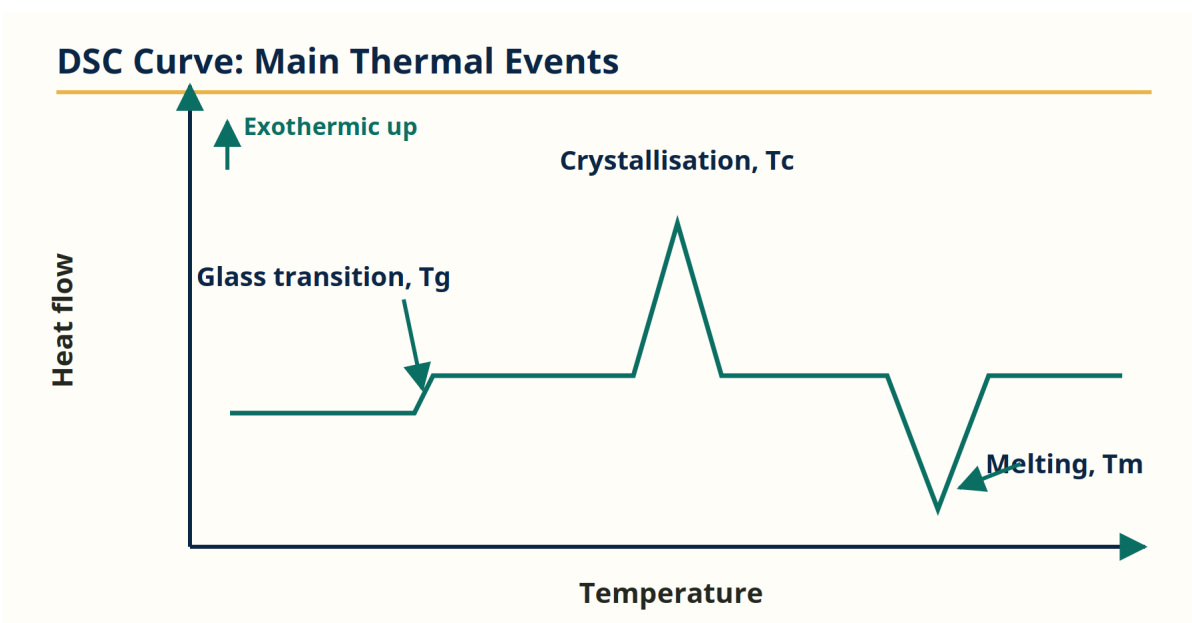


Figure: DSC curve with glass transition, crystallisation and melting events, exothermic direction up.

Differential scanning calorimetry (DSC): the sample and an inert reference are heated in separate pans, and the instrument measures the difference in heat flow needed to keep both at the same temperature as the furnace temperature rises at a linear rate. In the **power compensation** design two independent heaters supply that heat directly; in the **heat flux** design both pans sit on one heated disc and the temperature difference between them is converted into heat flow. The DSC curve plots heat flow against temperature: melting and other endothermic events appear as peaks in one direction, crystallisation and decomposition (exothermic events) in the other, and a **glass transition** appears as a step change in the baseline, the signature of an amorphous material. Two refinements from the syllabus: **modulated DSC** superimposes a small sinusoidal temperature oscillation on the ramp, which separates overlapping reversing events such as glass transition from non reversing events such as enthalpy relaxation and curing; **hyper DSC** uses very

fast heating rates (hundreds of degrees per minute) to increase sensitivity for tiny samples and weak transitions and to suppress reorganisation during the scan.

Experimental parameters that influence a DSC result, and which must be reported with it: sample preparation and mass (usually 2 to 10 mg, sealed, pin holed or open pan as the study needs), heating and cooling rate (faster rates raise observed transition temperatures and reduce resolution of close events, slower rates do the opposite), purge gas and its flow (nitrogen is standard; oxygen is used for oxidation studies), calibration with standards such as indium (melting point and heat of fusion) for temperature and enthalpy, and the thermal history of the sample itself, which is why a first heating run and a second heating run of the same sample can differ. Sources of error include poor pan contact, moisture loss mistaken for a transition, decomposition overlapping melting, and baseline drift.

Differential thermal analysis (DTA): the older and simpler relative of DSC. Sample and reference sit in the same furnace and the temperature difference between them (ΔT) is plotted against furnace temperature. When the sample melts it absorbs heat and lags behind the reference; when it crystallises or oxidises it gets ahead. DTA gives transition temperatures and a qualitative picture up to very high temperatures, but it does not measure energy as directly or as accurately as DSC. **Derivative DTA (DDTA)** plots the derivative of the DTA curve, which sharpens overlapping events and locates transition points more precisely.

Thermogravimetric analysis (TGA): a sensitive microbalance weighs the sample continuously while it is heated in a controlled atmosphere. The TGA curve plots mass (or percentage mass) against temperature, and its derivative (DTG) marks the temperatures of fastest mass loss. Mass falls in steps when volatile components leave: surface moisture first, then solvent of crystallisation, then decomposition products. Factors affecting TGA results: heating rate, atmosphere (air gives oxidative loss, nitrogen gives pyrolysis), sample mass and particle size, crucible type, and buoyancy effects at the start of the run. It measures only events that change mass, so melting without decomposition is invisible to TGA, which is exactly why DSC and TGA are run together as complementary methods.

Feature	DSC	DTA	TGA
Quantity measured	Difference in heat flow	Difference in temperature	Change in mass
Melting point	Yes, with enthalpy of fusion	Yes, temperature only	No, unless mass is lost
Glass transition	Yes, clear step	Weak or unclear	No
Moisture and solvent content	Indirect	Indirect	Yes, directly from mass loss steps
Polymorphism study	Yes	Yes	No
Main pharmaceutical uses	Melting, purity, polymorphism, compatibility, amorphous content	High temperature transitions, qualitative thermal profile	Drying loss, solvates and hydrates, decomposition, ash related work

Pharmaceutical applications of thermal methods, combined: drug excipient compatibility screening from changes in the drug melting peak, polymorph identification (different forms melt differently), determination of purity from melting point depression (the Van't Hoff DSC purity method), amorphous content measurement, stability prediction, characterisation of lipids, waxes and polymers used in formulations, and confirmation of hydrate and solvate stoichiometry by matching TGA mass loss with theory. Advantages: tiny samples, speed, and information about physical form that chemical assays miss. Disadvantages: destructive for the portion tested, results depend strongly on experimental conditions, and

overlapping events can be hard to assign without a supporting method such as hot stage microscopy or XRPD from Section 5.3.

Frequently Asked Questions

Q: Write the Beer-Lambert law and list any four reasons for deviation from it. The law states $A = \epsilon \times b \times c$, where absorbance is directly proportional to molar absorptivity, path length and concentration. Deviations occur when the radiation is not monochromatic, when stray light reaches the detector, when the solution is too concentrated so molecules interact with each other, and when the solute associates, dissociates or reacts with the solvent or changes form with pH.

Q: What are the advantages of FT-IR over a dispersive IR spectrophotometer? FT-IR measures all frequencies at once through an interferometer (Fellgett or multiplex advantage), passes more radiation to the detector because it has no narrow slits (Jacquinot or throughput advantage), and uses a laser reference for a highly accurate wavenumber scale (Connes advantage). The result is a spectrum in seconds with a much better signal to noise ratio.

Q: What is chemical shift? Why is TMS used as the reference in NMR? Chemical shift is the position of an NMR signal on the delta scale in ppm, measured relative to TMS, and it tells the electronic environment of the protons. TMS is used because its 12 protons are equivalent and give one sharp strong signal, it is chemically inert and volatile so it can be removed easily, and its signal appears upfield of almost all sample signals.

Q: A proton NMR signal is a quartet and another is a triplet, with the same J value. What does this tell you? The two groups are coupled to each other. By the n+1 rule, the quartet has three equivalent neighbouring protons and the triplet has two, the classic picture of an ethyl group, CH₃ giving the triplet and CH₂ giving the quartet. Equal J values confirm they are mutual coupling partners.

Q: Compare EI and ESI ionisation in mass spectroscopy. Electron impact is a hard ionisation using 70 eV electrons on vaporised samples; it fragments the molecule heavily, which is useful for structure work but the molecular ion may be weak or absent, and the sample must be volatile. Electrospray is a soft ionisation from liquid solution at atmospheric pressure; it gives strong molecular species, often multiply charged, with little fragmentation, and it suits polar, large and heat sensitive molecules in LC-MS.

Q: How does the presence of chlorine or bromine show up in a mass spectrum? Chlorine occurs as mass 35 and 37 in about a 3:1 ratio, so a compound with one chlorine shows M and M+2 peaks in that ratio. Bromine occurs as mass 79 and 81 in about a 1:1 ratio, so one bromine gives M and M+2 peaks of almost equal height. These isotope patterns are the quickest way to spot a halogen in an unknown compound.

Q: Define resolution, plate number and HETP. What resolution value means baseline separation? Plate number N measures column efficiency, $N = 16 (t_R/W)^2$. HETP is the column length divided by N, and a smaller HETP means a better column. Resolution R_s compares the separation of two peak maxima with their widths, and R_s of 1.5 or more means the two peaks are separated to the baseline, the standard target in pharmaceutical methods.

Q: Why is reversed phase HPLC used more than normal phase HPLC in quality control? In reversed phase HPLC a non polar C18 stationary phase is used with a water based polar mobile phase, which suits most drugs, gives reproducible retention, is compatible with aqueous samples and dissolution media, and the columns are robust and long lived. Normal phase with bare silica is very sensitive to traces of water in the mobile phase, which makes retention drift, so it is reserved for special cases.

Q: State Bragg law and give two pharmaceutical applications of X-ray diffraction. Bragg law states $n\lambda = 2d \sin \theta$, relating the wavelength, the spacing d between crystal planes and the angle θ at which diffraction occurs. Its main pharmaceutical uses are identification and quantification of polymorphs by powder diffraction, and complete three dimensional structure determination of drug molecules by single crystal diffraction.

Q: What is isoelectric focusing? Isoelectric focusing is electrophoresis in a stable pH gradient. A protein migrates until it reaches the pH equal to its isoelectric point, where its net charge becomes zero and it stops, focused into a sharp band. It separates proteins with very small differences in isoelectric point and is used for charge variant profiling of proteins and monoclonal antibodies.