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MPG101T Modern Pharmaceutical Analytical Techniques, M.Pharm Semester 1

Unit 5: Electrophoresis and X-Ray Crystallography

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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.

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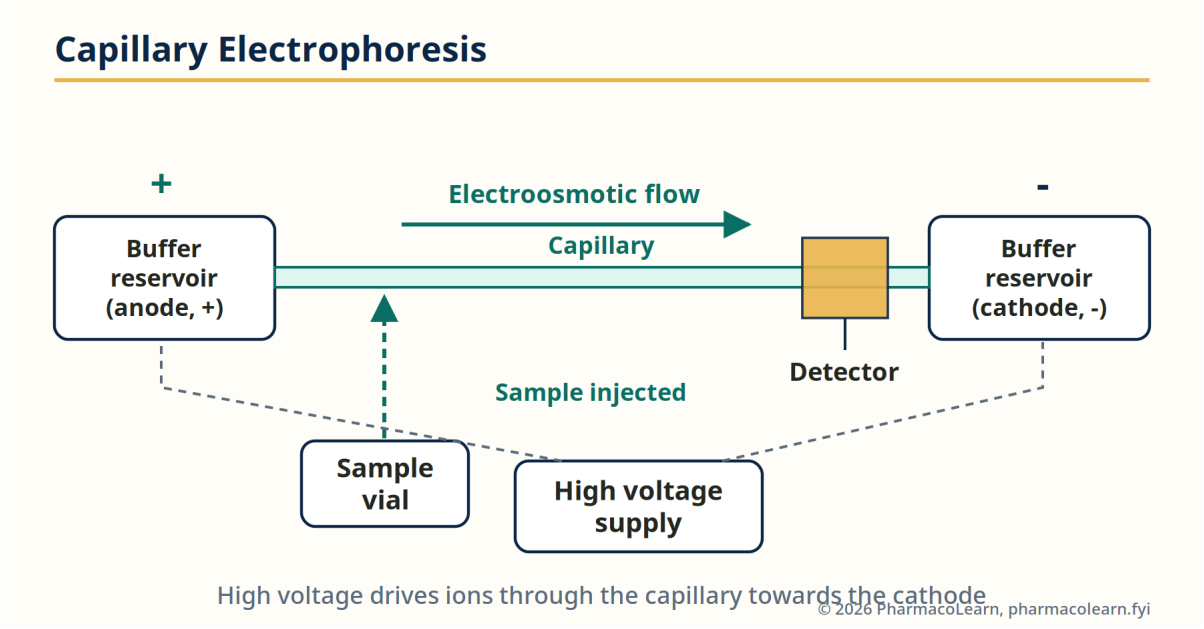

Figure: Capillary electrophoresis set up, electroosmotic flow carries the sample towards the cathode.

- **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.

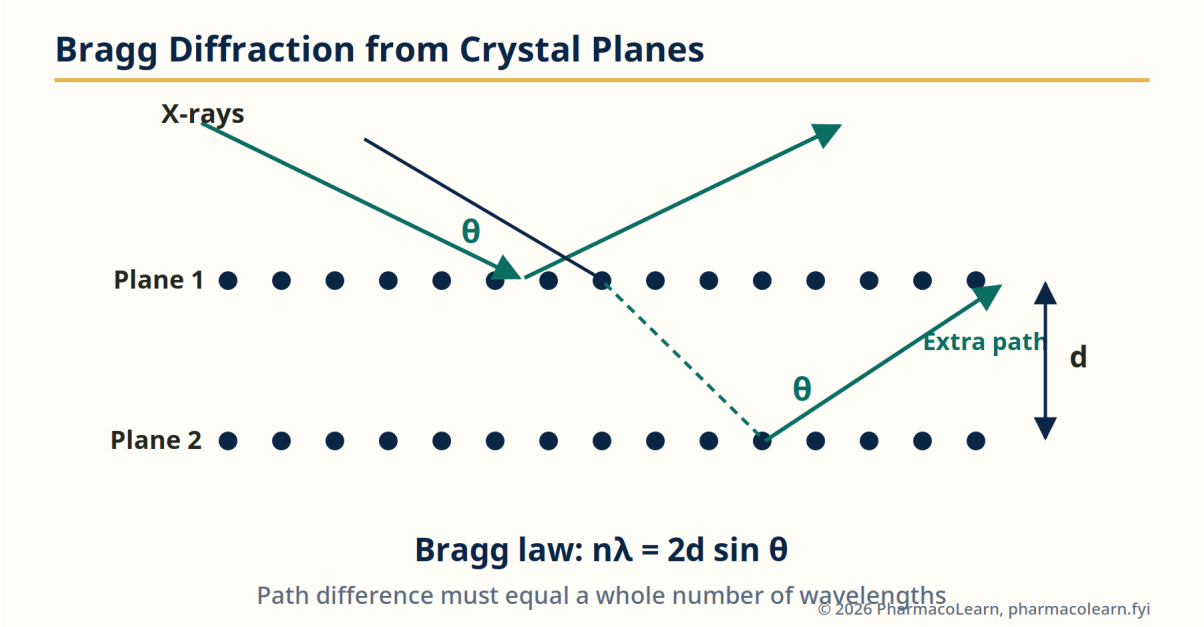

Figure: Bragg diffraction, reflected X-rays reinforce when the path difference equals a whole number of wavelengths.

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 x lambda = 2d sin theta

where n is the order of reflection, lambda is the wavelength, d is the interplanar spacing and theta 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 (alpha, beta, gamma); 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.

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