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MPC101T Modern Pharmaceutical Analytical Techniques, M.Pharm Semester 1

Unit 4: Chromatography

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4.1 Principle and basic terms

Chromatography is a separation technique in which the components of a mixture are distributed between two phases: a **stationary phase** (a solid, or a liquid coated on a solid, which does not move) and a **mobile phase** (a liquid or a gas, which flows over the stationary phase). Components separate because they have different affinities for the two phases: the component attracted more strongly to the stationary phase moves slowly, and the component attracted more to the mobile phase moves quickly. The underlying mechanisms are adsorption, partition, ion exchange, size exclusion and affinity, and most examination systems are still classified by which mechanism dominates.

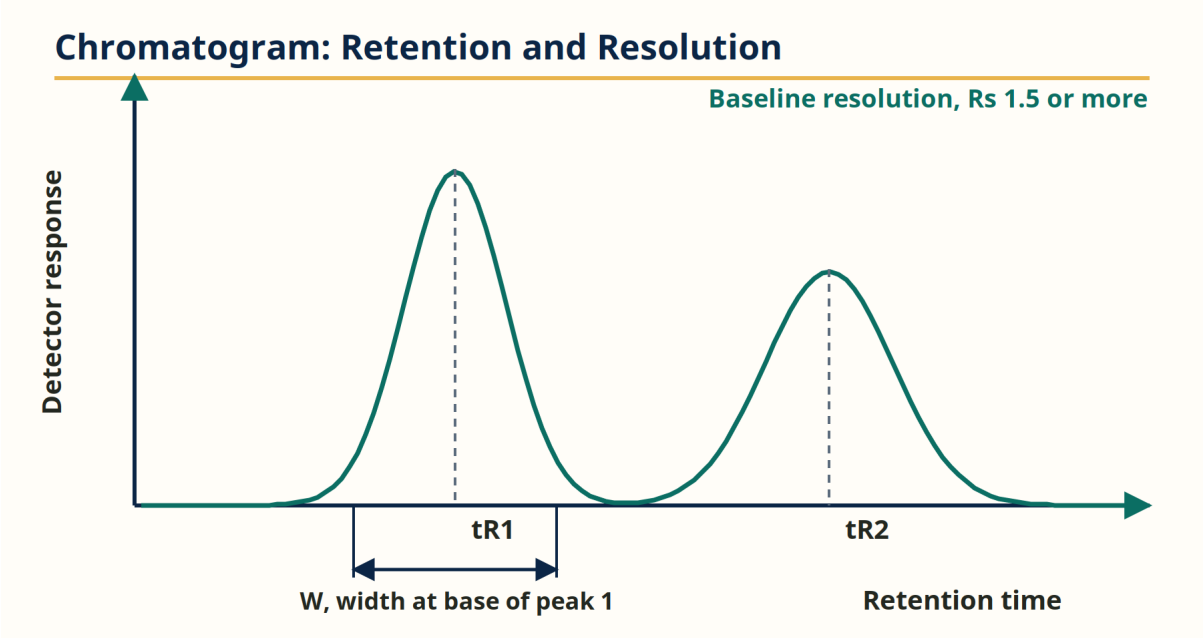

Figure: Chromatogram peaks, retention time, peak width at the base and baseline resolution.

Key chromatographic terms and parameters, used for every technique in this unit:

- **Retention time (tR):** the time from injection to the peak maximum. The time spent in the mobile phase alone, shown by an unretained marker, is the dead time (tM or t0).
- **Retention or capacity factor (k):** k = (tR minus tM) divided by tM. It measures how long a compound is held by the stationary phase; good separations usually need k between about 1 and 10.
- **Selectivity factor (alpha):** the ratio of the k values of two adjacent peaks. It depends on the chemistry of the phases and must be greater than 1 for any separation at all.
- **Efficiency and plate number (N):** the column behaves as if it contained N theoretical plates; N = 16 x (tR divided by peak width at base) squared, or 5.54 x (tR divided by width at half height) squared. Sharper peaks mean higher N.
- **Height equivalent to a theoretical plate (HETP or H):** H = column length L divided by N. Smaller H means a more efficient column.

- **Resolution (Rs):** Rs = 2 x (difference in retention times) divided by (sum of the two peak widths at base). Rs of 1.5 means baseline separation, the target in validated pharmaceutical methods.
- **Van Deemter equation:** H = A + (B divided by u) + C x u, where u is the mobile phase velocity, A is eddy diffusion (multiple flow paths), B is longitudinal diffusion (important in gases, dominant at low flow), and C is resistance to mass transfer (dominant at high flow). It explains why every column has an optimum flow rate at which H is minimum, and why small, uniform particles improve efficiency by reducing A and C.

Three factors control a separation, one for each term: efficiency improves resolution with the square root of N, selectivity is the most powerful lever, and retention must be kept in a useful range. In practice the analyst changes selectivity first (different stationary phase, solvent, pH or temperature), then efficiency, then retention.

4.2 Paper chromatography

In paper chromatography the stationary phase is water held in the fibres of a cellulose filter paper, and separation is mainly by partition. A spot of sample is applied near one edge, the edge is dipped in the solvent (mobile phase) in a closed chamber saturated with solvent vapour, and the solvent climbs by capillarity. In **ascending** development the solvent climbs up, in **descending** development it flows down with gravity assisting, in **radial or circular** development it spreads outwards from the centre, and in **two dimensional** development the paper is run in one solvent, dried, turned 90 degrees and run in a second solvent, which greatly increases the number of compounds that can be separated on one sheet. Common paper grades are Whatman No. 1 (routine) and thicker grades for preparative or heavily loaded work.

Results are expressed as the **Rf value**: distance travelled by the compound divided by distance travelled by the solvent front. Rf depends on the solvent system, temperature and paper, so standards are run on the same sheet. Compounds are detected by colour, by fluorescence or quenching under UV, or by spraying a locating reagent such as ninhydrin for amino acids. Applications today are mainly teaching, simple qualitative checks and some pharmacognostic screening; the method is cheap but slow, poorly reproducible and only semi quantitative.

4.3 Thin layer chromatography (TLC) and HPTLC

In TLC the stationary phase is a thin, uniform layer (about 0.25 mm) of adsorbent, most often silica gel G (silica with gypsum binder), alumina or cellulose, coated on a glass, aluminium or plastic plate. Silica is polar, so separation on normal phase TLC is by adsorption: polar compounds stick and run low, non polar compounds run high. A few microlitres of sample are spotted above the solvent level, the plate is developed in a closed, saturated tank over a short distance (usually 5 to 10 cm), dried, and visualised: coloured spots directly, UV active compounds at 254 nm on plates containing a fluorescent indicator (they appear as dark spots) or at 366 nm for fluorescent compounds, and the rest by spraying reagents or by iodine vapour. Rf is calculated as in paper chromatography, and identity is supported when sample and standard give the same Rf and the same colour reaction in at least two different solvent systems.

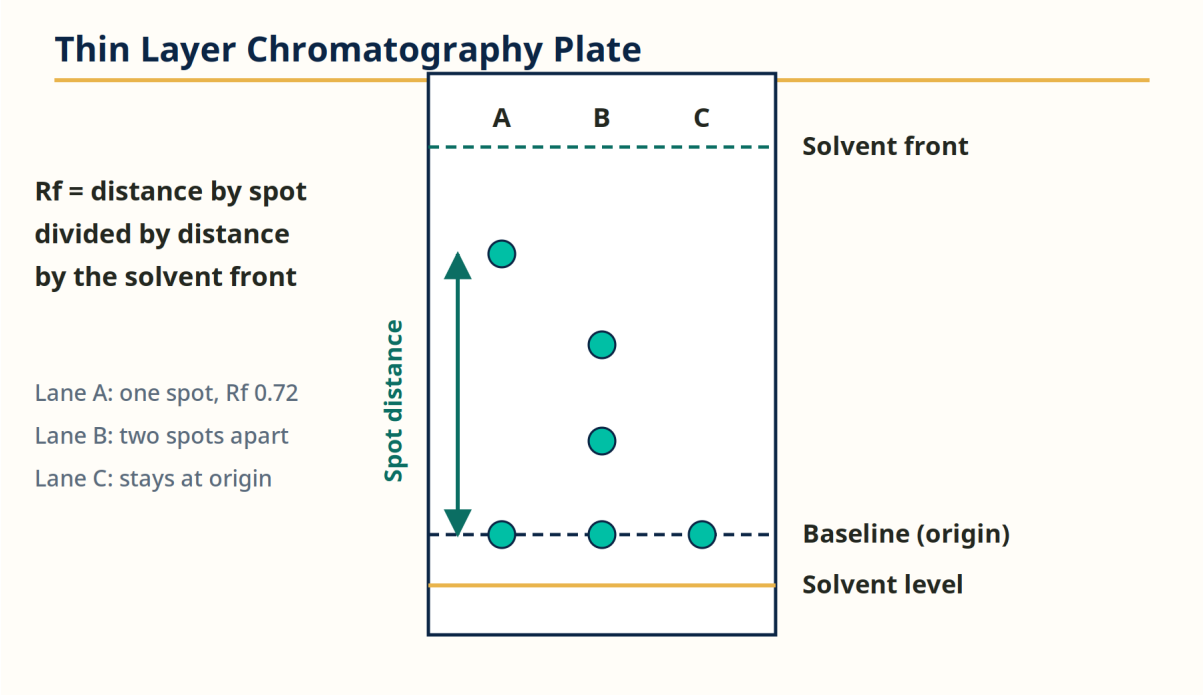

Figure: Developed TLC plate, Rf is the distance travelled by the spot divided by the distance travelled by the solvent front.

TLC remains a pharmacopoeial identity test for many drugs and herbal products because it is fast, cheap, parallel (many samples on one plate) and disposably clean, since the plate is used once and matrix interference is left at the origin. Its limits are moderate reproducibility and mainly qualitative output.

HPTLC (high performance TLC) uses plates with much smaller and more uniform particles (about 5 to 7 micrometres), thinner layers, automatic sample applicators that lay down narrow bands, controlled development chambers and densitometric scanning of the plate. The result is better resolution, lower detection limits and true quantification from spot intensity, used for herbal drug standardisation, fingerprinting of extracts and stability work.

4.4 Ion exchange chromatography

Ion exchange separates charged species on a resin carrying fixed ionic groups. A **cation exchanger** carries acidic groups (strong type: sulfonic acid, SO3 minus; weak type: carboxylic acid, COO minus) and retains cations. An **anion exchanger** carries basic groups (strong type: quaternary ammonium; weak type: amino groups) and retains anions. The sample ions compete with mobile phase ions for the resin sites, so elution is controlled by the pH and the ionic strength of the mobile phase: raising salt concentration or changing pH so the analyte loses its charge releases it from the resin.

Resin properties tested in exams: degree of cross linking (higher cross linking, harder resin, slower exchange but better selectivity for ions of different size), exchange capacity (milli equivalents per gram), and particle size. Applications: purification and assay of ionisable drugs and amino acids, removal of interfering ions, water demineralisation (an off line but standard example), separation of rare earths and of charged degradation products, and the amino acid analyser. In modern practice, ion chromatography with suppressed conductivity detection is the routine form for ions such as chloride, sulphate and counter ions in drug salts.

4.5 Column chromatography

Classical column chromatography packs the stationary phase, usually silica gel or alumina of 60 to 200 mesh, into a vertical glass column. The mixture is loaded on top, mobile phase is added, and components travel down at different rates by adsorption, collecting in fractions that are monitored by TLC. Elution can be

isocratic (one solvent throughout) or by a polarity gradient in steps. It is a preparative and purification technique rather than an assay technique: isolation of compounds from extracts, purification of synthetic intermediates, and clean up of samples before instrumental analysis. Flash chromatography is its faster modern form, using finer silica and moderate air pressure. The activity of alumina and silica depends on their water content (Brockmann activity grades), so adsorbents are activated by heating and standardised before use.

4.6 Gas chromatography (GC)

GC separates volatile and thermally stable compounds. The mobile phase is an inert carrier gas (helium, nitrogen or hydrogen) and the stationary phase is either a solid adsorbent (gas solid chromatography, for permanent gases) or, far more commonly, a thin film of a high boiling liquid coated inside a long capillary column (gas liquid chromatography). A modern capillary column is 15 to 60 m long, 0.25 mm internal diameter, with a film of a polysiloxane phase; non polar phases separate largely by boiling point, polar phases add selective interactions.

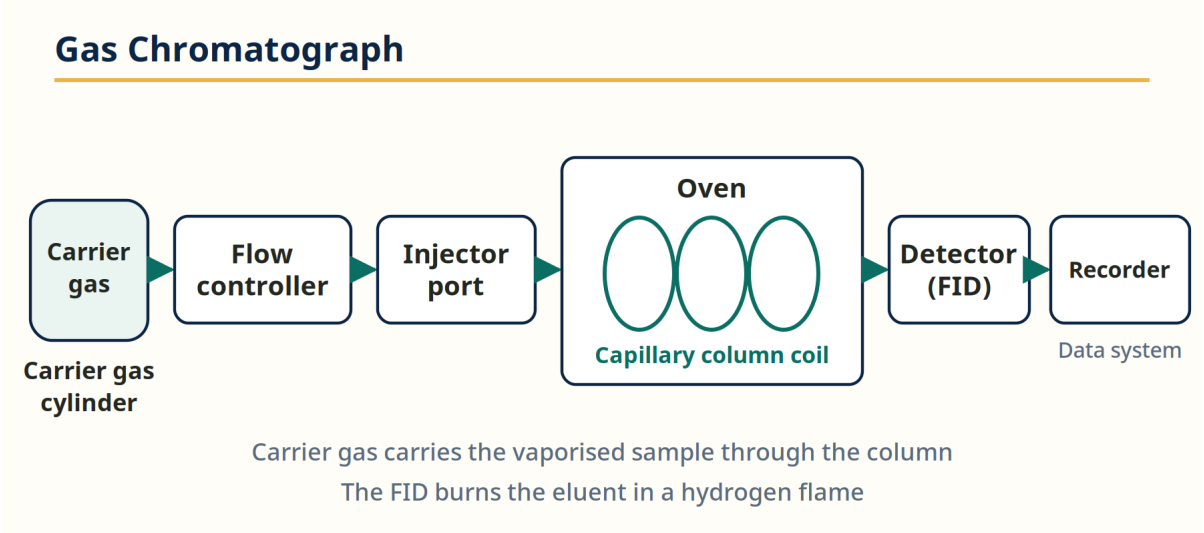

Figure: Gas chromatograph with a capillary column inside a temperature controlled oven.

The GC flow is:

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Carrier gas supply and flow controller -> Injector (heated, sample vaporises instantly)
-> Capillary column inside a temperature programmed oven -> Detector -> Data system
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- **Injector:** split injection sends only a small fraction of the sample to the column (for concentrated samples), splitless injection sends nearly all of it (for trace analysis). The injector is kept hot enough to vaporise the sample without decomposing it.
- **Oven programme:** temperature is raised in a controlled ramp so that both volatile and heavy components elute in reasonable time with good resolution.
- **Detectors:** the **flame ionisation detector (FID)** burns the eluent in a hydrogen flame and measures ions formed from carbon compounds; it is sensitive, robust, nearly universal for organics, but destroys the sample and does not respond to water or permanent gases. The **thermal conductivity detector (TCD)** is universal and non destructive but insensitive, used for gases. The **electron capture detector (ECD)** is extremely sensitive to halogenated and nitro compounds, used for pesticide and chlorinated drug residues. Other detectors: nitrogen phosphorus detector for N and P compounds, flame photometric detector for S and P, and the mass spectrometer as the most informative detector (GC-MS).

Applications: residual solvents in drug substances (a regulatory must), volatile impurities and essential oil analysis, fatty acid profiling after derivatisation, alcohol content, and analysis of anaesthetic gases. Compounds that are not volatile, such as most drug salts and sugars, need derivatisation, for example silylation, before GC.

4.7 High performance liquid chromatography (HPLC)

HPLC is the most important technique in this whole subject for a working pharmacist, because most drugs are non volatile, polar or heat sensitive, exactly the compounds GC cannot handle. A high pressure pump forces a liquid mobile phase through a short column packed with very fine particles, giving high efficiency in minutes.

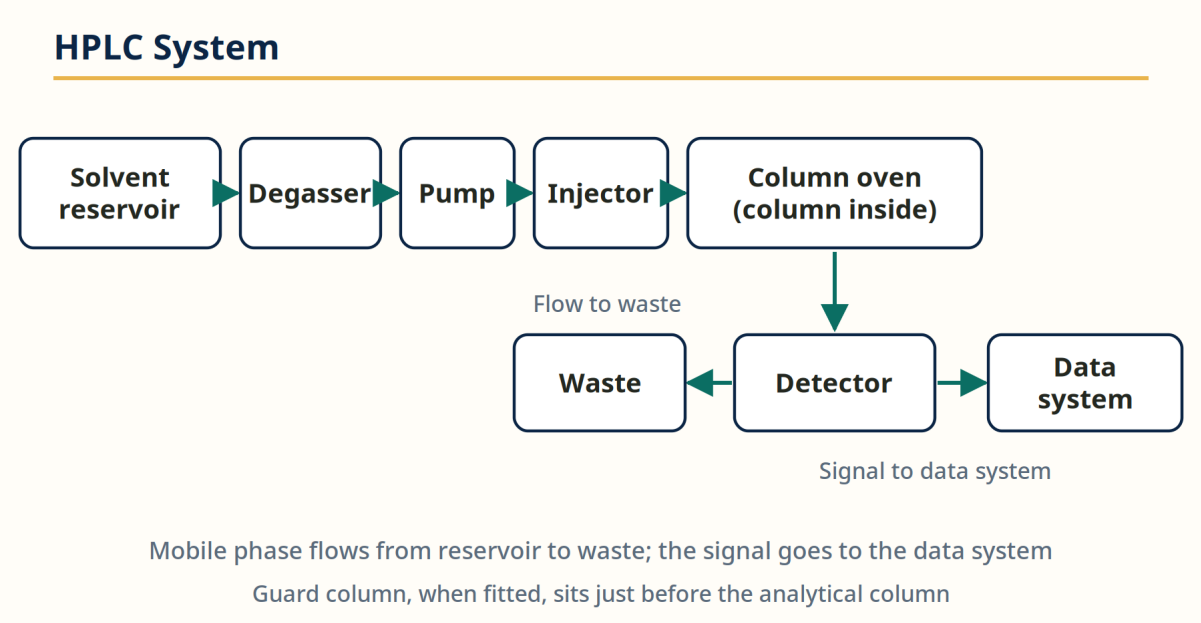

Figure: HPLC system, mobile phase flows from the reservoir through the column and detector to waste.

The HPLC flow is:

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Solvent reservoirs (with degasser) -> High pressure pump -> Injector or autosampler
-> Guard column -> Analytical column (in a column oven) -> Detector -> Data system
     (waste out, or fraction collector in preparative HPLC)
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- **Pumps** deliver a pulse free, constant flow, typically 0.1 to 2 mL per minute at pressures up to about 400 bar in conventional HPLC, in either isocratic mode (fixed mobile phase composition) or gradient mode (composition changes during the run to elute strongly retained compounds).
- **Columns:** stainless steel, commonly 150 or 250 mm long and 4.6 mm internal diameter, packed with 3 to 5 micrometre particles. The workhorse stationary phase is C18 (octadecylsilane bonded silica, reversed phase), followed by C8, phenyl, cyano and amino phases. In **reversed phase** HPLC the stationary phase is non polar and the mobile phase is polar (water with methanol or acetonitrile, usually buffered); polar compounds elute first. This is the reverse of classical normal phase chromatography, where a polar stationary phase such as bare silica is used with a non polar mobile phase.
- **Detectors:** the **UV and photodiode array (PDA) detector** is standard; PDA records the full spectrum of each peak, so peak purity can be checked. Others: refractive index (universal but insensitive and gradient incompatible), fluorescence (very sensitive for fluorescent analytes), electrochemical, evaporative light scattering and charged aerosol detectors for compounds without a chromophore, and the mass spectrometer (LC-MS).

- **Method points that decide success:** mobile phase pH must control ionisation of the analyte (an ionised drug is barely retained on C18), buffer choice and concentration, organic modifier percentage, column temperature, and system suitability tests (plates, tailing factor, resolution and repeatability) that pharmacopoeias require before results are accepted.

Applications: assay and related substances testing of almost every modern drug product, dissolution and stability studies (stability indicating methods), bioanalysis, cleaning validation and pharmacopoeial monograph methods. In short, if a quality control laboratory could keep only one instrument from this unit, it would keep the HPLC.

4.8 Ultra high performance liquid chromatography (UHPLC)

UHPLC uses columns packed with sub 2 micrometre particles and instruments built for pressures up to about 1000 to 1500 bar. From the Van Deemter equation, smaller particles give lower H and, importantly, keep that efficiency at higher flow rates, so runs become several times faster with equal or better resolution and much lower solvent use. The costs are the need for low dead volume fittings, very clean samples and filtration, and higher instrument cost. Methods transfer from HPLC to UHPLC by scaling column dimensions, flow and gradient time in proportion to column volume and particle size.

4.9 Affinity chromatography and gel chromatography

Affinity chromatography is the most selective of all: the stationary phase carries a ligand that binds one target molecule by a specific biological interaction, for example an antibody binding its antigen, an enzyme binding its inhibitor, or protein A binding the Fc region of antibodies. Everything else washes through; the bound target is then eluted by changing pH, ionic strength or adding a competing ligand. It is used for purification of monoclonal antibodies, enzymes, vaccines and plasma proteins, and for removing specific impurities. It gives purification in a single step that other methods need many steps for, at the cost of expensive and delicate stationary phases.

Gel chromatography, also called size exclusion or gel permeation (for organic solvents) and gel filtration (for aqueous systems), separates purely by molecular size. The stationary phase is a porous gel (dextran, agarose, polyacrylamide or porous silica). Large molecules cannot enter the pores, travel only in the space between particles and elute first; small molecules enter the pores, take a longer path and elute later, so elution order is strictly large to small, the reverse of intuition for students. Nothing is adsorbed ideally, so the mobile phase composition affects elution little. Uses: molecular weight estimation of polymers and proteins, desalting and buffer exchange, separation of aggregates from monomers in biopharmaceuticals, and polymer molecular weight distribution in gel permeation mode.

4.10 Unit 4 comparison table

Technique	Stationary phase	Separation basis	Typical pharmaceutical use
Paper chromatography	Water held on cellulose paper	Partition	Simple qualitative checks, teaching
TLC and HPTLC	Silica gel layer on a plate	Adsorption	Identity tests, herbal fingerprinting, quick screening
Ion exchange	Resin with fixed charged groups	Exchange of ions	Ionisable drugs, amino acids, counter ions
Column chromatography	Silica or alumina in a glass column	Adsorption	Preparative purification and isolation
Gas chromatography	Liquid film in a capillary column	Partition, volatility	Residual solvents, volatile compounds, essential oils

Technique	Stationary phase	Separation basis	Typical pharmaceutical use
HPLC	Bonded phase (C18) packed column	Partition, mainly reversed phase	Assay, impurities, stability, the QC workhorse
UHPLC	Sub 2 micrometre packed column	Partition, reversed phase	Fast, high resolution assay and screening
Affinity chromatography	Immobilised specific ligand	Biospecific binding	Antibodies, enzymes, vaccines purification
Gel chromatography	Porous gel beads	Molecular size	Molecular weight, aggregates, desalting