Unit-4

Instrumental Methods of Analysis

B.Pharma 7 Sem Notes

Unit: 4

- **Gas chromatography** – Introduction, theory, instrumentation, derivatization, temperature programming, advantages, disadvantages and applications.
- **High performance liquid chromatography (HPLC)-** Introduction, theory, instrumentation, advantages and applications.

GAS CHROMATOGRAPHY (GC)

Gas Chromatography (GC)

Introduction

Gas Chromatography (GC) is a powerful separation technique used to separate, identify, and quantify volatile and thermally stable compounds. It is called 'gas' chromatography because the MOBILE PHASE is an inert gas (called the carrier gas). The sample must be converted to the gas/vapour phase for separation to occur.

Simple Analogy — How GC Works
Imagine releasing a mix of gases into a long tunnel packed with sticky material. Different gases stick
to the walls with different strengths. Some zip through quickly; others take much longer to emerge.
When each gas exits the tunnel, a detector records it. The result is a chromatogram with separate peaks.
The tunnel = GC column | sticky material = stationary phase | carrier gas = mobile phase.

GC was first developed by Martin and James in 1952 (Nobel Prize-winning work). Today it is one of the most widely used analytical instruments in pharmaceutical, environmental, food, and forensic laboratories.

Types of Gas Chromatography

Type	Stationary Phase	Abbreviation	Best Used For
Gas–Solid Chromatography	Active solid (alumina, silica, molecular sieves)	GSC	Permanent gases (O_2, N_2, CO, CO_2), light hydrocarbons
Gas–Liquid Chromatography	Liquid coated on an inert solid support	GLC	Most organic compounds; widely used in pharmacy

Point — Which Type is More Common?
GLC (Gas–Liquid Chromatography) is by far the most widely used type.
When people say 'Gas Chromatography' or 'GC', they almost always mean GLC.
Separation in GLC is based on PARTITION — the solute distributes itself between the
gas (mobile) phase and the liquid (stationary) phase according to its partition coefficient.

Theory of Gas Chromatography

A. Basis of Separation — Partition

In GLC, separation occurs because each compound has a different affinity (measured by the partition coefficient K) for the liquid stationary phase vs the gas mobile phase.

K = Concentration of solute in stationary phase / Concentration of solute in gas phase

- HIGH K value: Compound strongly prefers the stationary phase → travels SLOWLY → elutes LATE → LONG retention time.
- LOW K value: Compound prefers the gas phase → travels QUICKLY → elutes EARLY → SHORT retention time.
- Factors determining K: Volatility (boiling point), polarity matching between solute and stationary phase.

B. Retention Time and the Chromatogram

When a sample is injected, each compound travels through the column at a different speed. The detector records a signal (peak) each time a compound exits the column. This record is called a CHROMATOGRAM.

Term	Definition	Significance
Retention Time (tR)	Total time from injection to peak maximum at the detector	Used to identify compounds (each compound has a characteristic tR under fixed conditions)
Dead Time / Hold-up Time (tM)	Time for a non-retained compound (e.g., air) to travel from injector to detector	Represents only the gas phase flow time
Adjusted Retention Time (t′R)	t′R = tR − tM	Reflects only the time spent in the stationary phase
Relative Retention (α)	tR2 / tR1 (ratio of two retention times)	Measure of how well two compounds are separated; α > 1 for separation
Retention Volume (VR)	VR = tR × F (F = flow rate in mL/min)	Volume of carrier gas needed to elute a compound
Capacity Factor (k′)	k′ = t′R / tM	Measures how long compound stays in stationary phase vs mobile phase

C. Column Efficiency — Theoretical Plates

Column efficiency describes how sharp and narrow the peaks are. Narrower peaks = better efficiency = better ability to separate closely-eluting compounds.

Number of Theoretical Plates (N):

$$N = 16 \times (tR / wb)^2 \quad OR \quad N = 5.545 \times (tR / w_{½})^2$$

Where: tR = retention time; wb = base width of peak; w½ = peak width at half-height

Height Equivalent to a Theoretical Plate (HETP or H):

$$H = L / N$$

Where: L = column length. Smaller H = More theoretical plates per unit length = Better efficiency

D. The Van Deemter Equation (Most Important Theoretical Concept)

The Van Deemter equation explains how various factors contribute to band broadening (peak spreading) in a GC column. Band broadening is undesirable because it reduces resolution.

Van Deemter Equation
$H = A + B/u + Cu$
H = Height Equivalent to a Theoretical Plate (smaller is better)
u = Linear velocity of carrier gas (cm/s)
A = Eddy Diffusion (multiple flow path) term
B = Longitudinal (molecular) diffusion term
C = Mass transfer resistance term (includes Cs and CM terms)

Term	Name	Cause	How to Minimise
A (Eddy Diffusion)	Multiple Flow Path Term	Solute molecules take different path lengths around stationary phase particles	Use CAPILLARY (open tubular) columns (A=0); use small, uniform particle size in packed columns
B/u (Longitudinal Diffusion)	Molecular Diffusion Term	Solute molecules diffuse along the column axis (from high to low concentration zone)	Use HIGHER carrier gas velocity; use heavier carrier gas (N_2 > He for this term)
Cu (Mass Transfer)	Resistance to Mass Transfer	Slow equilibration of solute between gas and liquid phases; solute spends unequal times in each phase	Use THIN liquid film; use smaller particles; use optimal (not excessive) flow rate

Van Deemter Curve — What It Tells Us
When H is plotted against flow rate (u), a hyperbola-shaped curve is obtained.
At LOW flow rates: B/u term dominates — longitudinal diffusion causes broad peaks.
At HIGH flow rates: Cu term dominates — mass transfer resistance causes broad peaks.
At the MINIMUM of the curve: Optimum flow rate (u_opt) — best efficiency (lowest H = sharpest peaks).
Practical tip: Run the GC at the optimal flow rate for best resolution.

Helium as carrier gas gives the optimal point at HIGHER flow rates than nitrogen — faster analysis!

E. Resolution (Rs)

Resolution measures how completely two adjacent peaks are separated from each other.

$$Rs = 2(tR2 - tR1) / (wb1 + wb2)$$

Resolution Value	Interpretation
Rs < 1.0	Peaks overlap significantly; poor separation
Rs = 1.0	Peaks are :94% separated (touching peaks)
Rs = 1.5	Baseline separation (complete separation); gold standard
Rs > 2.0	Over-resolved; wasted time / unnecessarily long analysis

Instrumentation of Gas Chromatography

A GC instrument consists of the following major components, each playing a critical role:

GC Instrument Block Diagram
Carrier Gas Cylinder → Pressure/Flow Regulators → Sample Injector → Column (in Oven)
→ Detector → Signal Amplifier → Data System / Recorder / Integrator

Component 1: Carrier Gas (Mobile Phase)

The carrier gas is the inert mobile phase that carries sample vapours through the column. It must be chemically inert (non-reactive with sample, stationary phase, or column), pure (99.995%+), and dry.

Carrier Gas	Mol. Weight	Thermal Conductivity	Detectors	Advantages	Disadvantages
Helium (He)	4	High	TCD, FID, MS, all detectors	Fast analysis; safe; excellent for capillary GC	Expensive; imported
Nitrogen (N_2)	28	Medium	TCD, FID, ECD	Inexpensive; readily available	Slower optimal velocity; poor for MS
Hydrogen (H_2)	2	Highest	TCD, FID	Fastest analysis; high efficiency; cheapest	Flammable; explosion risk; needs precautions
Argon (Ar)	40	Low	ECD only	Excellent for ECD detector	Limited to specific detectors

- Flow rate: Typically 1–10 mL/min for packed columns; 1–2 mL/min for capillary columns.
- Pressure regulators and mass flow controllers ensure stable, reproducible flow rates.
- Molecular sieves and moisture traps in the gas line remove water and impurities.

Component 2: Sample Injection System

The injector introduces the sample into the carrier gas stream and instantly vaporises it. The injection must be FAST (to ensure a sharp starting band) and REPRODUCIBLE.

A. Split / Splitless Injector (Most Common for Capillary GC)

The split/splitless injector is a heated metal block (150–320°C) where the sample is vaporised. It has a split vent that allows only a fraction of the sample vapour to enter the column.

Mode	Split Ratio	Sample Amount Entering Column	Used For
Split Mode	1:10 to 1:500	Only 1/10 to 1/500 of vaporised sample enters column	Concentrated samples (>0.1%); fast, avoids column overloading
Splitless Mode	No splitting; all sample enters	100% of vaporised sample enters column	Trace analysis (ppm/ppb level); dilute samples

B. On-Column Injection

- The liquid sample is injected directly into the cool column without prior vaporisation.
- Column is initially cool (below boiling point of solvent) to refocus the sample band.
- Best for thermally labile compounds that decompose at high temperatures; avoids thermal degradation.

C. Packed Column Injector

- Simpler injector for packed columns. Sample injected through a septum (silicone rubber disc) via a microsyringe.
- Vaporised at 20–50°C above column temperature. All vapour enters the packed column.

Practical Tips for Injection
Use a µL-range microsyringe (0.5–10 µL typical). Inject quickly in one smooth motion.
Inject 0.1–2 µL for capillary GC; 1–10 µL for packed column GC.
Injector temperature must be high enough to vaporise all components but not cause decomposition.
Replace the septum regularly — septum bleed causes ghost peaks in the chromatogram.

Component 3: The GC Column (Heart of the Instrument)

The column is where separation actually occurs. It is housed inside a thermostatically controlled oven. Two main types exist:

A. Packed Columns

- Material: Stainless steel or glass, 1–3 m long, 2–4 mm internal diameter.
- Packed with: Inert support material (Chromosorb, Celite) coated with stationary liquid phase (typically 3–20% by weight).
- Advantages: Large sample capacity; simpler to use; good for preparative work.
- Disadvantages: Lower efficiency (few thousand theoretical plates); slower analysis; more bleed.

B. Capillary (Open Tubular) Columns — Modern Standard

- Material: Fused silica (glass) with polyimide outer coating for flexibility.
- Dimensions: 10–100 m long, 0.1–0.53 mm internal diameter — very long and narrow!

- The A (Eddy diffusion) term = 0 because there is no packing; sample flows only through the open tube.
- Result: MUCH higher efficiency (100,000–500,000 theoretical plates!) compared to packed columns.

Type of Capillary Column	Abbreviation	Stationary Phase Location	Features
Wall-Coated Open Tubular	WCOT	Liquid stationary phase coated directly on inner wall	Most common; highest efficiency; thin film (0.1–3 µm)
Support-Coated Open Tubular	SCOT	Solid support coated with liquid phase on inner wall	Higher sample capacity than WCOT; intermediate efficiency
Porous-Layer Open Tubular	PLOT	Solid porous adsorbent layer on inner wall	For gases, light hydrocarbons (GSC principle in capillary form)

C. Common Stationary Phases and Their Polarity

Stationary Phase	Polarity	Max Temp (°C)	Used For
Squalane	Non-polar	150	Hydrocarbons, non-polar compounds
OV-1 / SE-30 (100% PDMS)	Non-polar	350	Hydrocarbons, drugs, flavours (most universal)
OV-17 (50% Phenyl PDMS)	Medium	300	Drugs, steroids, pesticides
OV-225 (Cyanopropyl phenyl)	Polar	250	Fatty acid methyl esters, polar drugs
Carbowax 20M (PEG)	Highly polar	250	Alcohols, organic acids, flavour compounds
Cyanosilicone (SP-2340)	Highly polar	240	Fatty acids, polyunsaturated lipids

Rule for Choosing Stationary Phase
LIKE DISSOLVES LIKE principle applies.
For NON-POLAR compounds → use a NON-POLAR stationary phase (OV-1, squalane).
For POLAR compounds → use a POLAR stationary phase (Carbowax, cyanosilicone).
Non-polar stationary phases separate compounds in ORDER OF BOILING POINT.
Polar stationary phases also separate by POLARITY in addition to boiling point.

Component 4: Column Oven

The column is housed in a thermostated oven that precisely controls the column temperature. Temperature control is critical because it directly affects the partition coefficient and thus retention and resolution.

- Temperature range: Typically 50–350°C (some go up to 450°C for high-boiling compounds).
- Temperature accuracy: ±0.1°C for reproducibility.
- Lower temperature: Better separation (more difference in K values); but SLOWER analysis.
- Higher temperature: Faster elution; shorter analysis time; but poorer separation.
- The oven enables both isothermal and temperature programming operations.

Component 5: Detectors

The detector continuously monitors the column effluent (the gas exiting the column) and generates an electrical signal proportional to the amount of each compound. An ideal detector should be highly sensitive, have a wide linear dynamic range, be stable, and respond to all compounds (or specific compounds for selective detectors).

A. Flame Ionisation Detector (FID) — Most Widely Used

FID — Principle and Working
The column effluent is mixed with hydrogen and air and burned in a small hydrogen–air flame.
Carbon-containing compounds produce ions and electrons when burned in the flame.
The ions are collected by a collector electrode above the flame.
The ion current (proportional to the number of carbon atoms burned) is measured and amplified.
Result: A signal proportional to the mass of carbon in the compound.

FID Property	Details
Sensitivity	Very high: 10^{-12} g/s (detects nanograms)
Linear range	10^7 (excellent for quantitation)
Responds to	All compounds containing C–H bonds
Does NOT respond to	H_2O, CO_2, CO, N_2, O_2, noble gases, CS_2 (no C–H bonds)
Destructive?	Yes — sample is burned
Carrier gas compatible	He, N_2 (H_2 and air needed for flame)
Primary use	Universal detector for organic compounds in pharmaceutical, food, environmental analysis

B. Thermal Conductivity Detector (TCD) — Universal, Non-Destructive

TCD — Principle and Working
A heated filament (tungsten–rhenium wire or thermistor) sits in the carrier gas stream.

The electrical resistance of the filament depends on temperature, which depends on thermal conductivity of gas.
Pure carrier gas (He or H_2) has HIGH thermal conductivity — cools the filament efficiently — low resistance.
When a sample compound (lower thermal conductivity) exits the column, it reduces heat loss from filament.
The filament gets hotter → resistance increases → this change is detected using a Wheatstone bridge circuit.
Signal is proportional to concentration of eluting compound.

TCD Property	Details
Sensitivity	Lower than FID: :10^{-7} g/mL
Linear range	10^5
Responds to	ALL compounds including permanent gases (universal)
Carrier gas	Helium or Hydrogen (high thermal conductivity — gives best sensitivity)
Destructive?	NO — sample is not destroyed (good for preparative work)
Primary use	Gas analysis (O_2, N_2, CO_2); when sample must be recovered; water analysis

C. Electron Capture Detector (ECD) — Highly Selective

ECD — Principle and Working
A radioactive source (^{63}Ni— a β-emitter) ionises the carrier gas (N_2 or Ar/CH_4) producing a steady
standing current of electrons (baseline current).
When a compound that can CAPTURE electrons (halogenated compounds, nitro compounds, conjugated
carbonyls) enters the detector, it absorbs free electrons — REDUCING the standing current.
The drop in current is proportional to the concentration of the electron-capturing compound.
Compounds WITHOUT electron-capturing ability pass through undetected.

ECD Property	Details
Sensitivity	EXTREMELY high: 10^{-14} g/s (detects femtograms)
Selectivity	Highly selective — responds ONLY to electron-capturing compounds

ECD Property	Details
Responds to	Halogenated compounds (Cl, Br, F, I), nitro compounds, conjugated carbonyls, organometallics
Does NOT respond to	Hydrocarbons, alcohols, amines (NOT electron-capturing)
Carrier gas	N_2 or Ar/5%CH_4 (pure, dried)
Primary use	Pesticide residues, chlorinated solvents, PCBs, drugs with halogens, environmental pollutants

D. Flame Photometric Detector (FPD)

- Burns the effluent in a H_2–air flame. Compounds containing sulphur emit light at 394 nm; phosphorus compounds emit at 526 nm.
- A photomultiplier tube with appropriate optical filters detects the emission.
- SELECTIVE for S and P compounds. Signal ∝ [concentration]² for sulphur.
- Used for: Organophosphate and organosulphur pesticides; sulphur drugs; petroleum sulphur content.

E. Nitrogen–Phosphorus Detector (NPD) / Thermionic Detector

- Modified FID with an alkali metal bead (rubidium silicate) above the flame.
- Selectively detects nitrogen- and phosphorus-containing compounds with 500–10,000× greater sensitivity than FID for these compounds.
- Used for: N-containing drugs (alkaloids, antihistamines), organophosphate pesticides.

F. Mass Spectrometry Detector (MSD / GC-MS)

- The most powerful GC detector. The mass spectrometer separates ions by their mass-to-charge ratio (m/z).
- Gives both QUANTITATIVE data (peak area) and QUALITATIVE data (mass spectrum for structural identification).
- Can identify unknowns by searching mass spectra against library databases (NIST, Wiley).
- GC-MS is the GOLD STANDARD for confirmatory identification and quantification of drugs, metabolites, and unknowns.

Detector	Principle	Selectivity	Sensitivity	Key Application
FID	Ion production in H_2 flame	Universal (C–H)	High	Most organic analyses; pharma
TCD	Thermal conductivity change	Universal (all compounds)	Moderate	Gases; non-destructive analysis
ECD	Electron capture by analyte	^{3}Ni β source; selective	Ultra-high (femtogram)	Halogenated pesticides, PCBs

Detector	Principle	Selectivity	Sensitivity	Key Application
FPD	Flame photometry	S and P selective	High	Organophosphate/organo-S pesticides
NPD	Thermionic emission	N and P selective	Very high	N/P-containing drugs, pesticides
MS (GC-MS)	Mass-to-charge ratio	Universal + structure	Very high	Unknown ID, confirmatory testing

Derivatization in Gas Chromatography

Many compounds of pharmaceutical interest are NOT directly suitable for GC analysis. They may be non-volatile, thermally unstable, strongly polar, or give poor peak shapes. Derivatization is a chemical reaction that converts such compounds into derivatives that are more GC-friendly.

Why Derivatization is Needed
Non-volatile compounds: High boiling point or ionic — cannot evaporate into gas phase.
Thermally labile: Decompose in the hot injector before reaching the column.
Strongly polar: Adsorb onto the column or give tailing (asymmetric) peaks.
Enhancing detection: Convert compound into a form detectable by ECD (add halogen group).
Improve chromatography: Give sharper, more symmetrical peaks and better resolution.

Types of Derivatization Reactions

A. Silylation — Most Widely Used

Silylation replaces active hydrogen atoms (–OH, –COOH, $–NH_2$, –SH) with a trimethylsilyl (TMS) group, forming a TMS derivative.

$$R–OH + (CH_3)_3SiCl \rightarrow R–O–Si(CH_3)_3 + HCl$$

Silylating Agent	Abbreviation	Reactivity	Notes
Trimethylchlorosilane	TMCS	Low	Requires a base catalyst; mildest agent
Hexamethyldisilazane	HMDS	Moderate	Used for hydroxyl groups; often mixed with TMCS
Bis(trimethylsilyl)trifluoroacetamide	BSTFA	High	Most reactive silylating agent; reacts with most OH, NH, COOH

Silylating Agent	Abbreviation	Reactivity	Notes
N-Methyl-N-TMS-trifluoroacetamide	MSTFA	Very high	Even more reactive; for hindered groups

- Compounds silylated: Amino acids, sugars, steroids, bile acids, prostaglandins, nucleosides, drugs with OH or NH groups.
- Advantages: One-pot reaction, rapid, increases volatility dramatically, gives sharp symmetric peaks.

B. Acylation

Acylation reacts –OH and –NH_2 groups with acyl halides or anhydrides to form esters and amides.

$$R–OH + (CF_3CO)_2O \rightarrow R–O–CO–CF_3 + CF_3COOH$$

Reagent	Abbreviation	Product	Advantage
Acetic anhydride	Ac_2O	Acetate ester	Simple; makes compounds less polar
Trifluoroacetic anhydride	TFAA	Trifluoroacetate ester	Halogenated → ECD-detectable; very volatile product
Pentafluoropropionic anhydride	PFPA	PFP ester	Even more halogenated; ultra-sensitive ECD detection
Heptafluorobutyric anhydride	HFBA	HFB ester	Maximum ECD sensitivity; used for drugs of abuse

- Special feature of halogenated acylation agents: They introduce fluorine atoms → products are detectable by ECD with ultra-high sensitivity.
- Used for: Catecholamines, amphetamines, barbiturates, steroids, drugs of abuse testing.

C. Alkylation (Esterification)

Replaces acidic protons with alkyl groups, most commonly converting carboxylic acids to their methyl esters (methyl esters are volatile and stable).

$$R–COOH + CH_2N_2 \rightarrow R–COOCH_3 + N_2$$

Reagent	Reaction	Used For
Diazomethane (CH_2N_2)	Methylation of COOH, OH	Fatty acids, prostaglandins, bile acids; very fast; toxic — use with care

Reagent	Reaction	Used For
BF_3–methanol	Acid-catalysed esterification	Fatty acid methyl esters (FAME) from triglycerides/lipids
Trimethylphenylammonium hydroxide (TMAH)	Flash methylation	Direct injection; COOH → methyl ester in the injector

- Most important application: FAME (Fatty Acid Methyl Ester) analysis for food, cosmetics, biofuels, clinical lipid profiles.

D. Oxime Formation

- Converts ketones and aldehydes (C=O groups) to oximes (C=N–OH) using hydroxylamine.
- Followed by silylation or acylation of the oxime → stable, volatile derivative.
- Used for: Steroids, corticosteroids, ketone-containing drugs, urinary steroids analysis.

Derivatization Type	Functional Groups Converted	Reagents	Applications
Silylation	OH, COOH, NH_2, NH, SH	BSTFA, TMCS, HMDS	Sugars, amino acids, steroids, prostaglandins, drugs
Acylation	OH, NH_2	Acetic anhydride, TFAA, PFPA, HFBA	Drugs of abuse (ECD); catecholamines; amphetamines
Alkylation (Esterification)	COOH (forms esters)	Diazomethane, BF_3/MeOH, TMAH	Fatty acids (FAME), prostaglandins, bile acids
Oxime formation	C=O (ketones, aldehydes)	Hydroxylamine + silylation	Steroids, corticosteroids, ketones

Temperature Programming in GC

Temperature programming means that the column oven temperature is INCREASED systematically during the GC run, rather than being kept constant.

Isothermal GC (Constant Temperature)	Temperature Programmed GC
Oven temperature stays constant throughout the run	Oven temperature increases during the run
Good for samples with NARROW boiling point range	Ideal for samples with WIDE boiling point range
Low-boiling compounds elute quickly (may overlap)	Early eluters are resolved at low starting temperature

Isothermal GC (Constant Temperature)	Temperature Programmed GC
High-boiling compounds take very long to elute	High-boiling compounds elute faster at high temperature
General-purpose analyses where all compounds are similar	Real-world samples: plant extracts, essential oils, blood samples

Why Temperature Programming is Needed — The General Elution Problem
Problem: In any complex mixture, compounds have different boiling points. If you use a LOW isothermal
temperature — early-eluting (low BP) compounds separate well, but high BP compounds take HOURS to elute
with very broad, flat peaks. If you use a HIGH isothermal temperature — high BP compounds elute quickly
but low BP compounds all rush out together at the front (poor resolution).
Solution: START at a low temperature (resolve early-eluting compounds), then INCREASE temperature
gradually so that later-eluting compounds are 'pushed out' as the temperature rises.
Result: All peaks are resolved AND elute in a reasonable time with similar peak widths.

Types of Temperature Programmes

Programme Type	Description	Example	When Used
Linear Ramp	Constant rate of temperature increase	30°C → 280°C at 5°C/min	Most common; works for most samples
Multi-Ramp	Different rates in different stages	30°C (hold 2 min) → 150°C at 10°C/min → 280°C at 5°C/min	Complex samples needing different separation conditions at different stages
Isothermal Hold + Ramp	Hold at initial temp, then ramp	40°C (hold 5 min) → ramp to 300°C	When solvent peak needs to elute before gradient begins
Step Programme	Abrupt jumps to higher temperature	200°C → 250°C (jump) → 300°C (jump)	Rarely used; specialised applications

Advantages of Temperature Programming

- Resolves compounds with widely different boiling points in a single run.
- All peaks have similar widths (band broadening equalised) — easier quantitation.
- Shorter total analysis time compared to using a high isothermal temperature.
- Better sensitivity for high-boiling compounds (they elute as sharper peaks).
- Simulates gradient elution in HPLC — universally applicable to complex real-world samples.

Disadvantages of Temperature Programming

- Requires re-equilibration of the column at the starting temperature between runs — adds time.
- Increased column bleed at high temperatures reduces column lifetime.
- Greater risk of stationary phase decomposition if maximum temperature is exceeded.
- Increased baseline drift during temperature ramp (especially with TCD).

Advantages of Gas Chromatography

- Excellent resolution: Capillary columns give hundreds of thousands of theoretical plates — can separate very similar compounds.
- High sensitivity: FID detects nanograms; ECD detects femtograms of appropriate compounds.
- Fast analysis: Most analyses complete in 5–60 minutes.
- Versatile: Can analyse gases, liquids, volatile solids. Wide range of stationary phases available.
- Highly quantitative: Wide linear range; reproducible peak areas for accurate quantitation.
- Small sample requirement: µL volumes of liquid sample are sufficient.
- Coupling with MS (GC-MS): Provides definitive structural identification of unknowns — extremely powerful.
- Automated: Autosamplers enable high-throughput analysis with minimal operator involvement.

Disadvantages of Gas Chromatography

- Sample must be VOLATILE: GC cannot analyse non-volatile compounds (ionic, high MW, thermally labile without derivatization).
- Sample decomposition: Thermally labile compounds may decompose in the hot injector.
- Derivatization required: For many pharmaceutical compounds — adds steps, time, and potential errors.
- Not suitable for inorganic compounds and most biomolecules without derivatization.
- Temperature limitation: Maximum ~350–450°C — limits analysis of very high boiling compounds.
- Carrier gas cost: Helium is expensive and becoming scarce; hydrogen requires safety precautions.

Applications of Gas Chromatography

Application Field	Specific Examples
Pharmaceutical Analysis	Residual solvent testing (ICH Q3C), assay of volatile drugs (anaesthetics), purity of essential oils, enantiomeric purity (chiral GC)
Drug Testing & Toxicology	GC-MS for drugs of abuse in urine/blood (confirmatory test), blood alcohol level determination
Clinical & Forensic	Volatile fatty acids in biological fluids, blood alcohol, toxicological screening, arson investigation

Application Field	Specific Examples
Environmental Analysis	Pesticide residues in food and water (ECD), VOCs (volatile organic compounds) in air/water, PCBs
Food & Flavour Analysis	FAME analysis for food labelling, flavour & aroma compounds (essential oils), food adulteration detection
Petroleum Industry	Composition of natural gas, fuel quality control, identification of hydrocarbons
Polymer Industry	Residual monomers in polymers, plasticiser content, polymer decomposition products

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High Performance Liquid Chromatography (HPLC)

Introduction

High Performance Liquid Chromatography (HPLC) — also known as High Pressure Liquid Chromatography — is the most widely used analytical technique in the pharmaceutical industry. It separates, identifies, and quantifies components of a mixture using a LIQUID mobile phase pumped at HIGH PRESSURE through a column packed with very small, uniform particles of stationary phase.

Why HPLC is so Powerful
Traditional liquid column chromatography used large (>100 μm) particles and gravity flow — SLOW.
HPLC uses extremely small particles (1.7–5 μm) which give MUCH higher efficiency.
But small particles cause high resistance — liquid cannot flow through by gravity.
Solution: Use a HIGH-PRESSURE PUMP (up to 400–6000 bar) to force the mobile phase through.
Result: Excellent efficiency, speed (20–30 min runs), sensitivity, and reproducibility.
HPLC can analyse NON-VOLATILE compounds — complementary to GC.

HPLC was developed in the late 1960s–1970s by Csaba Horvath, Jack Kirkland, and Lloyd Snyder. Today, newer versions include UHPLC (Ultra-High Performance LC) using sub-2-μm particles and pressures up to 1000+ bar.

Theory of HPLC

A. Basis of Separation

Separation in HPLC is based on the differential distribution of solute molecules between the stationary phase and the flowing liquid mobile phase. Different modes of HPLC exploit different intermolecular interactions:

HPLC Mode	Stationary Phase	Mobile Phase	Separation Mechanism	Used For
Reversed Phase (RP-HPLC)	Non-polar C18, C8, phenyl (on silica)	Polar: Water + organic modifier (MeOH, ACN)	Hydrophobic interactions; non-polar compounds retained longer	Most pharmaceutical drugs; 70–80% of all HPLC analyses
Normal Phase (NP-HPLC)	Polar: Bare silica, CN, NH_2	Non-polar: Hexane, dichloromethane	Adsorption; polar compounds retained longer	Fat-soluble vitamins, lipids, chiral separations
Ion Exchange (IEC)	Ionic groups on polymer/silica (cation or anion exchanger)	Aqueous buffers of defined ionic strength/pH	Electrostatic attraction between oppositely charged solute and stationary phase	Amino acids, proteins, nucleotides, inorganic ions
Size Exclusion (SEC/GFC)	Porous gel beads (defined pore size)	Aqueous (GFC) or organic (GPC)	Molecular sieving by size; no chemical interaction	Polymer MW distribution; protein analysis (GFC)
Affinity HPLC	Ligand immobilised on support (antibody, receptor)	Aqueous buffers	Highly specific biological interaction	Protein purification, receptor binding studies

Reversed Phase HPLC — The Dominant Mode
In RP-HPLC, the stationary phase is NON-POLAR (hydrophobic) and the mobile phase is POLAR (water + organic).
This is the REVERSE of normal phase — hence 'reversed phase'.
Non-polar (hydrophobic) compounds interact strongly with the non-polar stationary phase — elute LATE.
Polar (hydrophilic) compounds prefer the polar mobile phase — elute EARLY.
Elution order: Most polar → least polar.
Mobile phase: Aqueous buffer + organic modifier (methanol or acetonitrile).
Increasing organic modifier % → increases mobile phase strength → reduces retention of non-polar compounds.

B. HPLC Column Efficiency — Theoretical Plates

The same theoretical plate concept applies in HPLC. The Van Deemter equation is modified for liquids:

$$H = A + B/u + (Cs + CM) \times u$$

In HPLC with small particles: The A term is minimised by small, uniform particles; B term is small in liquid (diffusion is slow); C term dominates at high flow rates. Using sub-2-µm particles (UHPLC) dramatically reduces H and gives very high efficiency.

C. Gradient Elution vs Isocratic Elution

Isocratic Elution	Gradient Elution
Mobile phase composition is CONSTANT throughout the run	Mobile phase composition CHANGES during the run
Simple; reproducible; good for simple mixtures	Better for complex mixtures with wide polarity range
Late-eluting peaks can be very broad and slow	Late-eluting peaks remain sharp even for non-polar compounds
No re-equilibration needed between runs	Column must re-equilibrate at initial conditions between runs
Used in routine QC assays (simpler, validated)	Used in method development, impurity profiling, complex samples

Instrumentation of HPLC

A modern HPLC system consists of the following components:

HPLC Instrument Block Diagram
Solvent Reservoir(s) → Degasser → High-Pressure Pump → Gradient Mixer
→ Auto-sampler (Sample Injection) → Guard Column → Analytical Column
→ Detector → Data System / Workstation

Component A: Solvent Reservoir and Degasser

- Solvent reservoirs: Glass bottles (0.5–2 L) contain the mobile phase solvents. Stainless steel frits on the inlet lines filter out particulate matter.
- Degasser (online degasser or sparging): Dissolved gases (O_2, N_2, CO_2) in the mobile phase cause baseline noise, bubble formation in the pump, and instability in UV detection at 200–210 nm. Degassing removes these.
  - Methods of degassing: Helium sparging (bubbling He through solvent), vacuum degassing (online membrane degasser — most common in modern HPLC), sonication.

Component B: High-Pressure Pump

The pump is the mechanical heart of the HPLC system. It must deliver the mobile phase at precise, constant, and reproducible flow rates against high back-pressure.

Requirements of an HPLC Pump:

- Flow rate: 0.01–5 mL/min (analytical); 50–500 mL/min (preparative).
- Pressure capability: 400–6000 psi (conventional HPLC); up to 15,000+ psi (UHPLC).
- Flow rate reproducibility: ±0.1–0.5% RSD — critical for retention time reproducibility.
- Pulse-free flow: Pulsations cause baseline noise; pulse dampeners are used.

Types of HPLC Pumps:

Pump Type	Principle	Features	Use
Reciprocating Piston Pump	Piston moves in cylinder; inlet/outlet check valves	Most common; dual-piston design minimises pulses; flow rate easily programmable	Standard HPLC; gradient capable with multiple pumps
Syringe Pump	Motor-driven syringe; continuous (pulse-free) delivery	Excellent flow precision; limited reservoir volume	Micro/nano-HPLC; low-flow applications
Pneumatic (gas-driven) Pump	Gas pressure forces solvent	Simple; no moving parts; not suitable for gradients	Older/simple systems; isocratic only

Gradient Pumping Systems:

System	Type	Principle	Advantage
High-Pressure Mixing (Binary/Quaternary pump)	High-pressure	Two or four separate pumps mix solvents AFTER pressurisation	Precise; fast gradient changes; standard in modern HPLC
Low-Pressure Mixing	Low-pressure	Solvents mixed BEFORE the pump using proportioning valves	Simpler; one pump; less expensive; slightly slower gradients

Component C: Sample Injection System

The sample introduction device introduces a precise, reproducible volume of sample into the high-pressure mobile phase stream without disrupting the flow.

Rheodyne Rotary Valve (Manual Injector):

- A 6-port, 2-position rotary valve with an external sample loop.
- LOAD position: Sample is loaded into the loop from the syringe at atmospheric pressure. Mobile phase flows through the column.
- INJECT position: Valve rotates to bring the sample loop in-line with the high-pressure mobile phase flow. Sample is carried onto the column.
- Loop sizes: 5–20 µL for analytical work.

Autosampler (Automatic Injector):

- Robotic system that automatically draws sample from vials, injects precise volumes, and returns needle.
- Advantages: High throughput (can process 100s of samples unattended), excellent reproducibility (±1% RSD injection volume), programmable wash sequences.
- Modern autosamplers can also perform online dilution, standard addition, and sample preparation.

Component D: The HPLC Column

The column is the heart of the separation. HPLC columns are precise engineered devices packed with very small, uniform particles under high pressure.

Column Parameter	Typical Values	Effect on Separation
Column Length	50–300 mm (most common: 150 mm)	Longer column → more theoretical plates → better resolution; but slower
Internal Diameter	1.0–4.6 mm (standard analytical: 4.6 mm)	Narrower column → less solvent used; higher sensitivity per unit mass
Particle Size	1.7–µm (UHPLC) to 5 µm (standard)	Smaller particles → much higher efficiency; requires higher pressure
Pore Size	60–Å (small molecules) to 300–Å+ (proteins)	Larger pores for large molecules; smaller pores for small molecules

Types of Column Packing (Stationary Phase Particles):

Particle Type	Description	Advantage
Fully Porous Silica Particles	Porous throughout; 3–5 µm	Classic standard; good capacity; wide range of chemistries
Sub-2-µm Particles (UHPLC)	1.7–1.9 µm porous particles	Ultra-high efficiency; very fast; requires UHPLC instrument
Core-Shell (Fused-Core) Particles	Solid silica core + thin porous shell	Near-UHPLC efficiency at lower pressure; fits in standard HPLC
Monolithic Columns	Continuous porous silica rod	Very fast (high flow rate at low pressure); low back-pressure
Polymer-Based Particles	PS-DVB or other polymers	pH stable 1–14; for strongly acidic/basic conditions

Common HPLC Stationary Phase Chemistries:

Stationary Phase	Code	Polarity	Applications
Octadecylsilane on silica	C18 (ODS)	Non-polar	Most widely used; drugs, vitamins, amino acids, lipids
Octylsilane on silica	C8	Non-polar (less)	Faster elution than C18; basic drugs
Phenyl phase	Ph	Intermediate	Aromatic compounds; complements C18
Cyano phase	CN	Polar	Can be used in NP or RP mode; pesticides
Amino phase	NH_2	Polar	Sugars, carbohydrates (NP mode)
Bare silica	Si	Very polar	Chiral separations, lipids (NP mode)

Guard Column:

- A short (5–10 mm), disposable column packed with the same stationary phase as the analytical column, placed before it.
- Protects the expensive analytical column from contamination by irreversibly adsorbing matrix components (proteins, particulates, strongly retained compounds).
- Replace the guard column regularly; analytical column lasts much longer as a result.

Component E: HPLC Detectors

The detector continuously monitors the column effluent and generates an electrical signal when a separated compound exits the column. Different detectors are chosen based on the properties of the analyte.

1. UV-Vis Absorbance Detector — Most Widely Used

UV-Vis Detector — Principle
Based on Beer–Lambert law: $A = \varepsilon \times c \times l$.
The column effluent flows through a small flow cell (8–10 µL volume, 1 cm path length).
UV or visible light passes through the flow cell. A photodiode measures the transmitted light.
When a UV-absorbing compound exits the column, absorbance increases → detector signal.
Wavelength is set to the absorption maximum of the analyte (λ max).

UV Detector Type	Wavelength Selection	Features
Fixed Wavelength	Single fixed wavelength (e.g., 254 nm from Hg lamp)	Simple, inexpensive; less flexible

UV Detector Type	Wavelength Selection	Features
Variable Wavelength Detector (VWD)	Any wavelength from deuterium/tungsten lamp (190–800 nm)	Flexible; set optimal wavelength for each compound
Photodiode Array Detector (DAD/PDA)	ALL wavelengths simultaneously (190–800 nm)	Captures full UV spectrum of every peak; peak purity assessment; identify unknowns by spectral comparison

2. Fluorescence Detector (FLD)

- Measures FLUORESCENCE (emission of light after excitation). Principle: Excite with UV light at λ_ex; measure emitted light at longer wavelength λ_em.
- MUCH more sensitive than UV (100–1000×): Can detect femtomoles. Also highly selective — only fluorescent compounds respond.
- Naturally fluorescent compounds: Aflatoxins, polycyclic aromatic hydrocarbons, quinine, riboflavin.
- Derivatization for non-fluorescent analytes: OPA (o-phthalaldehyde) for primary amines; FMOC for amino acids → enables fluorescence detection.
- Used for: Vitamins (B_2, B_6), drugs (warfarin, doxorubicin), amino acid analysis, aflatoxin in food.

3. Refractive Index Detector (RID)

- Measures the difference in REFRACTIVE INDEX between pure mobile phase and mobile phase containing the analyte.
- UNIVERSAL detector — responds to ALL compounds (that change RI).
- LIMITATION: Very sensitive to temperature and mobile phase composition changes — cannot be used with gradient elution. Also relatively insensitive.
- Used for: Sugars, carbohydrates, lipids, polymers — compounds with no UV absorption.

4. Evaporative Light Scattering Detector (ELSD)

- Column effluent is nebulised (converted to aerosol) and solvent is evaporated in a heated drift tube. Non-volatile analyte particles remain and scatter a laser beam.
- Universal (detects any non-volatile compound). Can be used with gradient elution (unlike RID).
- Used for: Lipids, carbohydrates, surfactants, non-UV-absorbing compounds.

5. Mass Spectrometer Detector (LC-MS)

- Most powerful and definitive HPLC detector. Provides BOTH quantitative data (peak area) and structural identification (mass spectrum, m/z values, fragmentation).
- Interface: Electrospray Ionisation (ESI) or Atmospheric Pressure Chemical Ionisation (APCI) interfaces are used to transfer liquid-phase analytes into the gas phase for MS analysis.
- LC-MS/MS (tandem MS): Even greater selectivity and sensitivity; gold standard for bioanalysis (drug levels in blood), metabolite identification, pharmacokinetics.
- Used for: Pharmacokinetic studies, metabolite identification, drug impurity characterisation, proteomics, forensic toxicology.

6. Electrochemical Detector (ECD)

- Measures current from oxidation or reduction of analyte at a working electrode.
- Highly sensitive and selective for electroactive compounds (phenols, catecholamines, thiols).
- Used for: Catecholamines (dopamine, adrenaline) in biological fluids, ascorbic acid (Vitamin C), thiols.

Detector	Principle	Sensitivity	Universal?	Gradient?	Primary Applications
UV-Vis (VWD)	Absorbance at set wavelength	High (ng)	No	Yes	Drugs, most pharmaceuticals
Photodiode Array (DAD)	Full UV spectrum simultaneously	High (ng)	No	Yes	Peak purity; unknown identification
Fluorescence (FLD)	Emission after excitation	Ultra-high (fg-pg)	No (selective)	Yes	Vitamins, aflatoxins, amino acids
Refractive Index (RID)	RI difference	Low (pg-ng)	Yes	NO	Sugars, polymers, lipids
ELSD	Light scattering by particles	Moderate	Yes	Yes	Lipids, carbohydrates, surfactants
Mass Spec (LC-MS)	m/z separation	Ultra-high (fg)	Yes + ID	Yes	Drug metabolism, proteomics, forensics
Electrochemical	Electrode reaction	Ultra-high	No (selective)	Limited	Catecholamines, Vit. C

HPLC Method Development — How to Build an HPLC Method

Developing an HPLC method involves selecting the right mode, column, mobile phase, and conditions. Here is a systematic approach:

Step	Decision	Guidelines
1. Define the goal	What do you need to measure? Identity, purity, assay?	Identify analyte, matrix, required sensitivity, throughput
2. Choose HPLC mode	Based on compound polarity and molecular weight	Most drugs → RP-HPLC (C18 column). Proteins → SEC or IEC.
3. Select column	Stationary phase + dimensions	Start with C18, 150×4.6 mm, 5 µm for most drugs
4. Choose mobile phase	Buffer, organic modifier, pH	Aqueous buffer (pH 2–7) + ACN or MeOH; pH 2 units from analyte pKa
5. Optimise	Run scouting gradients; adjust isocratic conditions	Target: Rf 0.2–0.8 equivalent; resolution ≥1.5; reasonable run time
6. Validate	Per ICH Q2(R1) guidelines	Linearity, accuracy, precision, LOD, LOQ, robustness, specificity

Mobile Phase Considerations in RP-HPLC

A. Organic Modifiers

Organic Modifier	UV Cutoff (nm)	Eluting Strength	Advantages	Disadvantages
Acetonitrile (ACN)	190 nm	Strong	Low viscosity; excellent UV transparency; fast equilibration	More expensive; toxic
Methanol (MeOH)	205 nm	Moderate	Inexpensive; less toxic; widely available	Higher viscosity; higher back-pressure
Tetrahydrofuran (THF)	210 nm	Very strong	Useful for specific separations	High viscosity; forms peroxides; restricted use
Isopropanol (IPA)	205 nm	Strong	Useful for lipid analysis, protein denaturation	Very high viscosity

B. Aqueous Component — Buffer Selection

- pH control is critical in RP-HPLC. Ionisable compounds (acids, bases) must be in the SAME ionisation state throughout the run to give reproducible, sharp peaks.

- Rule: Buffer pH should be at least 2 pH units away from pKa of the analyte to ensure >99% in one form.
- Weak acids: Use pH 2–3 (keeps them in non-ionised form, retained on C18).
- Weak bases: Use pH 7–8 (suppress ionisation for retention) or use ion-pairing agents.

Buffer	pH Range	UV Cutoff	Notes
Phosphate buffer	1.5–8.0	<200 nm	Most widely used; excellent UV transparency; not MS-compatible
Acetate buffer	3.6–5.8	<210 nm	MS-compatible at low concentration
Formate buffer	2.5–4.5	<210 nm	MS-compatible (volatile); ideal for LC-MS
Ammonium bicarbonate	6.8–8.2	<210 nm	Volatile; MS-compatible; for basic compounds
Trifluoroacetic acid (TFA)	1.0–2.0	<210 nm	Strong ion-pair; sharpens peaks of basic drugs; suppresses LC-MS

Advantages of HPLC

- Versatile: Can analyse virtually ANY compound — volatile or non-volatile, thermally stable or labile, ionic or neutral. No need for derivatization in most cases.
- High resolution and efficiency: Separates structurally very similar compounds (isomers, enantiomers with chiral columns, related impurities).
- Highly sensitive: With FLD or LC-MS, can detect femtogram amounts in complex matrices.
- Quantitative precision: Excellent linearity and reproducibility (RSD < 2% for validated assays).
- Non-destructive (mostly): The separated fraction can be collected for further analysis or biological testing.
- Aqueous/buffered mobile phases: Water-based solvents are compatible with biological matrices (plasma, urine).
- Automated: Autosamplers enable 24/7 operation; LIMS integration for pharmaceutical QC.
- Multiple detection options: UV, FLD, MS, ELSD — match detector to analyte for optimal sensitivity and selectivity.
- IP/BP/USP adopted: HPLC is the primary method in all major pharmacopoeias for drug assay and purity testing.

Applications of HPLC

Application Area	Specific Examples
Pharmaceutical Assay & QC	Content uniformity, dissolution testing, assay of bulk drugs and formulations — IP/BP/USP methods
Impurity Profiling	Related substances, degradation products testing (ICH Q3A/B); genotoxic impurity control
Bioanalysis / Pharmacokinetics	Drug + metabolite levels in plasma, urine, tissue (LC-MS/MS method); PK/PD studies
Stability Studies	Monitor drug degradation over time under accelerated conditions (ICH Q1A)
Natural Product Analysis	Alkaloids, flavonoids, terpenes, polyphenols in plant extracts; herbal drug standardisation
Vitamin Analysis	Water-soluble vitamins (B1, B2, B6, B12, C) and fat-soluble vitamins (A, D, E, K)
Amino Acid Analysis	Protein hydrolysate composition; nutritional supplement labelling (with derivatisation or AAA)
Pesticide Residue Analysis	Multi-residue analysis in food and agricultural products
Clinical Diagnostics	Therapeutic drug monitoring (TDM) — immunosuppressants, antibiotics, antiepileptics
Forensic Toxicology	LC-MS/MS detection of drugs, poisons, metabolites in biological specimens

COMPARATIVE — GC vs HPLC

GAS CHROMATOGRAPHY (GC)	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)
Mobile Phase: Inert GAS (He, N_2, H_2)	Mobile Phase: LIQUID (aqueous/organic solvent)
Sample must be VOLATILE and thermally stable	Sample can be non-volatile, ionic, thermally labile
Derivatization often needed for polar/ionic compounds	Usually NO derivatization needed
Column: Packed or capillary (fused silica)	Column: Stainless steel packed with small particles
Oven temperature: 50–450°C; temperature programming used	Column temperature: Usually room temp to 60°C
Detectors: FID, TCD, ECD, FPD, NPD, MS	Detectors: UV, FLD, RID, ELSD, MS, ECD

GAS CHROMATOGRAPHY (GC)	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)
Resolution: Extremely high (capillary: 100,000+ plates)	Resolution: Very high (5000–20,000 plates typical)
Best for: Volatile organics, gases, fatty acids, solvents, drugs of abuse	Best for: Non-volatile drugs, biomolecules, polar compounds, ionic species
Key advantages: Speed, very high efficiency, GC-MS	Key advantages: Universal applicability, aqueous compatibility, LC-MS
Primary pharmaceutical use: Residual solvents (ICH Q3C), volatiles	Primary pharmaceutical use: Assay, purity testing, bioanalysis

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