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B.PHARMA 8TH SEMESTER

BP811ET — ADVANCED INSTRUMENTATION TECHNIQUES

UNIT IV — COMPLETE PREMIUM NOTES

Radioimmunoassay (RIA) + Solid Phase Extraction (SPE) + Liquid-Liquid Extraction (LLE)

★ PREMIUM PAID NOTES ★ AKTU Aligned ★

Unit IV Contents at a Glance
PART A — RADIOIMMUNOASSAY (RIA): Importance | Components (Antigen, Antibody, Radiolabelled Antigen, Separation Systems) | Principle | Competitive Binding | Methods (Equilibrium Dialysis, Double Antibody, PEG method, Solid Phase) | Limitations | Applications
PART B — EXTRACTION TECHNIQUES: Solid Phase Extraction (SPE) — Principle, Sorbent Types, Step-by-Step Procedure, Modes | Liquid-Liquid Extraction (LLE) — Principle, Distribution Coefficient, Procedure, Continuous & Back-Extraction, Comparison

INTRODUCTION AND IMPORTANCE OF RIA

Radioimmunoassay (RIA)?

Radioimmunoassay (RIA) is an extremely sensitive and specific in vitro analytical technique that combines the **specificity of immunological reactions** (antigen-antibody binding) with the **sensitivity of radioactive detection** to measure minute quantities of substances — typically in the nanogram (10^{-9} g) to picogram (10^{-12} g) range.

Discoverers: RIA was developed independently by **Rosalyn Yalow and Solomon Berson** in 1959 for measurement of **plasma insulin** using ^{131}I-labelled insulin. Rosalyn Yalow was awarded the **Nobel Prize in Physiology or Medicine in 1977** for this discovery.

Feature	RIA Capability	Comparison
Sensitivity	1–10 pg/mL (picogram range)	10,000× more sensitive than standard colorimetric methods
Specificity	Measures only the antigen the antibody is raised against	Distinguishes structurally similar molecules (e.g., hormones from their precursors)
Sample volume	50–200 µL	Small sample — critical for paediatric samples, rare specimens
Sample matrix	Serum, plasma, urine, CSF, tissue extracts	Works in complex biological matrices without extensive purification
Throughput	100–1000 samples/day in batch mode	High throughput — suitable for large clinical laboratory volumes
Detection range	Femtomol (10^{-15}) to nanomol	Spans 6–8 orders of magnitude with appropriate dilution

Importance of RIA in Pharmacy and Medicine

- **Hormone assay:** Insulin, growth hormone, thyroid hormones (T3, T4, TSH), cortisol, progesterone, estrogen, LH, FSH, hCG — all routinely measured by RIA or RIA-derived techniques
- **Therapeutic drug monitoring (TDM):** Digoxin, cyclosporine, methotrexate, phenytoin, theophylline at therapeutic and toxic blood levels
- **Tumour markers:** AFP (alpha-fetoprotein), CEA, PSA, CA-125 — cancer diagnosis and monitoring
- **Drug abuse screening:** Marijuana (THC), cocaine metabolites, opiates, amphetamines in urine
- **Infectious disease serology:** HBsAg (hepatitis B surface antigen), HIV antibodies, TORCH panel
- **Vitamins and nutritional markers:** Vitamin B12, folate, ferritin, vitamin D
- **Research:** Measurement of pharmacokinetics, receptor binding, and endogenous ligand concentrations

Historical Significance: RIA revolutionised endocrinology. Before 1959, measuring plasma insulin required hundreds of mL of blood and took weeks using bioassay. RIA reduced this to 1 mL of blood and hours, enabling the routine diagnosis of insulin disorders, leading to modern diabetes management.

COMPONENTS OF RIA

A complete RIA system requires four essential components. Each must be optimally selected and prepared for accurate, reproducible results.

Component 1 — Antigen (Analyte)

The **antigen (Ag)** is the substance being measured. It can be any molecule that:

- Is present in biological fluids at very low concentrations (ng–pg range)
- Has a specific antigenic determinant (epitope) that an antibody can bind
- Can be radioactively labelled without destroying its immunological activity

Category	Examples	Molecular Size
Proteins and peptides	Insulin, growth hormone, LH, FSH, hCG, PTH, glucagon	Large — inherently immunogenic
Haptens (small molecules)	Steroid hormones (cortisol, estrogen, progesterone), drugs (digoxin, theophylline), vitamins	Small — must be conjugated to carrier protein (BSA, KLH) to raise antibody
Nucleotides / DNA	cAMP, cGMP (second messengers); nucleoside drugs	Medium — specific antibodies available
Tumour markers	CEA, AFP, PSA, CA-125, CA 19-9	Variable

Component 2 — Specific Antibody (Antiserum)

The **antibody (Ab)** or antiserum provides the specificity of RIA. It must bind exclusively to the antigen of interest and not cross-react with structurally similar molecules.

Requirements for RIA Antibody

- **High affinity:** Association constant Ka ≥ 10^9 L/mol — strong, stable binding
- **High specificity / low cross-reactivity:** < 1% cross-reaction with structurally related molecules
- **High titre:** Antibody should be effective at high dilution (1:5000 to 1:100,000)
- **Consistent quality:** Polyclonal (most classical RIA) or monoclonal (more specific, modern assays)

Antibody Production

- **For proteins:** Inject immunogen (e.g., insulin) + Freund's adjuvant into rabbit, sheep, guinea pig → collect antiserum after 6–8 weeks → booster injections → harvest when titre is adequate

- **For haptens:** Conjugate small molecule (drug/steroid) to carrier protein (BSA) using bifunctional reagent → inject conjugate → raise antibody against the hapten portion
- **Monoclonal antibodies:** Hybridoma technology (mouse immunised → B cells fused with myeloma cells → immortal hybrid secretes single, specific antibody) — used in IRMA and ELISA

Property	Polyclonal Antibody	Monoclonal Antibody
Source	Multiple B-cell clones; animal antiserum	Single B-cell clone; hybridoma cell line
Specificity	Multiple epitopes recognised	Single specific epitope only
Cross-reactivity	Higher (may cross-react)	Lower (very specific)
Affinity variability	Variable between batches	Consistent, reproducible
Production	Simpler — immunise animal, bleed	Complex — hybridoma technology
Use in RIA	Classical competitive RIA	IRMA, sandwich assays, modern immunoassays

Component 3 — Radiolabelled Antigen (Tracer)

The **radiolabelled antigen (Ag*) **— also called the** tracer** — is the same antigen as the analyte but tagged with a radioactive isotope. The tracer competes with unlabelled analyte for binding to the limited antibody.

Requirements for the Tracer

- **Identical immunological reactivity** to unlabelled antigen — the antibody must not distinguish between Ag and Ag*
- **High specific activity** — maximum radioactive disintegrations per unit mass → better sensitivity
- **Stability** — tracer must not degrade rapidly (sufficient shelf life)
- **Safe handling** — use minimum effective specific activity; appropriate containment

Isotope	Half-life	Type of Emission	Specific Activity	Labelling Method	RIA Use
^{125}I (Iodine-125)	60.1 days	Gamma (35 keV)	Very high (~2200 Ci/mmol max)	Iodination of tyrosine residues (Chloramine-T, Iodogen, Lactoperoxidase)	MOST COMMON — peptides, proteins, hormones. Measured by gamma counter.
^{131}I (Iodine-131)	8 days	Beta + Gamma (364 keV)	High	Iodination	Now less used (shorter t½, higher energy hazard). Historical use.

Isotope	Half-life	Type of Emission	Specific Activity	Labelling Method	RIA Use
^{3}H (Tritium)	12.3 years	Beta (18 keV, weak)	Lower	Biosynthetic labelling or reduction	Steroids, drugs, small molecules. Measured by liquid scintillation counter (LSC).
^{14}C (Carbon-14)	5730 years	Beta (156 keV)	Very low	Chemical synthesis	Research, metabolic studies. LSC detection. Very long half-life.
^{57}Co (Cobalt-57)	271 days	Gamma	Moderate	Cobalt labelling of vitamin B12	Specific to vitamin B12 RIA

⚡ Exam Trap — Most Used Isotope: ^{125}I is the most widely used RIA isotope. Reasons: (1) 60-day half-life is long enough for routine use but short enough for safe disposal; (2) gamma emission detected easily by sodium iodide crystal gamma counter; (3) very high specific activity gives excellent sensitivity; (4) easy to label proteins via tyrosine iodination. ^{3}H is used when iodination would destroy immunoreactivity (small molecules, steroids).

Component 4 — Separation System

After the competitive binding reaction reaches equilibrium, **free Ag* (unbound) must be physically separated from bound Ag* (Ag*-Ab complex)**. This separation is critical — the radioactivity in either fraction is then counted. Several separation methods are used:

Separation Method	Principle	Procedure	Advantages	Disadvantages
Activated Charcoal / Dextran-Coated Charcoal (DCC)	Charcoal adsorbs small free molecules (Ag*); large Ag*-Ab complexes remain in solution	Add charcoal suspension → centrifuge → count supernatant (bound fraction)	Simple, fast, inexpensive	Non-specific; time-dependent; damages some antibodies; not suitable for large antigens
Double Antibody / Second Antibody Precipitation	Second antibody (anti-IgG) precipitates first antibody-antigen complex	Add second antibody → incubate → centrifuge → count precipitate (bound)	Complete precipitation; clean separation	Slow (overnight incubation); requires second antibody; PEG often added to accelerate
PEG (Polyethylene Glycol) Precipitation	High concentration PEG (15–25%) non-specifically precipitates immunoglobulins (Ab-Ag complexes)	Add PEG → centrifuge → count precipitate	Faster than double antibody; cheap	Non-specific precipitation of other proteins; variable efficiency
Solid Phase Separation	Antibody coated on solid support (tube,	Incubate → wash → bound fraction stays on	Simple washing; no centrifuge	Higher cost; coating must be optimised;

Separation Method	Principle	Procedure	Advantages	Disadvantages
	bead, microplate well)	solid phase → count tube/bead directly	needed; automatable	limited to Ab-coated solid phase
Protein A / Protein G Coated Beads	Protein A/G binds Fc region of IgG → separates Ab-Ag complex	Add Protein A beads → centrifuge → count bead bound fraction	Versatile for many antibodies	Species-specific Protein A/G binding
Ammonium Sulfate Precipitation	High salt concentration salts out immunoglobulins (Ab-Ag complexes)	Add $(NH_4)_2SO_4$ → centrifuge → wash pellet → count	Classic method; simple	Non-specific; moderate efficiency

PRINCIPLE OF RADIOIMMUNOASSAY

Competitive Binding Principle

RIA is based on the **competitive inhibition** of the binding of a radiolabelled antigen (Ag* = tracer) to a specific, **limited amount of antibody** (Ab) by an **unlabelled antigen** (the analyte). The antibody quantity is deliberately kept LIMITED — insufficient to bind all Ag* — so that competition occurs.

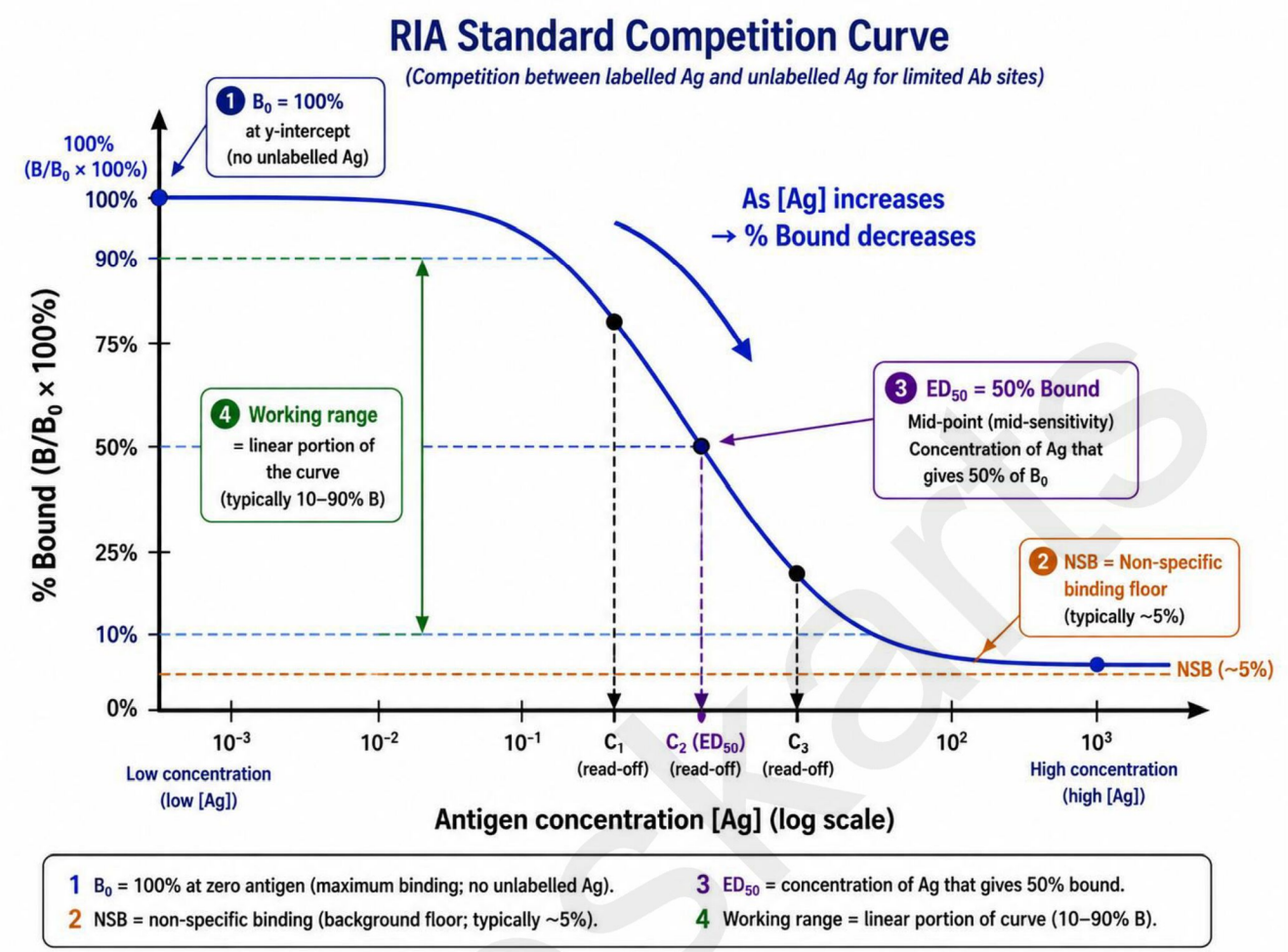
The fundamental competitive binding reaction:

$$\mathbf{Ag + Ag^* + Ab(limited) \rightleftharpoons Ag\text{-}Ab + Ag^*\text{-}Ab + Free\ Ag + Free\ Ag^*}$$

Inverse relationship: As the concentration of unlabelled Ag (analyte in sample) increases, more Ag competes for the limited Ab binding sites, displacing Ag* (tracer) from the antibody. Therefore:

Unlabelled Ag in Sample	Ag*-Ab Bound (B)	Free Ag* (F)	B/F or B/T Ratio	Signal Read
Zero (standard blank)	Maximum binding (B_0)	Minimum free Ag*	B/F = highest	B_0 is reference point
Low	High bound Ag*	Low free	B/F = high	Point on upper curve
Medium	Moderate bound	Moderate free	B/F = medium	Point on mid-curve
High	Low bound Ag*	High free Ag*	B/F = low	Point on lower curve
Saturating (excess)	Near zero binding	Maximum free Ag*	B/F = near zero	End-point of curve

The **standard curve (competition curve)** is constructed by plotting: **%B/B₀ (or B/T)** on Y-axis vs **standard concentration** on X-axis (logit-log transformation gives a straight line for interpolation).



Mathematical Basis of RIA

The binding of antigen to antibody follows the law of mass action:

$$Ka = [Ag\text{-}Ab] / ([Ag] \times [Ab])$$

For competitive RIA, Scatchard analysis plots B/F vs B to determine Ka and antibody concentration. The IC50 value (concentration giving 50% inhibition of tracer binding) is used to determine sensitivity.

Important RIA Parameters

- **B₀ (Maximum Binding):** Counts bound when zero unlabelled antigen is present (all Ab occupied by Ag*). Reference = 100%.
- **NSB (Non-Specific Binding):** Counts bound when no antibody is present (physical sticking of Ag* to tube). Subtracted from all values. NSB should be < 5% of total counts.
- **%B/B₀:** (Bound counts − NSB) / (B₀ − NSB) × 100% — normalised binding percentage plotted on standard curve.
- **B/T (Bound/Total):** Ratio of bound radioactivity to total radioactivity added.
- **ED50 (Effective Dose 50%):** Antigen concentration giving 50% of maximum binding — point of maximum sensitivity of the assay.
- **Working range:** The usable portion of the standard curve (typically 10–90% B/B₀) — where the curve is steep and sensitivity is highest.

Step-by-Step RIA Protocol

STEP 1: Prepare Standards and Samples — Prepare standard solutions of unlabelled antigen at 6–8 concentrations (e.g., 0, 0.1, 0.5, 1, 5, 10, 50, 100 ng/mL). Include zero standard (B_0) and non-specific binding (NSB) tubes. Pipette 50–200 µL of each standard or sample into labelled assay tubes.

STEP 2: Add Antibody — Add a fixed, predetermined volume of specific antiserum (diluted to working titre, e.g., 1:10,000) to all tubes EXCEPT NSB. Mix gently.

STEP 3: Add Tracer (Radiolabelled Ag*) — Add a fixed quantity of ^{125}I-labelled antigen (tracer) to ALL tubes including NSB. The quantity is chosen so that at B_0 (zero standard), approximately 30–50% of total tracer is bound.

STEP 4: Incubation (Equilibration) — Incubate at controlled temperature for sufficient time to reach equilibrium. Typical conditions: 4°C for 18–24 hours (overnight) OR 37°C for 2–4 hours. Equilibrium binding is essential for reproducible results.

STEP 5: Separation of Bound and Free Fractions — Separate Ag*-Ab complex (bound) from free Ag* using chosen separation method (DCC, double antibody, PEG, solid phase). Centrifuge if needed (3000 rpm, 15 min, 4°C).

STEP 6: Count Radioactivity — Decant supernatant or aspirate; count appropriate fraction in gamma counter (for ^{125}I) or liquid scintillation counter (for ^{3}H, ^{14}C). Count time: 1–5 min per tube. Record CPM (counts per minute).

STEP 7: Data Processing and Interpolation — Calculate %B/B_0 for each standard. Plot standard curve. Read unknown sample concentrations by interpolation from the standard curve. Apply dilution factors if samples were diluted.

RIA Component / Step	Typical Specification	Purpose
Antibody dilution	1:5,000 to 1:100,000	High dilution = still specific; preserves sensitivity
Tracer quantity	10,000–50,000 CPM per tube	Sufficient counts for statistical accuracy
Incubation (4°C)	18–24 hours (overnight)	Equilibrium achieved; temperature controls non-specific binding
NSB	< 5% of total counts	Measures physical sticking artefact — subtracted from all values
B_0 binding	30–50% of total counts	Enough dynamic range for inhibition curve
Duplicate/triplicate	All standards and samples run in duplicate minimum	CV between duplicates < 10% acceptable
Sensitivity (functional)	Concentration at 90% B/B_0	Lowest detectable concentration on steep part of curve

METHODS OF RIA

Several variations of the basic RIA format exist, each with specific advantages for particular analytes or laboratory settings:

Equilibrium (Standard Competitive) RIA

Aspect	Description
Format	All components (Ag, Ag*, Ab) added simultaneously; incubated to equilibrium
Principle	Classical competitive binding — unlabelled Ag and Ag* compete simultaneously for Ab
Advantage	True thermodynamic equilibrium; reproducible; most common format for plasma hormones
Disadvantage	Requires long incubation time for equilibrium; sensitivity limited by Ka
Examples	Insulin RIA, testosterone RIA, cortisol RIA

Sequential Saturation (Non-Equilibrium) RIA — Two-Incubation Method

Aspect	Description
Format	Step 1: Add Ab + unlabelled Ag (standard/sample) → incubate. THEN add Ag* → second incubation
Principle	Unlabelled Ag 'pre-occupies' Ab binding sites before tracer is added → enhanced sensitivity
Advantage	Greater sensitivity (2–5×) than equilibrium RIA; suitable for low-concentration analytes
Disadvantage	Two-step process = more pipetting steps = more variability; longer total assay time
Examples	Used for gonadotrophins (LH, FSH) where ultra-sensitivity is needed

Double Antibody RIA (Liquid-Phase Separation Method)

Aspect	Description
Format	Competitive RIA with double antibody separation. First antibody (specific anti-Ag) in excess. After equilibrium, add second antibody (anti-species IgG, e.g., anti-rabbit IgG in goat).
Principle	Second antibody precipitates first Ab-Ag complex → centrifuge → count precipitate (bound)
Advantage	Complete, clean precipitation; widely used for hormones; flexible — works with any primary Ab

Aspect	Description
Disadvantage	Requires second antibody (extra cost); overnight second incubation needed; PEG added to accelerate precipitation
PEG acceleration	Add 10–25% PEG with second antibody → precipitation complete in 30 min at 4°C
Examples	Classical insulin RIA, GH RIA, PTH RIA in research and clinical laboratories

Solid Phase RIA

Aspect	Description
Format	Antibody physically adsorbed or covalently coupled to solid support (plastic tube, bead, microplate well, membrane)
Principle	Competitive RIA on solid phase. Ag and Ag* compete for Ab on solid support. After washing, count solid phase (bound) directly
Advantage	Simple separation by washing (no centrifuge); automatable; low NSB; rapid
Disadvantage	Coating process must be optimised; limited Ab capacity per surface area; variable between batches
Examples	Tube-based solid phase RIA (antibody-coated tubes) — most popular format; bead-based (IMx/Axsym platforms)

Immunoradiometric Assay (IRMA) — Reverse Format

Aspect	Description
Format	REVERSE of classical RIA — the ANTIBODY (not antigen) is radiolabelled; excess antibody used; often two-site (sandwich) format
Principle	Step 1: Capture antibody (on solid phase) captures antigen. Step 2: Radiolabelled second antibody (Ag*-Ab sandwich) binds to captured Ag. Wash → count solid phase.
Key difference from RIA	IRMA: Labelled Ab (excess); direct relationship (more Ag → more counts). RIA: Labelled Ag (limited Ab); INVERSE relationship (more Ag → FEWER counts). This is the critical distinction!
Advantage	Better sensitivity and dynamic range; steeper dose-response (direct); lower NSB; suitable for large molecules with multiple epitopes
Disadvantage	Requires two specific antibodies (sandwich); hook effect at very high concentrations; more complex
Examples	TSH-IRMA (sensitive thyroid function); AFP-IRMA; PSA-IRMA — all large proteins

⚡ **CRITICAL Exam Trap — RIA vs IRMA:** In RIA: MORE Ag → LESS bound Ag* (inverse/competition). In IRMA: MORE Ag → MORE bound radiolabelled Ab (direct/sandwich). This inverse vs direct relationship is a GUARANTEED 2-mark question and MCQ. Also: RIA uses LABELLED ANTIGEN; IRMA uses LABELLED ANTIBODY.

Comparison of RIA Methods

Parameter	Classical RIA	Sequential RIA	Double Antibody RIA	Solid Phase RIA	IRMA
Labelled component	Ag* (antigen)	Ag* (antigen)	Ag* (antigen)	Ag* (antigen)	Ab* (antibody)
Ab quantity	Limited (key feature)	Limited	Limited	Limited (on surface)	EXCESS
Dose-response	Inverse (MORE Ag → LESS counts)	Inverse	Inverse	Inverse	Direct (MORE Ag → MORE counts)
Separation	DCC, charcoal, PEG	DCC, charcoal	2nd antibody + centrifuge	Wash	Wash (sandwich)
Sensitivity	Standard	Higher (2–5×)	Standard–high	Standard–high	Very high
Automation	Moderate	Moderate	Moderate	High	High
Format	Liquid phase	Liquid phase	Liquid phase	Solid phase	Solid phase (sandwich)

LIMITATIONS OF RIA

Despite its extreme sensitivity and specificity, RIA has several significant limitations that have led to the development of non-radioactive alternatives (ELISA, CLIA, FPIA):

Limitation	Description	Consequence / Solution
Radiation hazard	Use of radioactive isotopes (^{125}I, ^{3}H) creates radiation exposure risk for laboratory personnel	Shielding, dosimetry badges, restricted access, trained personnel; replaced by ELISA in many labs
Radioactive waste disposal	Radioactive waste must be stored until decay and disposed per radiation safety regulations	Expensive and complex waste management; disposal costs; regulatory compliance
Short shelf life of tracer	^{125}I-labelled antigen ($t_{½}$ = 60 days) degrades; assay kits expire in ~60–90 days	Frequent kit repurchase; cannot stockpile; logistics challenges
Specialised infrastructure	Requires gamma counter or liquid scintillation counter, radiation monitoring equipment, radioactive waste storage	High initial capital cost; restricted to licensed facilities
Non-specific binding (NSB)	Radiolabelled antigen sticks non-specifically to tube walls and other proteins	Increases background; reduces sensitivity; requires NSB correction in all calculations
Hook effect (high dose)	At very high antigen concentrations, immunoassay signal paradoxically DECREASES (both antibodies saturated)	False-negative or falsely low results; must dilute samples if hook effect suspected (in IRMA/sandwich especially)
Cross-reactivity	Antibody may cross-react with metabolites, precursors, or structurally similar molecules	False-positive results; must characterise antibody cross-reactivity profile thoroughly
Requires validated antibody	Antibody must be highly specific, high affinity, and reproducibly produced	Polyclonal antisera vary between bleeds; batch-to-batch variability
Regulatory complexity	Radioactive material requires licence from national radiation authority (e.g., BARC in India, NRC in USA)	Not available in all laboratories; additional compliance burden
Limited multiplexing	Each RIA tube typically measures one analyte	Low throughput per test; ELISA microplates and multiplex platforms now preferred for panels

📌 **Why RIA is being replaced:** ELISA (Enzyme-Linked Immunosorbent Assay) and CLIA (Chemiluminescence Immunoassay) have largely replaced RIA in clinical laboratories because they offer comparable sensitivity without radiation hazards. ELISA uses enzyme labels (HRP, AP); CLIA uses chemiluminescent labels (acridinium ester, luminol). However, RIA remains a gold standard for some analytes (aldosterone, renin activity) and in research.

APPLICATIONS OF RIA

Application Area	Specific Analytes Measured by RIA	Clinical/Pharmaceutical Significance
Endocrinology — Pituitary hormones	TSH, LH, FSH, ACTH, growth hormone, prolactin	Pituitary adenoma diagnosis; fertility workup; acromegaly diagnosis; hypothyroidism screening
Endocrinology — Thyroid hormones	Total T3, total T4, free T4, anti-TPO antibodies	Hypothyroidism, hyperthyroidism, Hashimoto's thyroiditis diagnosis
Endocrinology — Adrenal hormones	Cortisol, aldosterone, DHEA-S, catecholamines	Cushing's syndrome, Addison's disease, primary aldosteronism, pheochromocytoma
Endocrinology — Reproductive hormones	Estradiol, progesterone, testosterone, LH, FSH, hCG, inhibin B	Infertility, PCOS, menopause, pregnancy monitoring; doping in sports
Endocrinology — Pancreatic hormones	Insulin, C-peptide, glucagon, proinsulin	Diabetes diagnosis; insulinoma; monitoring insulin secretion; differentiating T1/T2 DM
Therapeutic Drug Monitoring (TDM)	Digoxin, cyclosporine A, methotrexate, phenytoin, tobramycin, carbamazepine	Narrow therapeutic index drugs; prevent toxicity; ensure efficacy; transplant immunosuppression
Tumour markers	AFP, CEA, PSA, CA-125, CA 19-9, beta-hCG, neuron-specific enolase	Cancer screening, staging, and monitoring of therapy response
Nutritional markers	Vitamin B12, folate, ferritin, 25-OH vitamin D, transferrin	Nutritional deficiency diagnosis; anaemia workup
Drug abuse screening	Cannabis (THC-COOH), cocaine metabolites, opiates, amphetamines	Forensic toxicology; workplace drug testing; athlete doping
Infectious disease serology	HBsAg (Hepatitis B surface antigen), TORCH titres, HIV core antigen	Blood bank screening; prenatal TORCH panel; early HIV diagnosis
Pharmacokinetic research	Measurement of drug and metabolite concentrations in plasma, urine, tissue	ADME studies; bioavailability; bioequivalence; drug-drug interactions
Receptor binding studies	Neurotransmitter receptor binding; hormone receptor characterisation	Drug mechanism studies; receptor pharmacology; CNS drug development

INTRODUCTION TO EXTRACTION

Extraction is a sample preparation technique used to **isolate and concentrate the analyte of interest** from a complex biological or environmental matrix (plasma, urine, tissue, food, water) prior to instrumental analysis (HPLC, GC, MS). Without extraction, matrix components interfere with detection and quantification.

Why Extraction is Needed	Example
Remove matrix interferents (proteins, lipids, salts, endogenous compounds)	Protein-free plasma needed for HPLC analysis of drugs
Concentrate the analyte (increase sensitivity)	Urine drug metabolites present at ng/mL → concentrate 10–100× for detection
Transfer analyte to compatible solvent	Move drug from aqueous plasma into organic solvent for reverse-phase HPLC injection
Remove ion-pairing agents or phospholipids	Phospholipid removal before LC-MS/MS to prevent ion suppression
Enrich trace analytes	Environmental monitoring: ppb-level pesticides from water samples concentrated by SPE

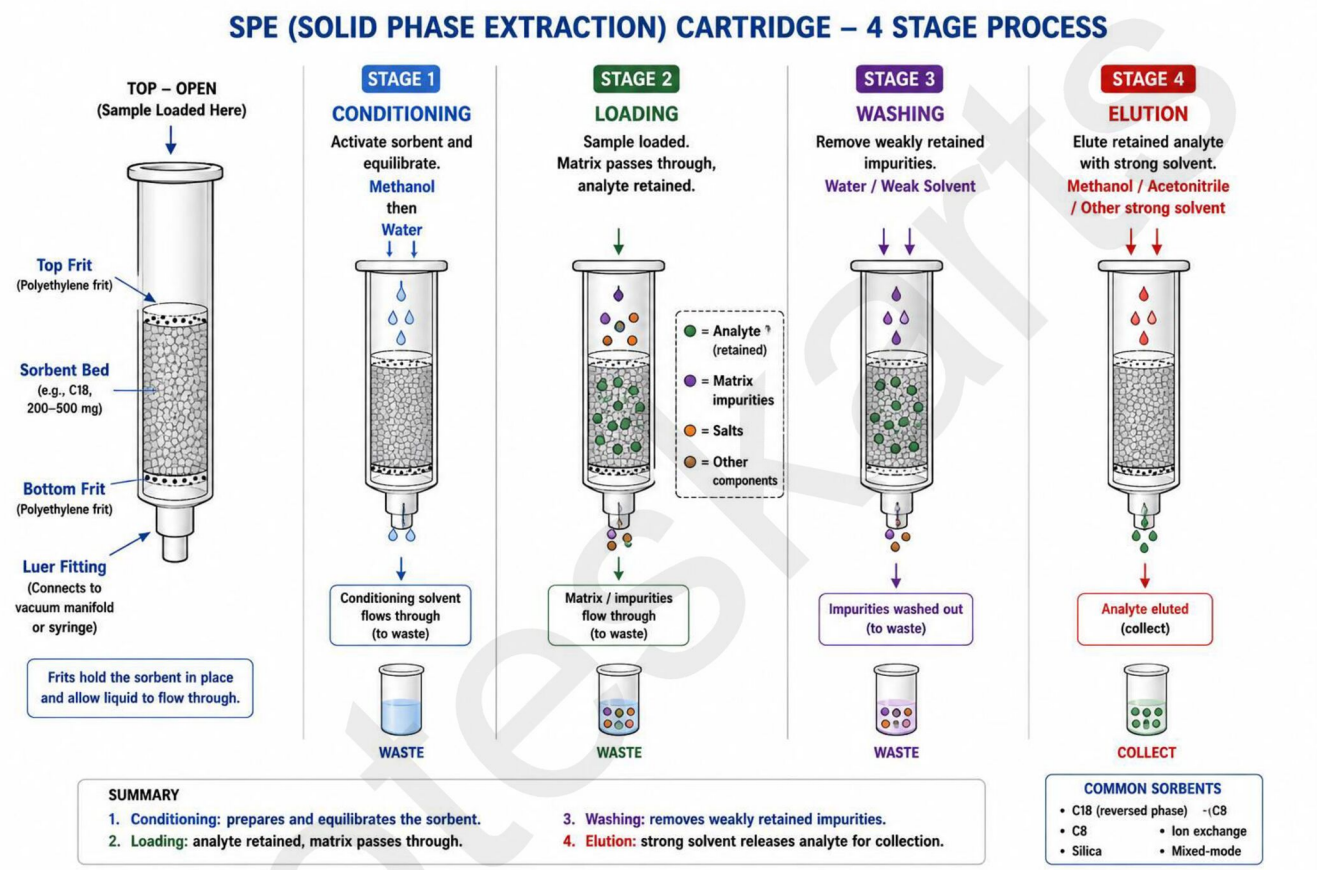
Extraction Technique	Basis of Separation	Typical Use
Solid Phase Extraction (SPE)	Differential adsorption to solid sorbent	Sample cleanup, concentration, desalting — drugs in plasma/urine
Liquid-Liquid Extraction (LLE)	Differential solubility in two immiscible liquids	Drug extraction from biological fluids; classical method
Protein Precipitation (PPT)	Denaturation of proteins by organic solvent or acid	Quick plasma cleanup (least selective; highest throughput)
Supported Liquid Extraction (SLE)	LLE on inert diatomaceous earth support	Hybrid of LLE and SPE; less emulsion formation
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe — modified LLE	Pesticide residue analysis in food

SOLID PHASE EXTRACTION (SPE)

Principle of SPE

Solid Phase Extraction (SPE) is a sample preparation technique that uses the **selective adsorption of analyte(s) from a liquid sample onto a solid sorbent material**, followed by selective elution of the analyte(s) with an appropriate solvent. SPE exploits the same interactions as HPLC (reversed phase, normal phase, ion exchange, mixed mode).

SPE concentrates the analyte AND removes matrix interferents in a single operation.



SPE Sorbent Types

The choice of sorbent determines the mechanism of retention and selectivity. Major sorbent categories:

Sorbent Type	Chemistry	Mechanism	Analytes Retained	Elution Solvent
Reversed Phase — C18 (Octadecyl)	18-carbon alkyl chains bonded to silica	Hydrophobic/non-polar interaction	Non-polar to moderately polar analytes: drugs, lipids, vitamins, steroids	Methanol, acetonitrile, or their mixtures
Reversed Phase — C8 (Octyl)	8-carbon alkyl chains	Hydrophobic (less retentive than C18)	Moderately polar analytes; faster elution	Methanol, acetonitrile

Sorbent Type	Chemistry	Mechanism	Analytes Retained	Elution Solvent
Reversed Phase — Phenyl	Phenyl groups bonded to silica	Hydrophobic + pi-pi interaction with aromatics	Aromatic compounds; better selectivity for PAH, drugs with aromatic rings	Methanol, acetonitrile
Polymer-based RP — HLB (Hydrophilic-Lipophilic Balance)	Copolymer of divinylbenzene + N-vinylpyrrolidone	Hydrophobic + hydrogen bonding	BROAD SPECTRUM — polar AND non-polar; excellent for drugs in urine/plasma	Methanol, acetonitrile
Normal Phase — Silica	Unmodified silica; polar surface	Polar interaction (adsorption)	Polar analytes; non-polar sample matrix	Hexane, chloroform with small % polar modifier
Normal Phase — Amino (NH_2)	Aminopropyl groups	Polar + weak anion exchange	Carbohydrates, weak acids, nucleotides	Dichloromethane, methanol
Ion Exchange — SCX (Strong Cation)	Sulfonic acid groups ($-SO_3H$)	Cation exchange	Basic drugs (amines, pKa > 8): amphetamines, opiates	High pH buffer or methanol + ammonia
Ion Exchange — SAX (Strong Anion)	Quaternary ammonium groups ($-N^+(CH_3)_3$)	Anion exchange	Acidic compounds (acids, phosphates): carboxylic acids, nucleotides	High concentration salt or acid buffer
Mixed Mode — MCX	C8 + sulfonic acid (RP + cation exchange)	Dual retention: hydrophobic + ionic	Basic drugs in complex matrices — very selective	Methanol, then methanol + 5% ammonia
Immunosorbent (IS)	Antibody covalently coupled to silica	Immunoaffinity	Ultra-selective; single analyte	Mild acid, organic solvent — elution disrupts Ab-Ag binding

📌 **Sorbent Selection Rule:** Match sorbent to analyte polarity: Non-polar drug → C18 (RP). Basic drug → SCX or MCX. Acidic drug → SAX. Broad spectrum (drugs in urine/plasma) → HLB polymer (most versatile). For forensic toxicology (multiple drugs) → MCX (mixed mode cation exchange) is gold standard.

SPE Step-by-Step Procedure — Complete Protocol

SPE is performed in **4 sequential steps** on a cartridge or 96-well plate under vacuum (manifold) or positive pressure:

STEP 1: Conditioning (Activation) — Wet the sorbent and prepare it for interaction with sample. **For C18:** Pass 2–3 mL methanol (wets non-polar chains) then 2–3 mL water or buffer (equilibrates to aqueous conditions). **Critical:** Never let sorbent dry between conditioning and loading — dry sorbent loses retention. Conditioning solvent must match sample matrix.

STEP 2: Loading (Sample Application) — Apply the prepared sample (plasma, urine, extract) to the conditioned cartridge. The analyte **adsorbs to the sorbent** while matrix components (proteins, salts, polar interferences) pass through in the **load/pass-through fraction** (DISCARD). Adjust sample pH, ionic strength, or dilution to maximise analyte retention. **Key point:** If analyte passes through during loading — check conditioning, sample pH, or switch sorbent.

STEP 3: Washing (Rinsing) — Remove remaining matrix impurities from the sorbent while keeping analyte retained. **For C18:** Wash with water or 5–10% methanol in water. **For MCX (basic drug):** Wash with water, then methanol. **The wash must be strong enough to remove interferences but NOT strong enough to elute the analyte** — requires careful optimisation. DISCARD wash fraction.

STEP 4: Elution (Recovery of Analyte) — Disrupt the analyte-sorbent interaction with a strong solvent to release the analyte. **For C18:** Elute with 1–2 mL methanol or acetonitrile. **For MCX (basic drug):** Elute with 2% ammonia in methanol (disrupts both hydrophobic + ionic interactions). COLLECT this fraction. Dry under nitrogen stream if needed → reconstitute in HPLC-compatible solvent.

SPE Step	Fluid Applied	Purpose	Fraction Action
1. Conditioning	Methanol → Water (RP)	Activate sorbent surface; equilibrate to sample matrix	Discard
2. Loading	Sample in aqueous matrix	Analyte binds to sorbent; matrix passes through	Discard (pass-through)
3. Washing	Weak solvent (water, 5–10% MeOH)	Remove polar matrix interferences; analyte stays	Discard
4. Elution	Strong solvent (MeOH, ACN, or ammonia/MeOH)	Release analyte from sorbent	COLLECT — contains analyte

SPE Modes and Formats

SPE Operating Modes

- **Positive pressure (syringe push):** Manual SPE using syringe plunger — simple, low-throughput
- **Vacuum manifold:** 12–24 cartridges processed simultaneously under vacuum — standard for bioanalytical labs
- **96-well plate (automated SPE):** 96 samples processed simultaneously; integrated with robotic liquid handlers — high-throughput bioanalysis

- **Online SPE (column switching):** SPE cartridge integrated directly into HPLC system; automated loading and elution within HPLC run — no manual steps

SPE Cartridge Formats

- **Syringe barrel cartridges:** 1, 3, 6 mL volume; 50–500 mg sorbent — standard format
- **Disk cartridges:** Sorbent embedded in polymer disk; high flow rate; large volume extraction
- **96-well plates:** High-throughput; compatible with liquid handling robots
- **Micro-elution plates:** Small elution volume (25–50 µL) → higher concentration factor → better sensitivity

Advantages and Disadvantages of SPE

Advantages of SPE	Disadvantages of SPE
Higher selectivity than LLE — many sorbent options	Higher cost per sample than LLE
Minimal or no emulsion formation (no phase separation issue)	More method development required — sorbent optimisation
Small solvent volumes (1–3 mL elution)	Cartridge-to-cartridge variability
Easily automated (96-well plates, robotics)	Cartridge may dry out if timing is poor
Concentrates analyte AND removes matrix in single step	Incomplete recovery possible if sorbent capacity exceeded
Applicable to polar AND non-polar analytes with right sorbent	Some sorbents retain analyte too strongly (incomplete elution)
Applicable online (in-line with HPLC)	Requires careful pH and ionic strength control

LIQUID-LIQUID EXTRACTION (LLE)

Principle of LLE

Liquid-Liquid Extraction (LLE) — also called **solvent extraction** — is based on the **differential solubility of the analyte** between two immiscible liquid phases (typically an aqueous phase and an organic phase). The analyte partitions between the two phases according to its relative affinity for each.

The fundamental principle is described by the **Nernst Distribution Law**:

$$Kd = C_{organic} / C_{aqueous}$$

Where **Kd = distribution coefficient** (or partition coefficient, P) = ratio of analyte concentration in organic phase to aqueous phase at equilibrium. For quantitative extraction, Kd must be large (Kd >> 1), meaning the analyte strongly prefers the organic phase.

Distribution Coefficient (Kd) and Extraction Efficiency

The percentage extraction efficiency (%E) for a single LLE is:

$$\% \text{ Extraction} = [Kd \times V_{org} / (Kd \times V_{org} + V_{aq})] \times 100$$

Where V_{org} = volume of organic phase; V_{aq} = volume of aqueous phase.

Key insight: Multiple smaller extractions are more efficient than one large extraction. Using the same total volume of organic solvent in 3 separate extractions gives better recovery than one single large-volume extraction.

Kd Value	Physical Meaning	Extraction Efficiency (1:1 volume ratio, single extraction)
Kd = 0.1	Analyte strongly prefers aqueous phase	9.1% — poor extraction; many stages needed
Kd = 1	Equal distribution between phases	50% — moderate; at least 3–4 extractions needed
Kd = 10	Analyte prefers organic phase	90.9% — good; 1–2 extractions sufficient
Kd = 100	Strongly prefers organic	99% — excellent; single extraction usually adequate
Kd > 1000	Almost completely in organic phase	~100% — quantitative in single extraction

Multiple extraction advantage: If Kd = 1 and $V_{org} = V_{aq} = 10$ mL. Single extraction gives 50% recovery. Three extractions with 10 mL each = $(1 - (1/(1+1))^3) \times 100\% = (1 - 0.125) \times 100\% =$ **87.5%** recovery. Five extractions = 97%!

Factors Affecting LLE

9.3.1 pH of Aqueous Phase — Most Critical Factor

For ionisable drugs (acids and bases), the **pH of the aqueous phase determines the ionisation state**, which directly controls extraction:

Drug Type	Ionisation Form	Polarity	Extraction Condition	Rationale
Acidic drugs (carboxylic acids, phenols) R-COOH / R-COO^-	Unionised (R-COOH) at low pH	Non-polar	Extract at low pH (pH 2–4)	Unionised acid is lipophilic → partitions into organic phase
Basic drugs (amines) R-NH_2 / R-NH_3^+	Unionised (R-NH_2) at high pH	Non-polar	Extract at high pH (pH 9–11)	Free base form is lipophilic → partitions into organic phase
Ionised form (at wrong pH)	R-COO^- or R-NH_3^+	Polar	Stays in aqueous phase	Charged = hydrophilic = not extracted by organic solvent

⚡ **pH Rule — Must Memorise:** ACIDS: extract at LOW pH (acid form = unionised = lipophilic). BASES: extract at HIGH pH (free base = unionised = lipophilic). The Henderson-Hasselbalch equation governs: at pH = pKa, 50% ionised. For >99% extraction: acidic drugs at pH < pKa − 2; basic drugs at pH > pKa + 2.

Choice of Organic Solvent

The organic solvent must be: (1) **immiscible with water**, (2) have high Kd for the analyte, (3) be volatile for evaporation and reconstitution.

Solvent	Log P	Polarity	Best For	Limitation
Diethyl ether	0.85	Low	Moderately non-polar drugs; steroids	Flammable; volatile; peroxide formation; forms emulsions
Ethyl acetate	0.73	Medium-low	Drugs of moderate polarity; hormones	Slightly water-miscible (saturate with water first); volatile
Dichloromethane (DCM)	0.93	Medium	Wide range; excellent for many drugs	Suspected carcinogen; chlorinated waste disposal
n-Hexane / Heptane	3.0	Very low (non-polar)	Very non-polar compounds; lipids; PAH	Poor for most drugs; limited solubility
MTBE (methyl tert-butyl ether)	1.1	Low-medium	Excellent universal drug extraction solvent	Forms peroxides; lower density makes pipetting easier

Solvent	Log P	Polarity	Best For	Limitation
Chloroform	0.97	Medium	Classical extraction solvent	Hepatotoxic, carcinogenic; regulated waste
n-Butyl chloride	2.7	Low	Basic drugs; amphetamines	Less common; less available

Other Factors Affecting LLE

- **Ionic strength / salting out:** Adding NaCl or Na_2SO_4 to aqueous phase increases ionic strength → reduces analyte solubility in water → increases Kd → better extraction (salting-out effect)
- **Temperature:** Higher temperature generally increases solubility in both phases — variable effect; usually extract at room temperature
- **Volume ratio (V_org/V_aq):** Larger organic volume → better single-extraction efficiency but more solvent use
- **Mixing time:** Adequate mixing (vortex 1–5 min; or rotary mixer) needed to maximise contact area → approach equilibrium
- **Emulsion formation:** Problem with biological fluids (plasma, blood) due to proteins and phospholipids → use centrifugation, addition of electrolyte, freeze-thaw cycle, anti-emulsifiers

LLE Step-by-Step Procedure

**Standard LLE procedure for drug extraction from plasma:

1. **pH adjustment:** Add appropriate buffer to plasma (1:1 ratio). For basic drug: add NaOH or carbonate buffer to pH 9–10. For acidic drug: add H_3PO_4 or acetate buffer to pH 2–3.
2. **Deproteinisation (if needed):** Add organic solvent or acetonitrile to precipitate proteins first — optional for some matrices.
3. **Add organic solvent:** Transfer pH-adjusted plasma to polypropylene tube; add 3–5× volume of organic solvent (e.g., 1 mL plasma + 5 mL ethyl acetate).
4. **Mixing:** Vortex mix vigorously for 1–3 minutes OR use end-over-end rotary mixer for 5–10 minutes to achieve equilibrium.
5. **Phase separation:** Centrifuge at 3000–5000 rpm for 5–10 minutes at room temperature. Two distinct layers form.
6. **Transfer organic layer:** Carefully transfer upper organic layer to new tube using pipette (for less-dense solvents like ether, MTBE) OR use lower organic layer (for denser solvents like DCM, chloroform).
7. **Back extraction (optional):** Re-extract the organic layer with aqueous phase at opposite pH to back-extract drug away from non-polar interferences (increases specificity).
8. **Evaporation:** Evaporate organic solvent under gentle nitrogen stream at 40–50°C to dryness.
9. **Reconstitution:** Dissolve dry residue in small volume of HPLC mobile phase (50–200 µL). Vortex, centrifuge briefly. Transfer to HPLC vial. Inject.

Back-Extraction (Re-Extraction / Stripping)

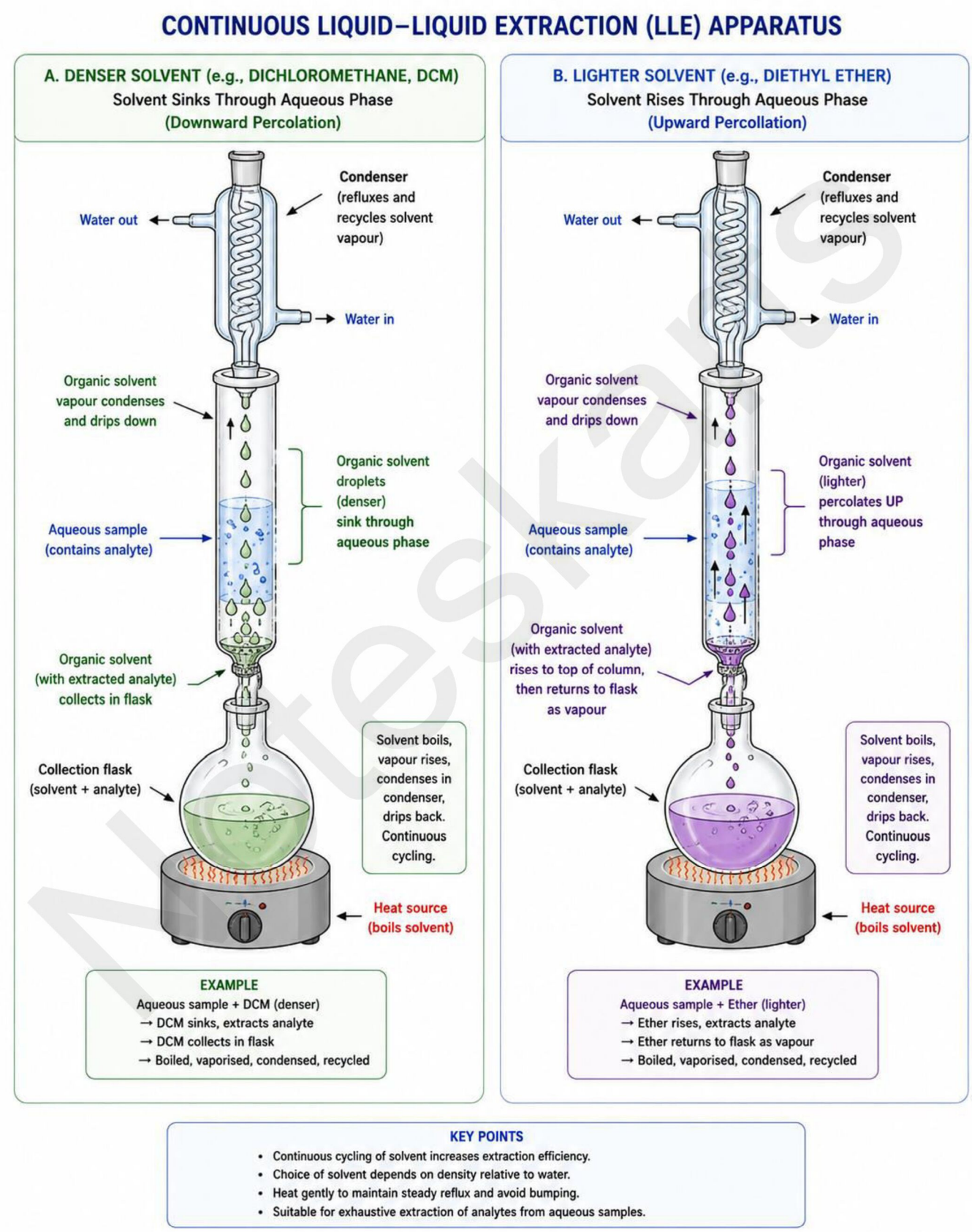
Back-extraction (also called re-extraction) is a two-step LLE process that improves specificity:

- **Step 1 — Forward extraction:** Extract drug from aqueous sample into organic at optimal pH (e.g., basic drug: pH 10 → organic)

- **Step 2 — Back extraction:** Transfer organic layer to new tube; add aqueous acid (e.g., 0.1 M HCl → ionises basic drug → goes back into aqueous). Discard organic (containing non-polar impurities).
- **Step 3 — Re-extraction:** Adjust aqueous back-extract to alkaline pH again; re-extract with fresh organic. Collect this organic layer.
- **Advantage:** Removes non-polar matrix interferences (lipids, fatty acids) that also co-extract in forward extraction
- **Application:** Forensic toxicology (urine drug screening); plasma sample cleanup for GC-MS or LC-MS/MS

Continuous Liquid-Liquid Extraction (Continuous LLE)

For analytes with low Kd (difficult to extract) or for large-volume aqueous samples (environmental monitoring), **continuous LLE** uses a special apparatus (liquidliquid extractor) where organic solvent continuously cycles through the aqueous phase, achieving multiple extractions automatically.



SPE vs LLE - Comparison

Parameter	Solid Phase Extraction (SPE)	Liquid-Liquid Extraction (LLE)
Principle	Adsorption of analyte to solid sorbent; selective retention and elution	Partition of analyte between two immiscible liquid phases (Nernst law)
Selectivity	Very high (many sorbent options: RP, IE, mixed mode)	Moderate (pH-dependent; can be improved by back-extraction)
Solvent volume	1–3 mL elution (very small)	3–10 mL per extraction (larger)
Number of steps	4 steps (condition, load, wash, elute)	5–9 steps (pH adjust, add solvent, mix, centrifuge, transfer, evaporate, reconstitute)
Emulsion formation	None (no liquid-liquid interface)	Common problem with biological matrices (proteins cause emulsions)
Automation	Easy — 96-well plates, robotics	Difficult — multiple liquid transfers; evaporation
Cost per sample	Higher (cartridge cost)	Lower (solvents only)
Recovery	High, reproducible (95–105% typical)	Variable (70–95% typical); multiple extractions improve
Method development time	Longer (sorbent selection + 4 steps to optimise)	Faster (mainly pH and solvent choice)
Throughput	Very high (96-well plate = 96 samples)	Lower (manual = 10–20 samples/hour)
Matrix effects in LC-MS	Lower (better cleanup)	Higher (lipids co-extract — ion suppression)
Best application	High-throughput bioanalysis; LC-MS/MS sample prep	Classical drug analysis; forensic toxicology; low-resource labs

IMPORTANT QUESTION BANK

A. 2-Mark Questions with Model Answers

Q1. Define RIA and state who discovered it.

Ans: **RIA (Radioimmunoassay)** is an extremely sensitive in vitro analytical technique that combines the specificity of immunological antigen-antibody reactions with the sensitivity of radioactive detection to quantify analytes at nanogram to picogram per mL concentrations. **Discovered by:** Rosalyn Yalow and Solomon Berson in 1959, initially for measuring plasma insulin using ^{131}I-labelled insulin. Yalow received the **Nobel Prize in Physiology or Medicine in 1977** for this discovery.

Q2. Name the 4 essential components of RIA.

Ans: The four essential components of RIA are: (1) **Antigen (Ag)** — the analyte being measured (e.g., insulin, cortisol, drug). (2) **Specific Antibody (Ab)** — raised against the antigen; must have high affinity and specificity. (3) **Radiolabelled Antigen (Ag* = Tracer) — **same as analyte but tagged with radioisotope (^{125}I or ^{3}H); competes with Ag for Ab binding. (4)** Separation System** — physically separates bound Ag*-Ab complex from free Ag* (DCC, double antibody, solid phase, PEG).

Q3. What is the principle of competitive binding in RIA? State the key inverse relationship.

Ans: **Principle:** RIA uses a **fixed, limited amount of antibody** that cannot bind all the tracer (Ag*). When unlabelled antigen (analyte) is added, it competes with the tracer (Ag*) for the limited antibody binding sites. **Key inverse relationship:** As the concentration of unlabelled analyte INCREASES, MORE Ag* is displaced from the antibody → LESS Ag*-Ab (bound fraction) → LESS radioactivity in bound fraction. Therefore: MORE sample antigen = LESS bound radioactivity counted. This inverse relationship forms the basis of the standard competition curve.

Q4. Name 3 radioisotopes used in RIA. Which is most common and why?

Ans: Three isotopes used in RIA: (1) **^{125}I (Iodine-125):** Half-life 60 days; gamma emitter; detected by gamma counter — MOST COMMON for protein/peptide hormones. (2) **^{3}H (Tritium):** Half-life 12.3 years; beta emitter; liquid scintillation counter — used for steroids and drugs. (3) **^{14}C:** Half-life 5730 years; beta emitter — research and metabolic studies. **^{125}I is most common because:** very high specific activity, convenient half-life (60 days), easy gamma counting, and easy labelling of tyrosine residues in proteins.

Q5. What is the principle of SPE? Name the 4 steps in order.

Ans: **Principle of SPE:** The analyte from a liquid sample is selectively adsorbed onto a solid sorbent based on differential interactions (hydrophobic, ionic, polar). Matrix interferents either do not bind or are removed by washing. The retained analyte is then selectively eluted with a strong solvent. **Four steps in order:** (1) **Conditioning** — activate sorbent with methanol then water. (2) **Loading** — apply sample; analyte adsorbs; matrix passes through (discard). (3) **Washing** — remove matrix impurities with weak solvent (discard). (4) **Elution** — release analyte with strong solvent (COLLECT this fraction).

Q6. State the Nernst Distribution Law and define the distribution coefficient (Kd).

Ans: **Nernst Distribution Law:** At constant temperature and pressure, when a solute distributes between two immiscible liquids, the ratio of concentrations at equilibrium is constant, regardless of the total amount of solute. **Distribution Coefficient (Kd):** Kd = C_organic / C_aqueous. Where C_organic = concentration in organic phase; C_aqueous = concentration in aqueous phase at equilibrium. **Significance:** High Kd = analyte prefers organic phase = efficient extraction. Kd ≥ 10 = >90.9% extracted in a single equal-volume extraction. Kd is influenced by analyte polarity, pH, ionic strength, and temperature.

Q7. State the pH rule for LLE with acidic and basic drugs.

Ans: **LLE pH Rule:** For extraction into organic solvent, the drug must be in its UNIONISED (non-polar) form. **Acidic drugs** (R-COOH): Extract at **LOW pH** (pH 2–4) — acid form is unionised → lipophilic → partitions into organic. At high pH, $R\text{-}COO^-$ (ionised) = hydrophilic = stays in aqueous. **Basic drugs** ($R\text{-}NH_2$): Extract at **HIGH pH** (pH 9–11) — free base form is unionised → lipophilic → partitions into organic. At low pH, $R\text{-}NH_3^+$ (ionised) = hydrophilic = stays in aqueous. **Mnemonic:** 'Acid-Low, Base-High'.

Q8. State 3 advantages of SPE over LLE.

Ans: Three advantages of SPE over LLE: (1) **No emulsion formation** — SPE uses solid-liquid contact; no immiscible liquid-liquid interface; biological fluids (plasma, urine) don't cause emulsions. (2) **Higher selectivity** — multiple sorbent types (C18, HLB, MCX, SAX) allow selective retention based on specific interactions; LLE selectivity limited to polarity and pH. (3) **Automation-compatible** — 96-well SPE plates easily integrated with robotic liquid handlers and online HPLC systems; LLE difficult to fully automate due to liquid transfers and evaporation steps.

B. 5-Mark Questions — Structured Answers

Q1. Describe the principle and components of RIA. Draw the RIA standard competition curve. (5 marks)

Q2. Describe the different methods used in RIA. Explain the principle, advantages, and limitations of each. (5 marks)

Q3. Explain the principle, sorbent types, and 4-step procedure of SPE. (5 marks)

Q4. Explain the principle and procedure of liquid-liquid extraction (LLE). Discuss the factors affecting LLE. (5 marks)

Q5. State the limitations of RIA and compare with ELISA. Why is ELISA replacing RIA? (5 marks)

C. 10-Mark Question Skeletons

Q1. Write a comprehensive note on Radioimmunoassay (RIA) — principle, components, methods, limitations, and applications. (10 marks)

Q2. Explain the principle and procedure of SPE and LLE. Compare the two techniques and discuss their pharmaceutical applications. (10 marks)

PREVIOUS YEAR-STYLE QUESTIONS — FULL SOLUTIONS

PYQ 1. Describe the principle, components, and applications of RIA. [AKTU-style, 7 marks]

PYQ 2. What is IRMA? How does it differ from classical RIA? [5 marks]

PYQ 3. Explain the procedure of SPE with special reference to sorbent types. [5 marks]

PYQ 4. What is liquid-liquid extraction? Explain the distribution coefficient and factors affecting LLE. [5 marks]

PYQ 5. Compare SPE and LLE. Discuss their limitations and pharmaceutical applications. [5 marks]

50 MCQs — UNIT IV (RIA + EXTRACTION TECHNIQUES)

1. RIA was discovered by Yalow and Berson in 1959 for measuring:
A. Cortisol
B. Insulin ✓
C. Growth hormone
D. TSH
Explanation: Rosalyn Yalow and Solomon Berson developed RIA in 1959 specifically for measuring plasma INSULIN using ^{131}I-labelled insulin. This allowed measurement of insulin in picogram quantities from small blood volumes. Yalow received the Nobel Prize in 1977 for this discovery (Berson died in 1972, before the Nobel was awarded).

2. The most commonly used radioisotope in RIA is:
A. ^{3}H (Tritium)
B. ^{14}C
C. ^{125}I ✓
D. ^{57}Co
Explanation: ^{125}I (Iodine-125) is the most widely used RIA isotope. Reasons: (1) Half-life 60.1 days — long enough for routine use, short enough for safe disposal; (2) Gamma emitter — simple gamma counter detection (no liquid scintillation needed); (3) Very high specific activity; (4) Easy labelling of proteins via tyrosine iodination using chloramine-T or iodogen.

3. In classical RIA, the dose-response relationship is:
A. Direct — more antigen, more counts
B. Inverse — more antigen, less bound radioactivity ✓
C. No relationship between antigen and counts
D. Exponential — dose squared proportional to counts
Explanation: Classical RIA uses COMPETITIVE INHIBITION: unlabelled antigen and tracer compete for limited antibody. MORE unlabelled antigen → MORE tracer displaced → LESS Ag-Ab complex formed → LESS radioactivity in bound fraction. This INVERSE relationship distinguishes RIA from IRMA (which is DIRECT).*

4. Which separation method in RIA uses dextran-coated charcoal?
A. To separate free antigen from bound antigen-antibody complex ✓
B. To precipitate the antibody
C. To radiolabel the antigen
D. To concentrate the antibody
Explanation: Dextran-Coated Charcoal (DCC) ADSORBS small FREE tracer molecules (Ag). Large Ag*-Ab complexes are too big to be adsorbed. After centrifugation: pellet (charcoal + free Ag*) discarded; SUPERNATANT counted = bound Ag*-Ab fraction. DCC is simple, fast, and inexpensive — but time-sensitive (centrifuge exactly at specified time) and non-specific (can damage some antibodies).*

5. The 4 essential components of RIA are:

A. Enzyme, substrate, antibody, sample

B. Antigen, antibody, radiolabelled antigen (tracer), separation system ✓

C. Antigen, detector, buffer, radioisotope

D. Antibody, gamma counter, incubation buffer, standard

Explanation: The 4 essential components: (1) Antigen (analyte being measured); (2) Specific Antibody (high affinity, high specificity); (3) Radiolabelled Antigen / Tracer (Ag = same as analyte but radiolabelled with ^{125}I or ^{3}H — competes with unlabelled Ag); (4) Separation System (physically separates bound Ag*-Ab from free Ag* — DCC, double antibody, solid phase, PEG).*

6. In IRMA (Immunoradiometric Assay), the dose-response relationship is:

A. Inverse — more antigen, less counts

B. No relationship

C. Direct — more antigen, more bound radiolabelled antibody ✓

D. Biphasic — increases then decreases

Explanation: IRMA uses EXCESS radiolabelled ANTIBODY (not antigen) in a two-site sandwich format. MORE antigen → MORE capture antibody-Ag sandwiches formed → MORE radiolabelled Ab captured → MORE radioactivity counted. DIRECT relationship (opposite to classical RIA). This makes IRMA easier to interpret and gives wider dynamic range.*

7. ^{3}H (tritium) is used in RIA for labelling:

A. Peptide hormones like insulin

B. Steroids and small molecule drugs (when iodination would destroy immunoreactivity) ✓

C. Large proteins only

D. Vitamin B12

Explanation: ^{3}H is used for STEROIDS (e.g., cortisol, testosterone, progesterone) and small DRUGS (e.g., morphine, theophylline) where: (1) No tyrosine residue available for iodination; (2) Iodination of the small molecule would change its structure and destroy immunoreactivity; (3) The molecule can be tritiated during synthesis. ^{3}H detected by liquid scintillation counter (LSC). ^{57}Co is used specifically for vitamin B12 RIA.

8. NSB in RIA stands for:

A. Normal Standard Background

B. Non-Specific Binding — radioactivity bound without antibody ✓

C. Normalised Signal Baseline

D. Negative Standard Blank

Explanation: NSB = Non-Specific Binding — the radioactivity retained in the tube in ABSENCE of specific antibody. Caused by physical sticking (adsorption) of tracer to tube walls, carrier proteins, or charcoal non-specifically. NSB represents background 'noise' — subtracted from all bound measurements: True binding = Raw CPM − NSB. NSB should be < 5% of total counts added to the tube.

9. The SPE step that removes matrix impurities while keeping analyte on the sorbent is:

A. Conditioning

B. Loading

C. Washing ✓

D. Elution

Explanation: WASHING is step 3 of SPE. After sample loading (step 2), the analyte is retained on the sorbent, but residual matrix impurities (slightly polar interferents, phospholipids) are still on the sorbent. The WASH (weak solvent: water or 5–10% MeOH for C18) removes these impurities while the analyte remains bound. The wash fraction is DISCARDED. The balance between removing impurities but not analyte requires careful optimisation.

10. The distribution coefficient (Kd) in LLE is defined as:

A. Kd = C_aqueous / C_organic

B. Kd = C_organic / C_aqueous ✓

C. Kd = C_organic × C_aqueous

D. Kd = (C_organic − C_aqueous) / C_aqueous

Explanation: Distribution coefficient Kd = C_organic / C_aqueous at equilibrium. High Kd = analyte prefers organic phase = efficient extraction. Kd = 1 means equal concentration in both phases (50% extracted in equal volumes). Kd = 10 means 10× more concentrated in organic than aqueous = 90.9% extracted per extraction (equal volumes).

11. For extraction of a basic drug (amine) by LLE, the aqueous phase should be adjusted to:

A. Low pH (pH 2–3) with HCl

B. Neutral pH (pH 7)

C. High pH (pH 9–11) with NaOH or buffer ✓

D. Any pH — basic drugs always extract well

Explanation: Basic drugs (amines, R-NH_2) must be in their FREE BASE (unionised) form to be lipophilic and extract into organic solvent. FREE BASE form exists at pH >> pKa. Therefore, aqueous phase must be at HIGH pH (9–11). At low pH, amine is protonated to R-NH_3^+ (charged, hydrophilic) → stays in aqueous → cannot extract. Rule: BASES extract at HIGH pH; ACIDS extract at LOW pH.

12. The most common C-18 SPE sorbent retains analytes by:

A. Ion exchange (ionic interaction)

B. Hydrogen bonding with OH groups

C. Hydrophobic (non-polar) interaction between C18 chains and non-polar analyte ✓

D. Size exclusion

Explanation: C18 (octadecyl silica) retains analytes by HYDROPHOBIC interaction — the 18-carbon alkyl chains on the silica surface attract non-polar analytes (van der Waals/London dispersion forces). Non-polar or moderately polar drugs partition into the C18 phase from the aqueous sample. Polar matrix compounds (salts, sugars, urea, some polar metabolites) do not interact with C18 and pass through during loading.

13. The SPE sorbent that is best for broad-spectrum extraction of both polar and non-polar drugs from urine is:

A. Silica (normal phase)

B. C18 (octadecyl)

C. HLB (Hydrophilic-Lipophilic Balance polymer) ✓

D. Strong anion exchange (SAX)

Explanation: HLB (Hydrophilic-Lipophilic Balance) sorbent from Waters Corp. is a copolymer of divinylbenzene (hydrophobic) + N-vinylpyrrolidone (hydrophilic). Water-wettable: doesn't need methanol pre-wetting; won't lose retention if dries out. Retains BOTH polar AND non-polar analytes. Best choice for broad-spectrum drug screening in urine. Standard for drugs of abuse, therapeutic drugs, and metabolites.

14. Which isotope is used specifically for vitamin B12 RIA?

A. ^{125}I

B. ^{3}H

C. ^{57}Co ✓

D. ^{14}C

Explanation: ^{57}Co (Cobalt-57, $t_{1/2}$ = 271 days, gamma emitter) is used specifically for Vitamin B12 (cobalamin) RIA because B12 naturally contains cobalt at its centre. The cobalt-labelled B12 (^{57}Co-cyanocobalamin) can be prepared and used as the tracer in B12 RIA. Sensitivity and specificity are excellent for measuring B12 in serum for nutritional deficiency diagnosis.

15. What does 'B_0' represent in an RIA standard curve?

A. The blank absorbance in spectrophotometry

B. Non-specific binding (NSB)

C. Maximum binding — radioactivity bound when zero unlabelled antigen is present ✓

D. The lowest point on the standard curve

Explanation: B_0 = MAXIMUM BINDING — radioactivity (CPM) in the bound fraction when ZERO unlabelled antigen is present in the standard (zero standard). At B_0, all antibody binding sites are occupied by tracer (Ag) — maximum competition by tracer. B_0 is set to 100% and all other standards/samples are expressed as %B/B_0. B_0 is the reference point for the entire standard curve.*

16. The 4 steps of SPE in correct order are:

A. Load → Condition → Wash → Elute

B. Condition → Load → Wash → Elute ✓

C. Condition → Wash → Load → Elute

D. Load → Wash → Condition → Elute

Explanation: Correct SPE order: (1) CONDITION: activate sorbent with methanol then water; (2) LOAD: apply sample — analyte adsorbs, matrix passes through; (3) WASH: remove residual matrix with weak solvent; (4) ELUTE: release analyte with strong solvent (COLLECT this fraction). Memory: 'Can Larry Wash Elephants?' = Condition, Load, Wash, Elute.

17. Back-extraction in LLE improves:

A. Sensitivity (lower LOD)

B. Specificity (removes non-polar matrix co-extractants) ✓

C. Precision (%RSD)

D. Extraction speed

Explanation: Back-extraction improves SPECIFICITY. Forward extraction (e.g., basic drug extracted into organic at pH 10) also co-extracts non-polar matrix lipids and fatty acids. Back-extraction: add aqueous acid to organic → re-ionises basic drug → goes back into aqueous. Discard organic (with lipid impurities). Re-extract aqueous at high pH with fresh organic. Net result: drug is cleaner from non-polar interferents.

18. The double antibody separation method in RIA uses:

A. DCC charcoal to adsorb free tracer

B. A second antibody (anti-species IgG) to precipitate the first Ab-Ag complex ✓

C. Polyethylene glycol alone

D. Solid phase antibody coating

Explanation: Double Antibody (DAB) method: The FIRST antibody (specific anti-Ag, e.g., anti-insulin from rabbit) forms Ag-Ab complex. A SECOND antibody (e.g., goat anti-rabbit IgG — raised against rabbit immunoglobulin) recognises the Fc region of the first Ab and precipitates the entire Ab-Ag complex. After centrifugation, count precipitate (bound fraction). PEG (polyethylene glycol) is often added with the second Ab to accelerate precipitation from overnight to 30 min.*

19. Adding sodium chloride (NaCl) to the aqueous phase in LLE is done to:

A. Decrease the pH for acid drug extraction

B. Increase ionic strength — salting-out effect increases Kd ✓

C. Prevent emulsion formation

D. React with the drug to form an extractable salt

Explanation: Adding NaCl (or Na_2SO_4) creates a SALTING-OUT EFFECT: increased ionic strength → water molecules more tightly associated with ions → less water available to dissolve the drug → drug pushed into organic phase → increased Kd → better extraction efficiency. Typically 10–20% NaCl (w/v) added to aqueous sample before LLE. Separate from (but may also help) emulsion prevention.

20. MCX SPE sorbent is best described as:

A. Strong anion exchange only

B. C18 reversed phase only

C. Mixed mode — cation exchange + reversed phase (C8 + sulfonic acid) ✓

D. Immunoaffinity sorbent

Explanation: MCX (Mixed Mode strong Cation eXchange) = C8 reversed-phase + sulfonic acid (strong cation exchanger). DUAL RETENTION of basic drugs: (1) Hydrophobic interaction with C8 chains; (2) Ionic interaction with sulfonic acid groups for positively charged amines. Elution requires BOTH interactions to be disrupted: first methanol (disrupts hydrophobic), then methanol + 5% ammonia (disrupts ionic by deprotonating amine). Very selective for basic drugs (amines) like opioids, amphetamines, antidepressants.

21. The hook effect in immunoassays occurs when:

A. Antigen concentration is too low to detect

B. Antibody concentration is too low (incomplete binding)

C. Antigen concentration is very high — both capture and detection Ab sites saturated — signal paradoxically decreases ✓

D. The tracer has degraded

Explanation: Hook effect (high-dose hook): At very HIGH antigen concentrations in sandwich assays (IRMA), there are so many antigen molecules that they SEPARATELY saturate BOTH the capture antibody AND the detection antibody — so few sandwiches are formed. Result: measured signal DECREASES despite high concentration → falsely LOW (or negative) result. Solution: dilute the sample before analysis and re-assay if hook effect is suspected.

22. Which detector is used for counting ^{125}I-labelled samples?

A. Liquid scintillation counter (LSC)

B. Geiger-Müller counter

C. Sodium iodide crystal gamma counter ✓

D. Proportional counter

Explanation: ^{125}I is a gamma emitter (35 keV). Detected by a SODIUM IODIDE (NaI:Tl) CRYSTAL GAMMA COUNTER (also called gamma well counter or gamma scintillation counter). The NaI crystal scintillates when hit by gamma photons

→ photomultiplier tube converts to electrical pulse → counted. ^{3}H and ^{14}C (beta emitters) require LIQUID SCINTILLATION COUNTER (LSC) — no solid crystal; sample dissolved in scintillation cocktail.

23. The purpose of the conditioning step in SPE is to:

A. Remove the analyte from the sorbent

B. Activate and wet the sorbent surface to allow interaction with the sample ✓

C. Wash away matrix impurities

D. Concentrate the analyte on the sorbent

Explanation: CONDITIONING (Step 1 of SPE) activates and prepares the sorbent surface for sample loading. For C18: methanol wets the non-polar octadecyl chains (opening/solvating them), followed by water equilibrating to aqueous conditions. If not conditioned: C18 chains remain collapsed in aqueous environment → analyte cannot interact → no retention → analyte passes through (lost). Critical: keep sorbent wet after conditioning — never let it dry before loading.

24. The principle of RIA involves competition between labelled and unlabelled antigen for:

A. The detector cell

B. The separation medium (DCC charcoal)

C. A limited and fixed amount of specific antibody ✓

D. The incubation buffer

Explanation: The ANTIBODY in RIA is deliberately used in LIMITED and FIXED quantity — there is never enough antibody to bind ALL the tracer. This creates a competition between unlabelled antigen (sample) and labelled tracer (Ag) for the available Ab binding sites. The limited Ab is the critical element that makes competition and the inverse dose-response possible.*

25. Cross-reactivity in RIA is defined as:

A. Radioactivity detected in wrong fraction after separation

B. Non-specific binding of tracer to the tube wall

C. Binding of the specific antibody to a molecule OTHER than the target antigen ✓

D. Interference from the separation system

Explanation: Cross-reactivity = the percentage binding of the ANTIBODY to a structurally SIMILAR but DIFFERENT molecule (not the target antigen). Example: an anti-cortisol antibody might cross-react 10–15% with cortisone (oxidised form). Cross-reactivity can cause falsely elevated results if cross-reactive molecules are present in sample. Antibody characterisation table (% cross-reactivity with related compounds) is essential in RIA kit validation.

26. An RIA has B_0 = 5000 CPM, NSB = 150 CPM, and a sample gives 2650 CPM. Calculate %B/B_0.

A. 52%

B. 50% ✓

C. 48%

D. 53%

Explanation: %B/B_0 = (Sample CPM − NSB) / (B_0 − NSB) × 100 = (2650 − 150) / (5000 − 150) × 100 = 2500/4850 × 100 = 51.5% ≈ 50% (nearest option). This would fall at the ED50 point on the standard curve — the concentration at which 50% of maximum specific binding is achieved. This is also the point of maximum analytical sensitivity (steepest slope of the curve).

27. Which SPE sorbent is the BEST choice for extracting ACIDIC drugs (pKa 3–5) from plasma?

A. MCX (mixed mode cation exchange)

B. C18 (reversed phase only)

C. SAX (strong anion exchange) or Oasis MAX ✓

D. SCX (strong cation exchange)

Explanation: Acidic drugs (carboxylic acids, pKa 3–5) are NEGATIVELY CHARGED at physiological pH (deprotonated). Best extraction: (1) SAX (Strong Anion Exchange) sorbent — quaternary ammonium groups attract anions; load at pH 5–7 (drug ionised = anion). (2) Oasis MAX = MAX (Mixed mode Anion exchange + RP). SCX and MCX are for BASIC drugs (cations). C18 only works for acidic drugs if pH is lowered to make them unionised (not always practical in plasma matrix).

28. In LLE, a basic drug has pKa = 9. At pH 10, approximately what % is in free base form?

A. 9%

B. 50%

C. 91% ✓

D. 99%

Explanation: Henderson-Hasselbalch for base: % unionised = 1/(1 + 10^(pKa−pH)) × 100. At pH 10, pKa = 9: % unionised = 1/(1 + 10^(9−10)) × 100 = 1/(1 + 10⁻¹) × 100 = 1/(1.1) × 100 = 90.9% ≈ 91%. So 91% is in free base form (extractable) and 9% is ionised (stays in aqueous). For >99% free base: need pH > pKa + 2 = 11. pH 10 gives good but not quantitative extraction.

29. The 'hook effect' is most problematic in which type of immunoassay?

A. Classical competitive RIA

B. Equilibrium dialysis RIA

C. Two-site sandwich IRMA ✓

D. Double antibody RIA

Explanation: Hook effect occurs primarily in TWO-SITE SANDWICH IRMA (and ELISA) at very HIGH antigen concentrations. When antigen is extremely concentrated: (1) It separately saturates the solid-phase CAPTURE antibody; (2) It separately saturates the free DETECTION antibody; (3) Few sandwiches form because all Ab sites are occupied separately. Classical RIA (competition format) is not prone to hook effect because it uses ONE antibody — at high Ag, the standard curve simply reaches its bottom plateau.*

30. For multiple LLE extractions using same total solvent volume, which approach gives better recovery?

A. One single large-volume extraction

B. Multiple smaller-volume extractions ✓

C. Both are identical in recovery

D. Depends on the solvent density

Explanation: MULTIPLE SMALLER EXTRACTIONS give better recovery. Proof: For Kd = 1, Vaq = 10 mL. One 30 mL extraction: %E = 1×30/(1×30+10) = 75%. Three 10 mL extractions: %E = 1 − (1/(1+1))³ = 1 − (0.5)³ = 1 − 0.125 = 87.5%. Mathematical principle: Each successive extraction equilibrates fresh solvent with remaining aqueous analyte, pushing equilibrium toward organic. More iterations = higher cumulative recovery.

31. The SPE format that allows processing 96 samples simultaneously is:

A. Single syringe barrel cartridge

B. Disk cartridge

C. 96-well plate format ✓

D. Online column-switching SPE

Explanation: 96-well SPE PLATES allow simultaneous processing of 96 samples in a microplate format matching standard lab automation. Compatible with robotic liquid handlers (Hamilton, Tecan), vacuum manifolds, and high-throughput bioanalysis workflows. Used in drug discovery for high-throughput PK screening and forensic toxicology confirmatory testing. Dramatically increases throughput compared to individual cartridges.

32. When conditioning a C18 SPE cartridge, the solvents applied in correct order are:

A. Water → Methanol

B. Methanol → Water ✓

C. Acetonitrile → Buffer → Water

D. Water only

Explanation: Correct C18 conditioning order: METHANOL FIRST (3 mL) → WATER SECOND (3 mL). Methanol solvates and wets the hydrophobic C18 chains, opening and activating them. Water then displaces methanol and equilibrates the sorbent to aqueous conditions matching the sample. Reversed order (water first) cannot wet the collapsed C18 chains — conditioning fails — analyte passes through on loading (lost recovery).

33. Which of these is NOT a limitation of RIA?

A. Radiation hazard

B. Short shelf life of ^{125}I-labelled tracer

C. Insensitive — cannot detect nanogram quantities ✓

D. Requires radioactive waste disposal

Explanation: RIA is EXTREMELY SENSITIVE (picogram to femtomolar range) — insensitivity is NOT a limitation. This was its revolutionary advantage! Limitations OF RIA: (1) Radiation hazard (A correct); (2) Short tracer shelf life due to ^{125}I 60-day half-life (B correct); (3) Radioactive waste disposal cost and complexity (D correct); (4) Specialised infrastructure; (5) NSB; (6) Hook effect (IRMA); (7) Cross-reactivity.

34. For an LLE with Kd = 5, Vorg = 5 mL, Vaq = 10 mL, calculate % extraction in a single step.

A. 71.4% ✓

B. 50%

C. 83.3%

D. 55%

Explanation: % Extraction = Kd×Vorg / (Kd×Vorg + Vaq) × 100 = 5×5 / (5×5 + 10) × 100 = 25 / (25+10) × 100 = 25/35 × 100 = 71.4%. Note: If Vorg were equal to Vaq (both 10 mL): %E = 5×10/(5×10+10) = 50/60 = 83.3%. Increasing Vorg relative to Vaq always improves single-extraction efficiency.

35. The primary purpose of using polyclonal antibody in classical RIA (vs monoclonal) is:

A. Polyclonal Ab has lower cost and recognises multiple epitopes — adequate for competitive RIA ✓

B. Polyclonal Ab is more specific than monoclonal

C. Monoclonal Ab cannot be used in RIA under any circumstances

D. Polyclonal Ab has longer shelf life

Explanation: In classical COMPETITIVE RIA, a polyclonal antiserum from immunised animals is typically used because: (1) LOWER COST than monoclonal Ab production; (2) Recognises MULTIPLE EPITOPES — still specific enough for RIA because the competition format inherently requires selectivity; (3) High affinity usually achieved in good antisera. Monoclonal Ab is preferred for IRMA (two-site sandwich) where single-epitope recognition is important. Both can be used in RIA, but polyclonal is classical.

36. An RIA with the following data: B_0=8000 CPM, NSB=400 CPM, standard at 10 ng/mL gives 3200 CPM. At this concentration, %B/B_0 equals:

A. 36.8%

B. 37.3% ✓

C. 40.0%

D. 35.9%

Explanation: %B/B_0 = (3200 − 400) / (8000 − 400) × 100 = 2800/7600 × 100 = 36.84% ≈ 37.3% (nearest option B). Always subtract NSB from both numerator and denominator. This concentration (10 ng/mL) represents ~37% binding — above the ED50 point — on the lower portion of the usable standard curve range.

37. A laboratory SPE method for basic drugs uses MCX cartridge. The elution sequence is: (1) Water wash, (2) Methanol wash, (3) Methanol + 5% ammonia solution. Why is the final step with ammonia necessary?

A. Ammonia lowers pH to protonate amine drugs for better elution

B. Ammonia provides basic conditions to deprotonate amine drugs — disrupts ionic interaction with sulfonic acid groups ✓

C. Ammonia washes off remaining proteins

D. Ammonia is needed to evaporate the solvent faster

Explanation: MCX retains basic drugs by TWO mechanisms: hydrophobic (C8) + ionic (sulfonic acid cation exchanger binds R-NH_3^+). Methanol alone (step 2) disrupts HYDROPHOBIC interaction but NOT ionic — drug still bound ionically. Ammonia in methanol RAISES pH → deprotonates amine (R-NH_3^+ → R-NH_2 = neutral) → disrupts ionic bond with –SO_3H → drug released. Without ammonia, basic drugs would remain on MCX despite methanol elution.

38. A forensic laboratory analyses urine for 30 classes of drugs using one SPE method. The best sorbent choice is:

A. C18 — retains all drugs

B. SCX — retains all ionic drugs

C. HLB or MCX — broad spectrum retention with clean elution ✓

D. Silica — retains polar drugs only

Explanation: For broad-spectrum MULTI-DRUG urine testing (30 drug classes including acids, bases, neutrals): HLB (retains polar + non-polar; water-wettable; excellent for acidic + neutral drugs) and MCX (retains basic drugs by dual mechanism). In practice: MCX is the gold standard for forensic urine drug testing because most drugs of abuse are basic amines (amphetamines, opiates, cocaine metabolites, benzodiazepines). C18 fails for polar metabolites; SCX only for bases; silica for polar only.

39. In RIA, if a 'wrong' separation technique is used (e.g., suboptimal incubation time with DCC), what error occurs?

A. Increased specific binding (B_0 increases)

B. NSB decreases to zero

C. Separation is incomplete — bound and free fractions mixed → imprecise results (poor CV) ✓

D. The standard curve becomes linear instead of sigmoid

Explanation: Separation is the most critical step after equilibration in RIA. Suboptimal DCC separation (too long contact → DCC strips some Ag from Ab; too short → insufficient free Ag* adsorbed) leads to INCOMPLETE SEPARATION — both bound and free fractions contaminate each other. Result: poor precision (high %CV between replicates), non-reproducible standard curve, incorrect analyte concentrations. Critical control: DCC centrifugation timing must be exactly reproducible (typically ±30 seconds).*

40. In LLE from plasma, severe emulsion formation is most likely caused by:

A. Adding too much NaCl

B. Very high pH adjustment

C. Plasma proteins and phospholipids at the organic-aqueous interface ✓

D. Using very large volumes of organic solvent

Explanation: PLASMA PROTEINS (albumin, globulins) and PHOSPHOLIPIDS are amphiphilic molecules — they have both hydrophilic and hydrophobic regions — and CONCENTRATE at the organic-aqueous INTERFACE, stabilising the emulsion droplets and preventing phase separation. Solutions: (1) Centrifugation at higher speed/longer time; (2) Add NaCl or Na_2SO_4 (destabilises protein-lipid film); (3) Protein precipitation with acetonitrile first, then LLE; (4) Use supported liquid extraction (SLE) instead; (5) Use SPE (no liquid-liquid interface).

41. RIA can detect insulin at a concentration of 10 pg/mL. If this is converted to molar concentration (MW insulin = 5808 g/mol), approximately what molarity is this?

A. 1.7×10^{-12} M ✓

B. 1.7×10^{-9} M

C. 1.7×10^{-15} M

D. 1.7×10^{-6} M

Explanation: 10 pg/mL = 10×10^{-12} g/mL = 10^{-11} g/mL. Convert to mol/L: (10^{-11} g/mL × 1000 mL/L) / 5808 g/mol = 10^{-8} / 5808 = 1.72×10^{-12} M = 1.7×10^{-12} mol/L (picomolar range = pM). This confirms RIA can detect in the picomolar range — far below most conventional analytical techniques. This concentration is below the LLOQ of most HPLC methods (typically nM range).

42. An LLE extraction is performed twice. First extraction at pH 3 using MTBE (for acidic drug): 70% recovery. Second back-extraction with aqueous base pH 11: drug goes back into aqueous. Third extraction of that aqueous at pH 3 with fresh MTBE: another 70% recovery. Cumulative recovery is:

A. 70%

B. 91%

C. 49% ✓

D. 100%

Explanation: BACK-EXTRACTION recovery: Forward extraction = 70%. Back-extraction re-partitions drug to aqueous. Third extraction at same Kd conditions = 70% of remaining 100% (all drug now in aqueous after back-extraction) = 70%. Net cumulative recovery = 70% × 1 × 70% = 0.70 × 0.70 = 49% of original. Back-extraction sacrifices recovery for SPECIFICITY. The 49% recovery is acceptable if the cleaner extract gives much better specificity and less matrix interference in the final analysis.

43. A C18 SPE cartridge is accidentally allowed to dry between the conditioning and loading steps. What happens?

A. No effect — the cartridge works normally

B. The analyte is retained more strongly — better extraction

C. C18 chains collapse — loss of retention — analyte passes through (lost recovery) ✓

D. The sorbent pH changes

Explanation: If C18 sorbent DRIES out between conditioning and loading: the hydrophobic C18 chains COLLAPSE back to their packed conformation, losing their activated, solvated state. When the aqueous sample is loaded onto dried C18, the non-polar chains cannot interact with the non-polar analyte in the aqueous environment — the analyte simply passes through without being retained (LOST). To recover: must re-condition from methanol again. Never allow sorbent to dry between SPE steps.

44. Which of the following applications BEST demonstrates RIA's advantage of sensitivity over UV-Vis spectrophotometry?

A. Aspirin tablet content assay (mg level)

B. Measurement of plasma cortisol at 5 ng/mL in 100 µL sample ✓

C. Water purity testing for chlorine

D. Alcohol breathalyser analysis

Explanation: Plasma cortisol at 5 ng/mL in 100 µL sample: Total cortisol mass = 5 ng/mL × 0.1 mL = 0.5 ng = 0.5 × 10^{-9} g. UV-Vis spectrophotometer LOD: ~0.1–1 µg/mL = 100–1000 ng/mL — completely unable to detect 5 ng/mL without extreme preconcentration. RIA can measure 0.1–1 ng/mL cortisol reliably. Aspirin content (mg level) and chlorine (ppm) are perfectly suited for UV-Vis — no RIA sensitivity advantage needed. Breathalyser uses electrochemical detection.

45. A pharmaceutical lab is developing an LLE method for a new basic drug (pKa = 8.5). The drug tends to form emulsions with plasma. Which modification would BEST improve phase separation?

A. Increase extraction pH to 12

B. Add 20% NaCl (w/v) to the aqueous phase before adding organic solvent ✓

C. Use a longer mixing time

D. Decrease the organic solvent volume

Explanation: Adding 20% NaCl (saturated/near-saturated salt solution) provides two benefits: (1) SALTING-OUT: increases ionic strength → pushes drug into organic → higher Kd → better recovery; (2) ANTI-EMULSION: high salt concentration DESTABILISES the protein-phospholipid film at the organic-aqueous interface that causes emulsions → cleaner phase separation → clear organic layer. Also assists centrifugation by increasing aqueous phase density difference. This is a standard modification for problematic biological matrices.

46. IRMA uses radiolabelled ANTIBODY rather than antigen. This means:

A. IRMA cannot detect antigens at physiological concentrations

B. IRMA has lower sensitivity than classical RIA

C. The antibody must have at least 2 epitopes for the antigen (sandwich format requires 2 non-overlapping Ab) ✓

D. The antigen must be radioactive

Explanation: Two-site sandwich IRMA requires TWO antibodies directed at TWO DIFFERENT, non-overlapping EPITOPES of the same antigen. This is why IRMA is NOT suitable for small haptens (drugs, steroids) — they have only ONE epitope, so no sandwich can form. Proteins (TSH, PSA, AFP) with multiple epitopes are ideal for IRMA. The two antibodies must not compete with each other for the same binding site — this requires careful epitope mapping during assay development.

47. An SPE cartridge sorbent with immunoaffinity (antibody-coated) is used to extract one specific drug from plasma. Compared to C18 SPE, this method offers:

A. Broader applicability — can extract many different drugs

B. Much lower cost per extraction

C. Ultimate selectivity — extract ONLY the one drug the antibody recognises ✓

D. Faster throughput with less method development

Explanation: Immunoaffinity SPE (IA-SPE) coats the sorbent with a specific antibody against the target analyte. Selectivity is ABSOLUTE — only the antibody's specific target is retained. All other matrix components (including closely related metabolites or drug isomers) pass through. Applications: clean-up of therapeutic antibiotics (mycotoxin IA columns), very clean extract for trace analysis. Trade-offs: VERY EXPENSIVE (per use of antibody sorbent); only one analyte per cartridge; requires specific antibody for each analyte.

48. In RIA, increasing the antibody dilution (using less antibody per tube) would affect the assay by:

A. Increasing sensitivity — lower limit of detection ✓

B. Decreasing sensitivity and reproducibility

C. Having no effect on the standard curve

D. Increasing non-specific binding

Explanation: Increasing antibody DILUTION (using LESS Ab) makes the limited antibody even more limiting → more competition at lower analyte concentrations → standard curve shifts to LEFT → assay can distinguish lower concentrations. This INCREASES SENSITIVITY (lower LOD). However: too high dilution (too little Ab) → B_0 falls too low (insufficient counts for statistical precision) → poor reproducibility. Optimal: Ab dilution giving B_0 = 30–50% of total counts added — balance between sensitivity and precision.

49. Which of the following LLE solvents is MOST problematic from a laboratory safety standpoint?

A. Ethyl acetate

B. MTBE (methyl tert-butyl ether)

C. Chloroform (trichloromethane) ✓

D. n-Hexane

Explanation: CHLOROFORM is the most problematic: (1) Hepatotoxic — damages liver with chronic exposure; (2) Potential CARCINOGEN — IARC Group 2B (possibly carcinogenic to humans); (3) Metabolised to phosgene (highly toxic) in vivo; (4) Dense liquid — dense organic layer means pipetting from BOTTOM of tube (spill risk). Regulated waste

disposal. Being replaced by DCM in many labs (similar properties, also suspect). MTBE forms peroxides (explosion risk on evaporation to dryness) — requires peroxide check; ethyl acetate and hexane have routine flammability hazards only.

50. A researcher wants to measure plasma levels of a drug in picomolar (pM) concentration in a preclinical pharmacokinetic study. The drug is a peptide (MW 2500 Da). Which technique is MOST appropriate?

A. UV-Vis spectrophotometry (absorbance at 280 nm)

B. Flame photometry

C. RIA or LC-MS/MS ✓

D. IR spectroscopy

Explanation: Picomolar levels of a peptide drug requires ULTRA-SENSITIVE detection: (1) RIA: Can detect pg/mL (pM range) of peptides using ^{125}I-labelled peptide tracer and specific antibody — classic approach for PK studies. (2) LC-MS/MS: After SPE cleanup, can achieve sub-pg/mL (femtomolar) detection for peptides with suitable MRM transitions — now preferred over RIA for regulatory PK submissions (no radiation). UV-Vis: LOD ~µM range — not sensitive enough by 6 orders of magnitude. Flame photometry: only for alkali metals. IR: structural ID, not trace quantification.