Unit-1

Instrumental Methods of Analysis

B.Pharma 7 Sem Notes

Unit: 1

UV Visible spectroscopy

- **Electronic transitions**, chromophores, auxochromes, spectral shifts, solvent effect on absorption spectra, Beer and Lambert's law, Derivation and deviations.
- **Instrumentation -** Sources of radiation, wavelength selectors, sample cells, detectors- Photo tube, Photomultiplier tube, Photo voltaic cell, Silicon Photodiode.
- **Applications -** Spectrophotometric titrations, Single component and multi component analysis

Fluorimetry:

- Theory, Concepts of singlet, doublet and triplet electronic states, internal and external conversions, factors affecting fluorescence, quenching, instrumentation and applications

UV-VISIBLE SPECTROSCOPY

Introduction

UV-Visible spectroscopy is an analytical technique based on the absorption of ultraviolet (UV) and visible radiation by molecules. When electromagnetic radiation interacts with matter, molecules absorb photons of specific energies, causing electronic transitions from ground state to excited state.

The UV region spans 200–400 nm and the visible region spans 400–800 nm. This technique is widely used in pharmaceutical analysis for identification, purity testing, and quantitative determination of drugs.

Electronic Transitions

When a molecule absorbs UV-Visible radiation, electrons undergo transitions between molecular orbitals. The types of electronic transitions are:

Transition Type	Description	Energy Required	Wavelength (nm)
$\sigma \rightarrow \sigma^*$	Sigma bonding to sigma antibonding; C-C, C-H bonds	Very High	< 150 nm (vacuum UV)
$n \rightarrow \sigma^*$	Non-bonding to sigma antibonding; O, N, S, halogens	High	150–250 nm
$\pi \rightarrow \pi^*$	Pi bonding to pi antibonding; C=C, C=O, aromatic	Moderate	170–250 nm
$n \rightarrow \pi^*$	Non-bonding to pi antibonding; C=O, -N=O, C=S	Low	270–350 nm

📌 Key Point:
The most analytically useful transitions are $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ as they occur in the accessible UV-Visible region (200–800 nm). Lone pair electrons (n) and π electrons are most easily excited.

Chromophores

A chromophore is a group or atom in a molecule responsible for absorbing UV-Visible radiation and producing colour or specific absorption.

Chromophore	Group	λmax (nm)	Transition
Carbonyl (Aldehyde)	-CHO	~290	$n \rightarrow \pi^*$
Carbonyl (Ketone)	>C=O	~270	$n \rightarrow \pi^*$
Carboxyl	-COOH	~205	$n \rightarrow \pi^*$

Nitro	-NO2	~210, 330	$\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$
Azo	-N=N-	~285–400	$n \rightarrow \pi^*$
Conjugated Diene	C=C-C=C	~217	$\pi \rightarrow \pi^*$
Benzene ring	C6H6	~184, 204, 255	$\pi \rightarrow \pi^*$

Auxochromes

An auxochrome is a functional group that by itself does not absorb UV-Visible radiation but, when attached to a chromophore, shifts the absorption maximum to a longer wavelength (bathochromic shift) and increases the intensity of absorption.

Common auxochromes include:

- -OH (hydroxyl)
- -NH2 (amino)
- -NHR, -NR2 (substituted amino)
- -OR (alkoxy)
- -SH (thiol)
- -Cl, -Br (halogens)

> 📌 **Mechanism of Auxochrome Effect:**
> *Auxochromes donate lone pair electrons to the π system through resonance, thereby lowering the energy of the π* orbital. This results in a reduced energy gap, causing absorption at longer wavelength.*

Spectral Shifts

Four types of spectral shifts are observed in UV-Visible spectroscopy:

Shift	Also Called	Direction	Cause
Bathochromic	Red Shift	Towards longer λ	Auxochromes, conjugation, polar solvents ($n \rightarrow \pi^*$)
Hypsochromic	Blue Shift	Towards shorter λ	Removal of conjugation, acidic conditions, non-polar solvents
Hyperchromic	Intensity increase	Increase in ε	Addition of auxochrome to chromophore
Hypochromic	Intensity decrease	Decrease in ε	Distortion of chromophore; steric crowding

Solvent Effects on Absorption Spectra

The choice of solvent affects the position and intensity of absorption bands:

Effect on π→π* Transition:

- Polar solvents cause BATHOCHROMIC (red) shift
- This is because polar solvents stabilize the excited π* state more than the ground state through dipole-dipole interactions
- Example: Benzene in hexane vs. ethanol — slight red shift in ethanol

Effect on n→π* Transition:

- Polar solvents cause HYPSOCHROMIC (blue) shift
- Polar solvents form hydrogen bonds with lone pairs (n), lowering energy of ground state more than excited state
- Greater energy gap → shorter wavelength absorption
- Classic example: Acetone: λmax = 279 nm (hexane) → 265 nm (water)

📌 **Ideal Solvents for UV Analysis:**

Ethanol (most common), hexane, cyclohexane, 1,4-dioxane, methanol. These solvents have low absorption in UV region (cutoff < 200 nm). NEVER use benzene, toluene, or acetone as they absorb strongly in UV.

Beer-Lambert's Law

Statement:

When a beam of monochromatic radiation passes through a homogeneous solution, the rate of decrease of intensity of radiation with thickness of the absorbing medium is proportional to the intensity of the incident radiation and the concentration of the solution.

Lambert's Law	Beer's Law
When concentration is constant: $A = \log(I_0/I) \propto l$ Where l = path length (cm)	When path length is constant: $A = \log(I_0/I) \propto C$ Where C = concentration (mol/L)

Combined Beer-Lambert Law:

$$A = \varepsilon \times C \times l = \log(I_0/I)$$

Where:

- A = Absorbance (dimensionless)
- ε = Molar absorptivity or molar extinction coefficient (L mol^{-1} cm^{-1})
- C = Concentration of solution (mol/L or mol/dm^3)
- l = Path length of the cuvette (cm)
- I_0 = Intensity of incident radiation
- I = Intensity of transmitted radiation
- T = I/I_0 = Transmittance; A = -log T = 2 - log %T

Derivation of Beer-Lambert Law:

Consider a solution of absorbing species in a cell of thickness l. Let I_0 be the intensity of incident monochromatic radiation and I be the intensity of transmitted radiation.

1. According to Lambert's Law: $dI/dl = -k_1 \times I$ (where k_1 is a constant)
2. On integration: $\log(I_0/I) = k_1 \times l$ ... (i)
3. According to Beer's Law: $\log(I_0/I) = k_2 \times C$ ... (ii)
4. Combining (i) and (ii): $\log(I_0/I) = \varepsilon \times C \times l$
5. Therefore: $A = \varepsilon \times C \times l$ (Beer-Lambert's Law)

Deviations from Beer-Lambert Law:

The law is strictly obeyed only under ideal conditions. Deviations may be real (chemical) or instrumental:

A. Real (Chemical) Deviations:

- Association, dissociation or reaction of the solute with the solvent alters the absorbing species
- At high concentrations (>0.01 M), electrostatic interactions between molecules change the refractive index and absorptivity
- Formation of dimers or aggregates at higher concentrations
- Equilibrium shifts due to concentration changes (e.g., pH-dependent species)

B. Instrumental Deviations:

- Use of polychromatic radiation (non-monochromatic light source) — Beer's law assumes monochromatic light
- Stray light reaching the detector without passing through sample
- Fluorescence by the sample leading to apparent lower absorbance
- Non-linearity of the detector response at high absorbance values
- Reflection and scattering losses in cuvettes

Instrumentation — UV-Visible Spectrophotometer

A UV-Visible spectrophotometer consists of the following components arranged in sequence:

Radiation Source	→	Wavelength Selector (Monochromator)	→	Sample Cell / Cuvette	→	Detector	→	Readout / Display

A. Sources of Radiation:

Source	Spectral Range	Wavelength	Notes

Deuterium (D_2) Lamp	UV Region	160–375 nm	Most common UV source; continuous spectrum
Tungsten-Halogen Lamp	Visible Region	320–2500 nm	Used for visible analysis; also called quartz-iodine lamp
Hydrogen Lamp	UV Region	180–375 nm	Continuous UV spectrum; older type
Xenon Arc Lamp	UV-Vis-NIR	200–2000 nm	High intensity; used in fluorescence spectroscopy

B. Wavelength Selectors (Monochromators):

Wavelength selectors are devices used to isolate a narrow band of wavelengths from the polychromatic radiation of the source.

Types:

- Filters (Interference filters, Absorption filters) — simple, low resolution
- Prisms — quartz prisms for UV; glass prisms for visible; high dispersion
- Diffraction Gratings — most commonly used; consists of ruled grooves on aluminum surface; provides uniform dispersion across entire spectrum

A monochromator consists of:

- Entrance slit — controls width of beam entering
- Collimating lens/mirror — produces parallel beam
- Dispersing element (prism/grating)
- Focusing lens/mirror — focuses dispersed beam
- Exit slit — isolates desired wavelength

C. Sample Cells (Cuvettes):

Material	Wavelength Range	Application	Characteristics
Quartz (Fused Silica)	150–3000 nm	UV and Visible	Most versatile; expensive
Glass	350–2000 nm	Visible only	Cheap; absorbs UV
Plastic (Polystyrene)	380–780 nm	Visible only	Disposable; low cost

Standard path length = 1 cm. Cells must be cleaned, matched, and free of scratches or fingerprints.

D. Detectors:

1. Phototube (Vacuum Phototube):

- Consists of a photosensitive cathode and an anode sealed in an evacuated glass envelope
- Radiation strikes the photoemissive cathode, ejecting electrons (photoelectric effect)
- Electrons are attracted to the anode, producing a photocurrent proportional to the radiation intensity
- Sensitivity range: 200–650 nm
- Limitations: Low sensitivity, requires amplification

2. Photomultiplier Tube (PMT):

- Most sensitive detector for UV-Visible spectroscopy
- Consists of a photocathode, 9–16 dynodes (electron multipliers), and an anode
- Each dynode amplifies the photoelectron signal (~4–6 times per stage)
- Total amplification: 10^6 to 10^8 times
- Fast response, high sensitivity, low noise
- Used in double-beam spectrophotometers and fluorimeters

> 📌 **PMT Working:**
> *Photon → Photocathode ejects electron → Electron hits Dynode 1 (ejects 4 electrons) → Each hits Dynode 2 (4×4=16 electrons) → ... Dynode 9 → Anode current. Overall gain = 4^9 = ~260,000*

3. Photovoltaic Cell (Barrier-Layer Cell):

- Consists of a thin layer of selenium deposited on iron or aluminum
- Top surface coated with transparent silver or gold film (collector electrode)
- Radiation causes generation of electron-hole pairs at the selenium-metal junction
- Electrons flow from selenium to silver, producing a current proportional to light intensity
- Self-generating — no external voltage required
- Used in simple, portable instruments and colorimeters
- Limitations: Fatigue at high intensities, not suitable for low light levels

4. Silicon Photodiode:

- P-N junction diode made of silicon
- Incident photons with energy > bandgap energy generate electron-hole pairs
- Applied reverse bias separates carriers, producing photocurrent
- Spectral range: 190–1100 nm
- Fast response time, rugged, compact, low cost
- Used in diode array detectors (DAD) and modern HPLC detectors
- Diode Array: 1024 photodiodes arranged in a row to detect entire spectrum simultaneously

Applications of UV-Visible Spectroscopy

A. Spectrophotometric Titrations:

Spectrophotometric titration involves following the progress of a titration by measuring the absorbance of the solution at a fixed wavelength as titrant is added.

Principle:

- If either the titrant, the analyte, or the product absorbs radiation, a plot of absorbance vs. volume of titrant gives a V-shaped or L-shaped curve
- The endpoint is identified at the inflection point or intersection of the two linear portions

Types:

- Where only the analyte absorbs — absorbance decreases to zero at endpoint
- Where only the product absorbs — absorbance increases to a plateau
- Both absorb — characteristic V-shaped curve

Advantages over potentiometric titrations: Can be used in turbid solutions, colored solutions, and for very dilute analytes.

B. Single Component Analysis:

Used for determination of a single drug/substance in a sample:

1. Prepare a series of standard solutions of known concentration
2. Measure absorbance of each standard at λmax
3. Plot a calibration curve (Absorbance vs. Concentration)
4. Measure absorbance of unknown sample
5. Read off concentration from calibration curve
6. Alternatively, use: $C_2 = (A_2/A_1) \times C_1$

Examples: Assay of paracetamol (λmax = 243 nm), aspirin (λmax = 265 nm), metronidazole (λmax = 278 nm)

C. Multi-Component Analysis:

Used when two or more components with overlapping spectra are present. Based on the principle of additivity of absorbances (Beer's law applied to each component):

At Wavelength λ_1:	At Wavelength λ_2:
$A_1 = \varepsilon_{1a}C_a l + \varepsilon_{1\beta}C_\beta l$	$A_2 = \varepsilon_{2a}C_a l + \varepsilon_{2\beta}C_\beta l$

Solving the two simultaneous equations gives the concentrations of both components A (C_a) and B (C_β). Wavelengths are usually chosen at the λmax of each component.

📌 Example:

Determination of aspirin and caffeine in combined tablets. Measure absorbance at 277 nm and 272 nm, then solve simultaneous equations using absorptivity values determined from individual standards.

FLUORIMETRY

Introduction to Photoluminescence

Photoluminescence is the emission of radiation by a substance after absorbing radiation. It includes two types: fluorescence and phosphorescence.

Fluorimetry (fluorescence spectrometry) is a technique based on the measurement of fluorescence emitted by a substance upon excitation by radiation of appropriate wavelength. It is more sensitive than UV-Visible absorption spectroscopy (up to 1000 times more sensitive).

Theory of Fluorescence

Fluorescence involves the following sequence of events:

1. A molecule absorbs a photon and is promoted from the ground electronic state (S_0) to an excited electronic state (S_1, S_2, etc.)
2. The molecule undergoes rapid vibrational relaxation within the excited state (10^{-12} s)
3. Internal conversion occurs from higher excited states to the lowest vibrational level of S_1
4. The molecule emits a photon and returns to the ground state — this emission is called FLUORESCENCE
5. Fluorescence is always at a longer wavelength (lower energy) than the excitation radiation — STOKES SHIFT

Electronic States: Singlet, Doublet, and Triplet

State	Electron Configuration	Multiplicity	Spin
Singlet Ground State (S_0)	All electrons paired; lowest energy	2(0)+1 = 1	Antiparallel (↑↓)
Singlet Excited State (S_1, S_2)	Electrons still paired but one promoted to higher orbital	2(0)+1 = 1	Antiparallel (↑↓)
Doublet State	Odd number of electrons (radical species)	2(1/2)+1 = 2	One unpaired
Triplet Excited State (T_1)	Electrons unpaired — one promoted with spin flip	2(1)+1 = 3	Parallel (↑↑)

📌 Key Concept:
The triplet state (T_1) is lower in energy than the corresponding singlet state (S_1) due to exchange energy (Hund's Rule). Transition from T_1 to S_0 is spin-forbidden and slow — this delayed emission is PHOSPHORESCENCE.

Jablonski Diagram

The Jablonski diagram is the most important diagram in fluorescence spectroscopy. It illustrates all photophysical processes occurring after light absorption:

Process	Transition	Time Scale	Radiation?
Absorption	$S_0 \rightarrow S_1, S_2$	10^{-15} s	Yes (excitation)
Vibrational Relaxation	$S_1^* \rightarrow S_1$ (v=0)	10^{-12} s	No (heat)
Internal Conversion (IC)	$S_2 \rightarrow S_1$	10^{-11}–10^{-9} s	No (heat)
Fluorescence	$S_1 \rightarrow S_0$	10^{-9}–10^{-7} s	Yes (emission)
Intersystem Crossing (ISC)	$S_1 \rightarrow T_1$	10^{-10}–10^{-8} s	No (forbidden)
Phosphorescence	$T_1 \rightarrow S_0$	10^{-3}–10^{2} s	Yes (delayed)
External Conversion	S_1 or $T_1 \rightarrow S_0$	Variable	No (collisions)

Internal and External Conversions

Internal Conversion (IC):

- Radiationless (non-radiative) transition between electronic states of the SAME multiplicity (singlet to singlet or triplet to triplet)
- Occurs when vibrational levels of the upper electronic state overlap with those of the lower state
- Very fast process: 10^{-11} to 10^{-9} seconds
- No photon is emitted — energy is lost as heat/vibrational energy
- Also called internal quenching

External Conversion:

- Also called collisional quenching or intermolecular energy transfer
- Excited molecule collides with solvent molecules or other species, transferring energy
- Results in deactivation of excited state without emission
- Increased by: higher temperature, high viscosity medium, presence of heavy atoms, concentration quenching

Intersystem Crossing (ISC):

- Radiationless transition between states of DIFFERENT multiplicity ($S_1 \rightarrow T_1$)
- Involves spin inversion — spin-forbidden but can occur through spin-orbit coupling
- Promoted by heavy atoms (I, Br) due to enhanced spin-orbit coupling
- Once in T_1, molecule may fluoresce rarely but can phosphoresce

Factors Affecting Fluorescence

A. Structural Factors:

- Rigid planar structure increases fluorescence (e.g., aromatic rings, polycyclic aromatics)
- Conjugation increases fluorescence intensity
- Low-lying n→π* states tend to decrease fluorescence (ISC competes)
- Substituent effects: electron-donating groups (-OH, -NH_2, -OR) enhance fluorescence; electron-withdrawing groups (-NO_2, -COOH) reduce fluorescence

B. Environmental Factors:

- Temperature: Increasing temperature decreases fluorescence (increased collisional quenching)
- Solvent viscosity: Higher viscosity reduces external conversion, increases fluorescence
- pH: pH affects ionization state of functional groups — changes fluorescence intensity
- Solvent polarity: Polar solvents generally reduce fluorescence of n→π* states
- Oxygen: Dissolved oxygen quenches fluorescence (paramagnetic quenching)

C. Concentration:

- At low concentrations: fluorescence is directly proportional to concentration ($F = k \times I_0 \times \varepsilon \times C \times l$)
- At high concentrations: concentration quenching and inner filter effect cause deviation from linearity

Quenching of Fluorescence

Quenching is any process that decreases the fluorescence intensity of a substance.

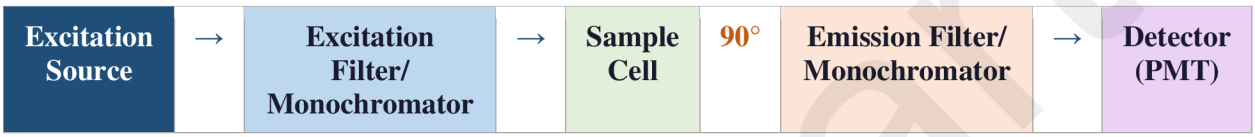
Type of Quenching	Mechanism	Examples/Notes
Static Quenching	Quencher forms non-fluorescent complex with fluorophore in ground state	Quinine-chloride complex; reversible with temperature increase
Dynamic (Collisional) Quenching	Quencher collides with excited fluorophore, deactivating it radiationlessly (Stern-Volmer mechanism)	O_2, I^-, acrylamide; follows Stern-Volmer equation: $F_0/F = 1 + kq\ \tau_0\ [Q]$
Self-Quenching (Concentration Quenching)	Fluorophore molecules interact with each other at high concentrations	Occurs above optimal concentration; non-linear calibration
Inner Filter Effect	Excitation radiation absorbed by the sample before reaching the fluorophore; or emitted light re-absorbed	Occurs at high absorbance values (A > 0.1); reduces apparent fluorescence

Heavy Atom Quenching	Heavy atoms (I, Br, Cs) enhance spin-orbit coupling, promoting ISC to triplet state	External heavy atom effect; reduces fluorescence quantum yield

📌 Stern-Volmer Equation:
$F_0/F = 1 + Ksv[Q]$, where F_0 = fluorescence without quencher, F = fluorescence with quencher, Ksv = Stern-Volmer quenching constant, [Q] = quencher concentration. A linear plot of F_0/F vs [Q] indicates dynamic quenching.

Instrumentation — Fluorimeter / Fluorescence Spectrophotometer

The basic components of a fluorimeter are:



NOTE: The detector is placed at 90° to the excitation beam to avoid interference from the excitation radiation.

Components:

- Excitation Source: Mercury arc lamp (line spectrum), Xenon arc lamp (300–700 nm continuous), High-pressure mercury lamp, Laser sources
- Excitation Monochromator: Selects wavelength for exciting the sample
- Sample Cell: Usually quartz fluorescence cuvette with 4 polished optical faces
- Emission Monochromator: Selects wavelength of emitted fluorescence for measurement
- Detector: Photomultiplier tube (PMT) — placed at 90° to the excitation beam
- Readout: Recorder, computer display

Applications of Fluorimetry

1. Pharmaceutical Analysis:

- Determination of quinine in tonic water (λex = 350 nm, λem = 450 nm)
- Assay of riboflavin (Vitamin B_2) — highly fluorescent (λex = 450, λem = 525 nm)
- Determination of thiamine, niacinamide, folic acid
- Assay of tetracyclines using metal chelation-induced fluorescence
- Determination of reserpine, chlorpromazine, and phenothiazines

2. Clinical and Biochemical Analysis:

- Determination of catecholamines (adrenaline, noradrenaline) in body fluids
- Serum albumin quantification using fluorescent dyes (fluorescamine)
- DNA quantification using intercalating dyes (ethidium bromide)
- Enzyme activity assays (fluorogenic substrates)

3. Environmental Analysis:

- Detection of polycyclic aromatic hydrocarbons (PAHs) in environmental samples
- Determination of pesticides and mycotoxins (aflatoxins are highly fluorescent)
- Water quality monitoring for petroleum products

4. Advantages of Fluorimetry:

- Sensitivity: 10–1000 times more sensitive than UV-Vis absorption (can detect ppb levels)
- Selectivity: Two wavelength parameters (excitation and emission) provide greater specificity
- Non-destructive technique
- Can be used for complex biological matrices without extensive sample cleanup

5. Limitations:

- Limited to fluorescent compounds only (~15% of organic molecules are naturally fluorescent)
- Quenching effects can cause errors
- Inner filter effect at high concentrations
- More expensive instrumentation than UV-Vis spectrophotometer