2. Fluorescence spectrophotometry

Introduction

- Fluorimetry is an analytical technique that relies on the emission of electromagnetic energy by molecules
- After the absorption of UV-Vis light, the excited molecular species are extremely short lived and deactivation occurs due to:
  - Cleavage of chemical bonds, initiating photochemical reactions
  - Re emission as light(luminescence)
- molecules, with a chromophore and a rigid structure, can be excited by UV/visible radiation, and will then emit the radiation absorbed at a longer wavelength
- The radiation emitted can then be measured

Deactivation: is a process by which an excited molecule returns to the ground state

Fluorescence

- Re –emission of light by molecules after absorption in the UV or visible region
- In fluorescence, the lifetime of the molecule in the excited singlet state is 10^{-6} to 10^{-9} sec
- Occurs in cyclic rigid compounds containing conjugated bonds and lone pair electrons

- Each molecule contains a series of closely spaced energy levels
- Absorption of a quantum of light energy by a molecule causes the transition of an electron from the singlet ground state to one of the number of vibrational levels of its first singlet state

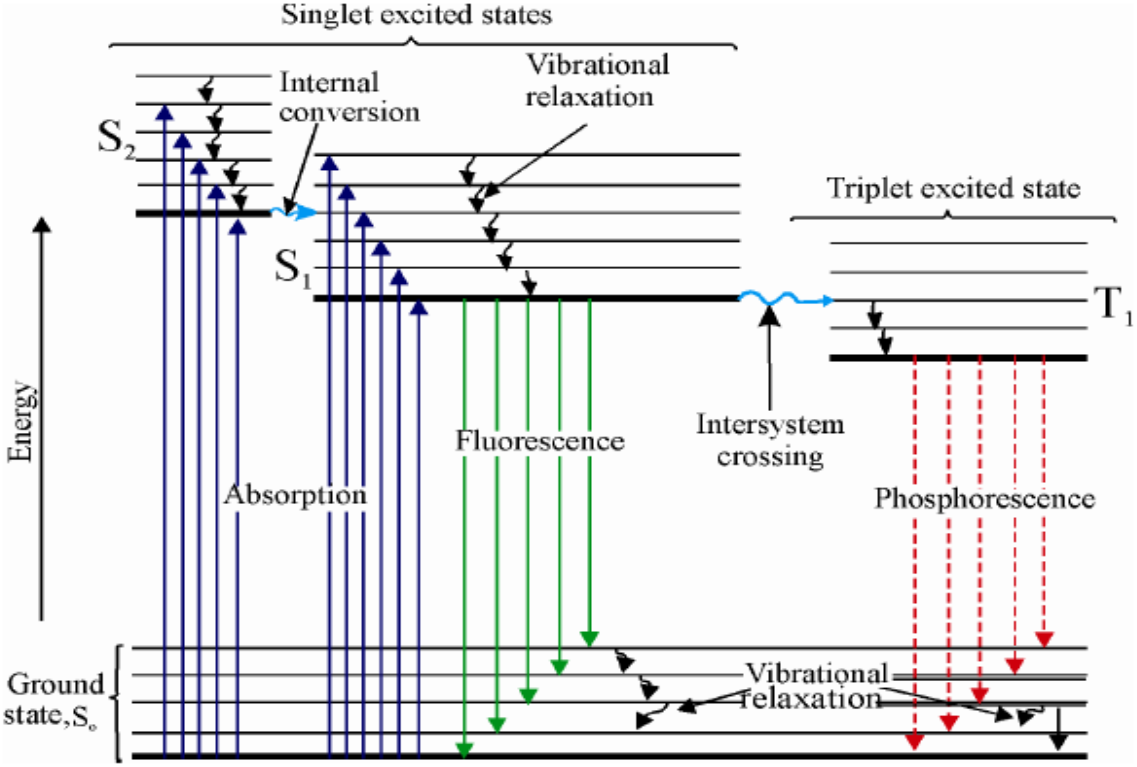


Fig. 5.1: The Jablonski diagram showing the phenomena of fluorescence and

Terms from energy level diagram

Vibrational relaxation:

- A form of radiationless relaxation and analyte moves from a higher vibrational energy level to a lower level
- Collisions of excited state analyte molecules with other molecules
- loss of excess vibrational energy and relaxation to lower vibrational levels
- Vibrational relaxation prohibit fluorescence
- The energy emitted is of lower energy than the energy absorbed
  - the excited electron moves to the lowest energy vibrational state in the excited state before returning to the ground state
- Thus fluorescence emission is typically shifted towards a longer wavelength

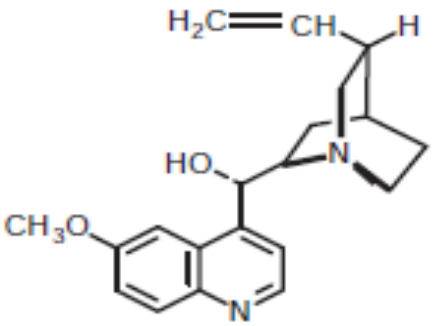
Fluorescence……..

- The light that is emitted by the sample is always of longer wavelength (i.e. lower energy) than the light absorbed by the molecule. This is known as Stokes' law

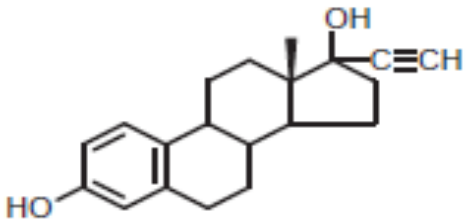
Order of λ_{max}

- λ (absorption) < λ (fluorescence)
- Fluorescence is observed at slightly higher wavelengths than the radiation used to excite the molecule

- Generally, fluorescence is associated with an extended chromophore/auxochrome system and a rigid structure
- Quinine
  - is an example of a strongly fluorescent molecule
  - has extended chromophore and rigid structure
  - An acidic solution of quinine displays a deep blue fluorescence
- Ethinylestradiol: the presence of aromatic ring, a phenolic hydroxyl group in combination with a rigid ring structure makes it fluorescent



Quinine



Ethinylestradiol

Fluorescence intensity

- All absorbed photons may not be emitted
- ϕ_f = no of photons emitted/ no of photons absorbed

  = I_f / I_{ab} , where ϕ_f - quantum yield of fluorescence

Quantum yield of fluorescence is less than unity

Quantum yield: The fraction of absorbed photons that produce a desired event, such as fluorescence or phosphorescence

$F = 2.3\ \phi_f\ I_0\ A = 2.3\ I_0\ abc\ \phi_f$

$F = Kc$

F = relative intensity

Φ = fluorescence efficiency (less than one)

Io = initial excitation intensity

a = Absorptivity

b = The path length

c = the concentration in mol/L

Fluorescence.....

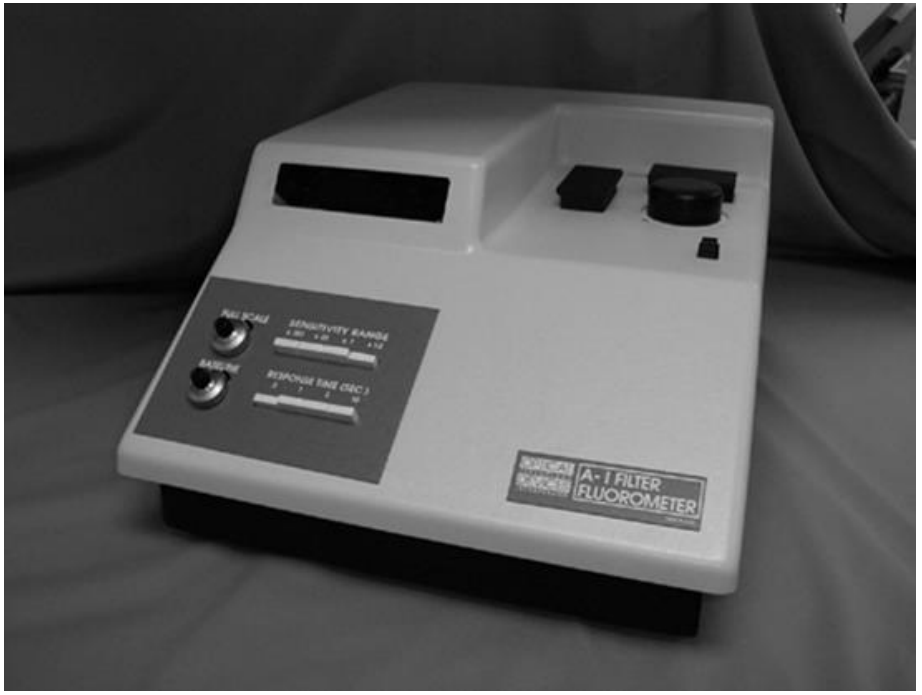
- at low concentrations fluorescence intensity is proportional to concentration
- Fluorescence intensity F is also proportional to
  - quantum yield of fluorescence
  - intensity of the excitation radiation
  - molar absorptivity of the compound
  - path length and the concentration of the compound

Instrumentation

Light sources

- Spectroflurimeter, require a high-energy light source (usually a xenon lamp) to deliver the energy required to excite the molecule.
- Source must produce high optical power(i.e. a large number of photons per unit time) $(F \propto I_0)$
- an intense source is required
- The sources used in most commercial fluorimeters is the mercury and xenon arc lamp

Instrumentation:



Fluorometer

Light source

Mercury Arc Lamp:

- Produce intense line spectrum above 350nm

Xenon Arc Lamp:

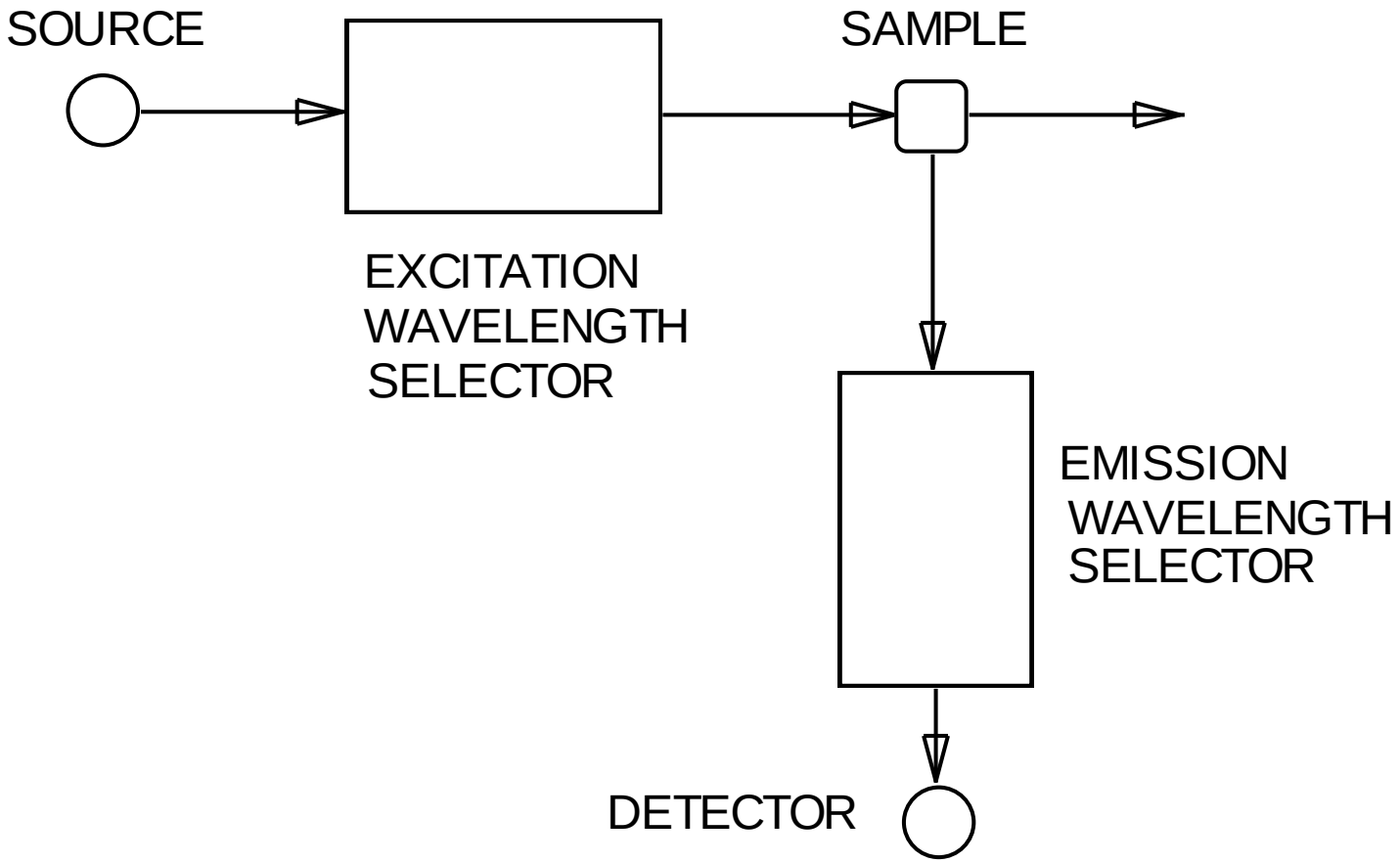
- emit an intense continuum of light in the whole UV region and in parts of the visible region
- Spectrum is continuous in the range between 250-600nm

Tungsten Lamp

- Intensity of the lamp is low
- If excitation is done in the visible region this lamp is used
- It does not offer UV radiation

A spectrofluorimeter requires two monochromators

- Two wavelength selectors are required
- Excitation monochromators:- isolates only the radiation which is absorbed by the molecule
- Emission monochromators:- isolates only the radiation emitted by the molecule
- Fluorescence is emitted in all directions but is normally measured at a 90 angle to the excitation radiation (to minimise interference from radiation used to excite the fluorescence)
- the detector is usually aligned at 90 to the source to minimize detection of light directly from the light source



Basic components of Spectrofluorometers include:

- an excitation source, an excitation monochromator, a cuvet, an emission monochromator, a detector

Cuvet:

- Generally quartz cells are used
- Path length is usually 1cm

Detectors

- Photomultiplier tube is used in most fluorescence spectrophotometers

Factors affecting fluorescence intensity

- Both molecular structure and chemical environment influences fluorescence

Temperature and viscosity:

- A rise in temperature decreases quantum efficiency of fluorescence
  - increase in the number of collisions between molecules increases the probability for deactivation by vibrational relaxation
- Increase in viscosity increases fluorescent intensity

Effect of other solutes:

- The solutes containing the halogens or heavy atoms decrease the fluorescence
- Heavy atoms decrease fluorescence by colliding with excited molecules so that their energy is dissipated
  - e.g. chloride, iodine or bromide ions in solution cause collisional quenching

Structure:

- fluorescence is favored in molecules that possesses rigid structures
- Lack of rigidity in a molecule probably causes and enhances internal conversion

Dissolved Oxygen:

- The presence of oxygen molecule decreases the fluorescence intensity
- The presence of oxygen may interfere by direct oxidation of the fluorescent substances to non fluorescent

Concentration

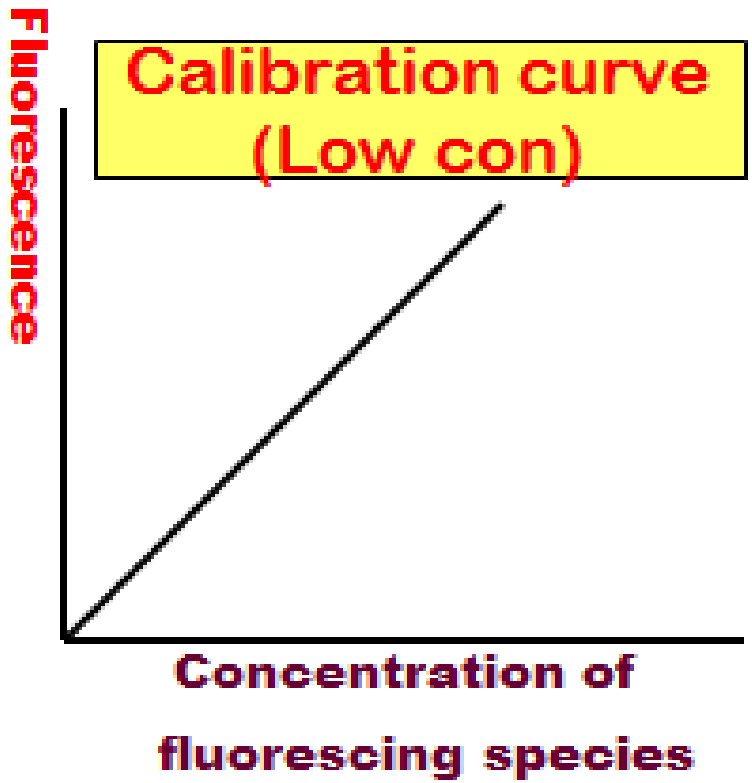
- Concentration is proportional to the emitted light
- At the maximum concentration, fluorescence peaks and may decrease

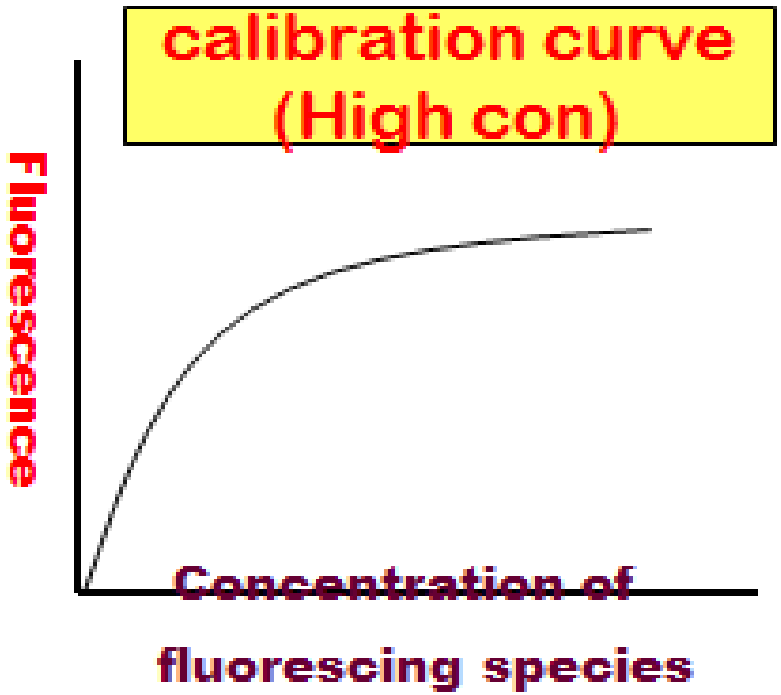
Quenching

- There are three types of quenching:
  - Self quenching
  - Chemical quenching
  - Static quenching

Self-quenching:

- observed when solutions of high concentrations do not show a proportional increase in fluorescence intensity as shown at low concentrations
- It is also called as concentration quenching
- Self-quenching is seen at high sample concentrations
- the deviation from a linear relationship b/n concentration and fluorescence
- A plot of intensity of light emitted versus concentration should be linear (obeying the Beer–Lambert law)
- If the linearity of the graph falls off at high concentration, self-quenching should be suspected





Deviations at higher concentrations can be attributed to self-quenching or self-absorption.

Static quenching:

- This occurs due to complex formation
  - Example: The riboflavin fluorescence intensity is decreased by the complex formation with the caffeine

Chemical quenching:

- decrease in fluorescence intensity due to the factors like change in pH, presence of oxygen, halides & heavy metals
- pH- aniline at pH 5-13 gives fluorescence but at pH <5 &>13 it does not exhibit fluorescence
- halides like chloride, bromide, iodide & electron withdrawing groups like NO2, COOH etc. leads to quenching
- Heavy metals leads to quenching, because of collisions of triplet ground state
- The most common quenching agents encountered in pharmaceutical analysis are halide ions (Cl, Br, I)

Advantages of fluorescence spectroscopy

1. Highly sensitive

- Fluorimetry is approximately 100 times more sensitive than UV spectroscopy
- is ideal for the analysis of very small amounts of potent drugs
- Examples : Digoxin Tablets BP and the contraceptive agent ethinylestradiol, which is present at levels of only 30 µg per tablet

2. Highly specific to the compounds

- Most of the compounds are non-fluorescent compounds
- fluorimetry is more specific than ultraviolet spectroscopy
- allows drugs to be assayed in the presence of other compounds that would interfere in an ultraviolet assay

Limitations of fluorescence spectroscopy

- The technique only applies to a limited number of molecules
  - All compounds are not fluorescent
- Fluorescence is subject to interference by heavy metals in solution, halides and is affected by temperature, pH and presence of oxygen

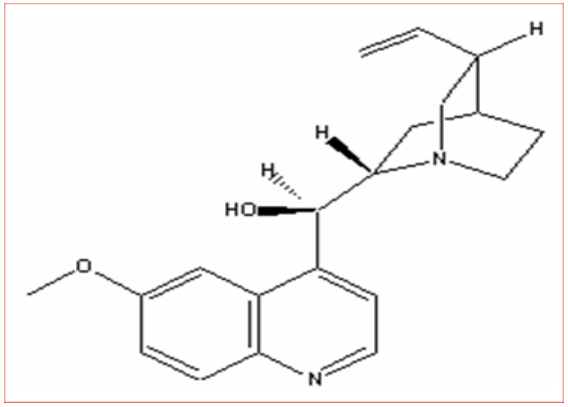
Applications of fluorescence spectrophotometry in pharmaceutical analysis

1. Determination of fluorescent drugs in low-dose formulations in the presence of nonfluorescent excipients

a. Direct method

- Uses the native fluorescence property of drugs
- Drugs should contain aromatic and conjugated aliphatic systems
- quinine can be detected at levels below 1 ppb.

Quinine



Used in the determination of the following drugs

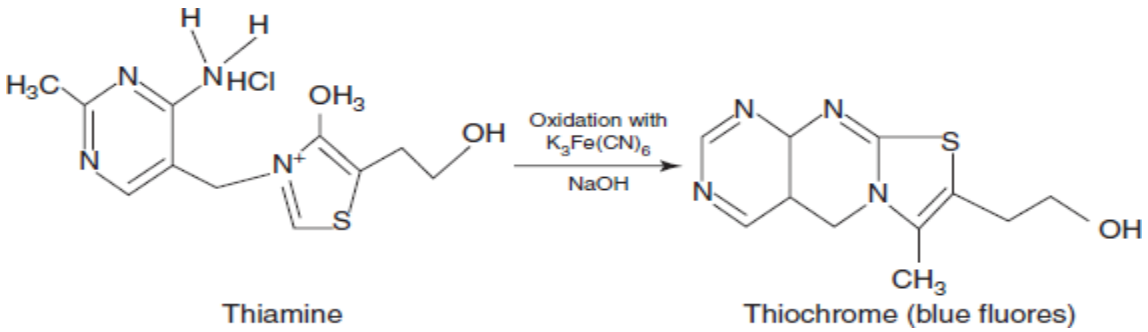
- Adrenaline
- Chloroquine
- Chlorpromazine
- Ergometrine
- Ethinylestradiol
- Folic acid
- Noradrenaline
- Phenobarbitone
- Dopamine
- Procaine
- procainamide
- Vitamins (vitamin A, vitamin B2, B6, B12 and vitamin E)
- Quinine

Determination of ethinylestradiol in tablets

- The BP utilises a fluorescence assay to determine ethinylestradiol in tablets.
- The tablets contain low dosages of the drug, so interference by excipients is likely to cause problems in UV/visible spectrophotometric measurements
- The sample is measured using an excitation wavelength of 280 nm and measuring the emission at 320 nm

b. Indirect methods

- For weakly fluorescence or non fluorescence drugs
- Accomplished by derivatization
  - Thiamine hydrochloride tablets oxidized to highly fluorescent thiochrome



  - Hydrocortisone when treated with 75% v/v H_2SO_4 in ethanol show fluorescence

2. In carrying out limit tests where the impurity is fluorescent

3. Fluorescence can be used as a detection method in liquid Chromatography (HPLC)