Pharmaceutical analysis II (phar 3122)

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1. Ultraviolet- Visible spectrophotometry

Outline

- Introduction
- Factors governing absorption of radiation in the UV/Visible region
- Instrumentation
- Qualitative & Quantitative spectrophotometry (Beer Lambert's law)
- Analysis of binary mixtures
- Derivative & Differential Spectrophotometry
- Colorimetry
- Applications in pharmaceutical analysis

After completing this course, students will be able to explain

- Principles of UV- Vis spectrophotometry
- the instrumentation of UV- Vis spectrophotometry
- their use in pharmaceutical analysis
- carry out analysis for different pharmaceuticals
- interpret and report data obtained from the analysis

SPECTROSCOPY

Introduction

- Spectroscopy is one of the valuable technique used to study atomic and molecular structure
- Spectroscopy is the branch of science that involves the study of interaction of electromagnetic radiations with atoms or molecules
- the consequence of this interaction is energy gets absorbed or emitted by atoms or molecules
- It is of two types
  - Emission spectroscopy
  - Absorption spectroscopy

Introduction……..

Emission spectroscopy

- It is the measurement of emitted light
- Example: Flame photometry, Fluorimetry

Absorption spectroscopy

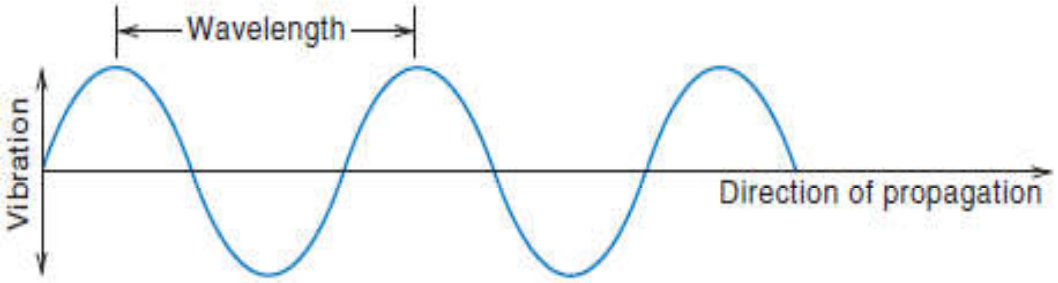
- It is the measurement of energy absorbed by an excited atom or molecule
- Example: IR, UV/VISIBLE, NMR
- spectroscopy can be can also be classified as :

  1. Atomic spectroscopy: deals with the interaction of EMR with atoms
  2. Molecular spectroscopy: deals with the interaction of EMR with molecules

Spectroscopy……..

Electromagnetic Radiation

- EMR consist of discrete packages of energy
- has wave like properties



Electromagnetic waves are usually described in terms of

1. wavelength (λ)
   - the distance between two successive peaks
   - The symbol for wavelength is (λ) the Greek letter ‘lambda’.
   - the unit for wave length is nm

Introduction

2. wave number

- the number of waves per unit of length
- is the reciprocal of the wavelength in centimeters
- is usually confined to infrared spectroscopy

3. frequency (v)

- the number of waves per second
- the unit for frequency is Hertz (Hz)

Introduction……

- The relationship between wavelength & frequency can be written as:

  c = ν λ, where c=*3* X *10^{8} m s^{-1} , where* λ= wavelength, ν= frequency

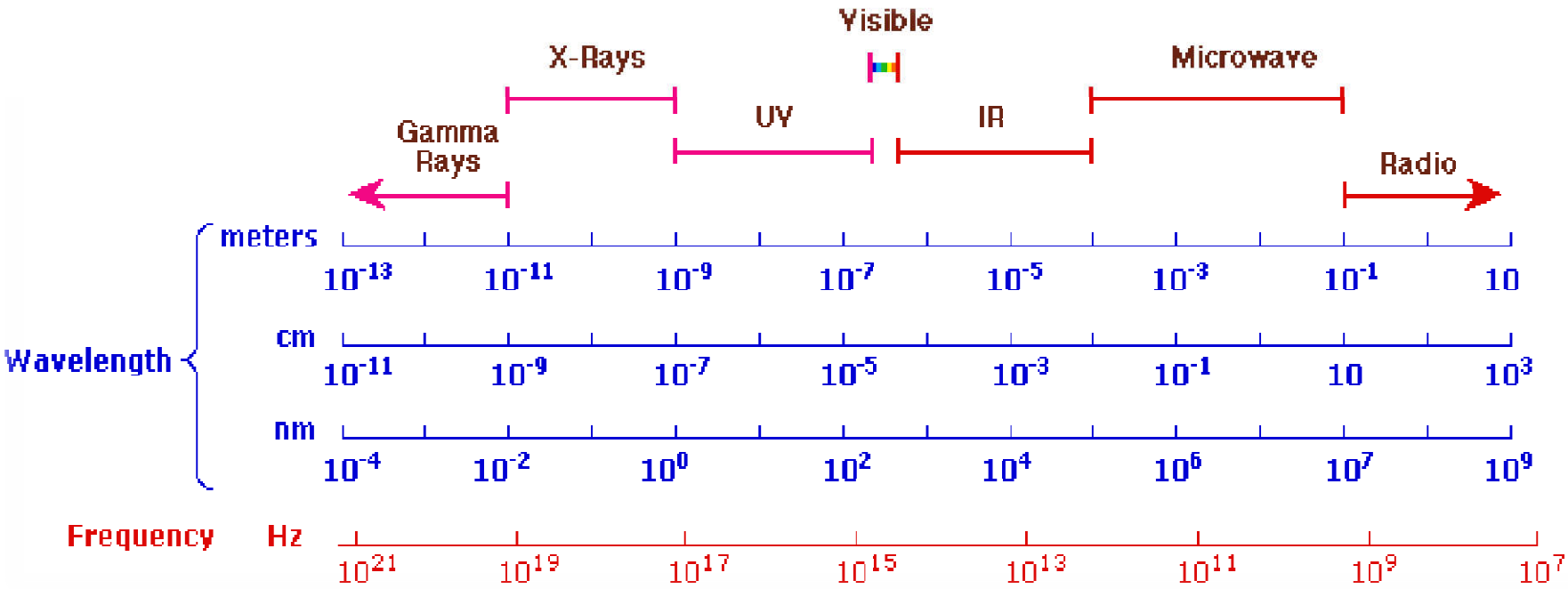
- Electromagnetic radiation possesses a certain amount of energy
- **E = h ν = h c / λ** where, h is Planck's constant (*6.63 x 10^{-34} Js*)
- The energy of EMR is directly proportional to frequency and inversely proportional to wave length

recall: $\nu = \frac{c}{\lambda}$ and $\lambda = \frac{c}{\nu}$

$\bar{\nu} = \frac{1}{\lambda}$ $\nu = c\bar{\nu}$

Electromagnetic spectrum

- The arrangement of all types of EMR in order of their increasing wavelength or decreasing frequency is known as electromagnetic spectrum
- Radio waves have low energies, while gamma rays and X-rays are high-energy, short-wavelength forms



1. Ultraviolet- Visible spectrophotometry

1.1. Introduction

- UV radiation is electromagnetic radiation in the wavelength region 200–400 nm
- This spectral range is used for UV spectrophotometry
- Visible wavelength cover a range from approximately 400 to 800 nm
- The visible spectrum
  - constitutes a small part of the total electromagnetic spectrum
  - electromagnetic radiation that can be perceived by the human eye
  - The longest visible wave length- red
  - The shortest- violet

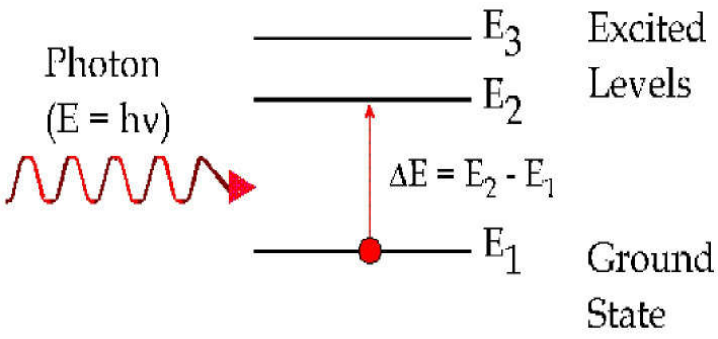
Visible spectrum…….

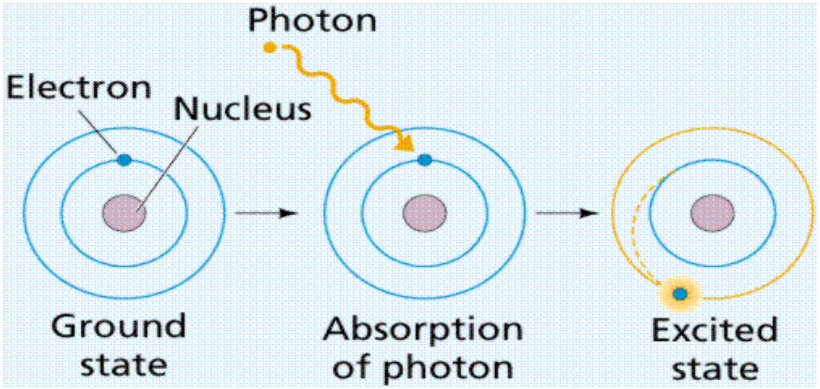
- Other common colors of the spectrum, in order of decreasing wave length, may be remembered by: **ROY G BIV**

Violet	400 - 420 nm	Yellow	570 - 585 nm
Indigo	420 - 440 nm	Orange	585 - 620 nm
Blue	440 - 490 nm	Red	620 - 780 nm
Green	490 - 570 nm		

1.2. Principles of UV -Vis Spectroscopy

- The absorption of energy by molecules forms the basis of molecular UV -Vis absorption spectroscopy
- The absorption of UV/visible radiation occurs through the excitation of electrons within the molecular structure to a higher energy state;
- Absorption of energy results in electronic transition of a molecule
- electrons are promoted from ground state to higher electronic states





1.3. Factors governing absorption of radiation in the UV/visible region

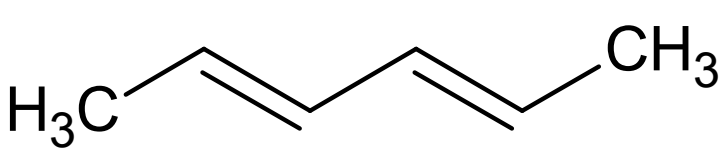
- There are various cardinal factors that govern measurement of absorption of radiant energy, namely:
  - Wave length
  - Chromophores
  - Presence of auxochrome
  - Solvent
  - PH of the solution
- The absorbance readings should preferably be at the wavelength where the analytes have their absorption maxima
- Quantitative measurements should be made at λ_{max} for maximum sensitivity and to achieve the best possible linear calibration curves

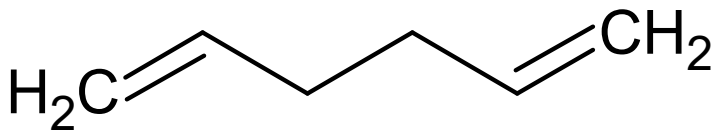
a. Chromophores

- part of the molecule that is responsible for the absorption of light
- consists of a region of double or triple bonds
- It is unsaturated group which is responsible for electronic absorption.
- E.g. N = N, N=O, C=O, C=N, C≡N, C=C, C=S
- The most common Chromophore found in drug molecules is a benzene ring
- As conjugation increases, absorption maxima shifts to higher wave length and becomes more intense

e.g. 1,5 - hexadiene has λ_{max} = 178 nm

2,4 - hexadiene has λ_{max} = 227 nm



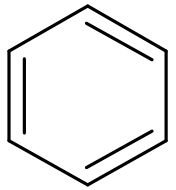


b. Auxochromes:

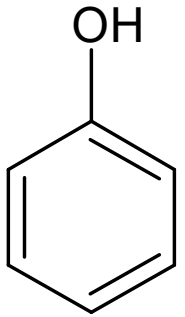
- a saturated functional group that does not absorb radiation in UV region but when attached to a chromophore alters both the wavelength & intensity of absorption
- Examples, -OH , OCH_3 -NH2 , -SH, Cl, Br and I
- have one or more non-bonding pair of electrons
- inters into resonance interaction with the chromophore
  - thus increase the extent of conjugation, and shift the absorption maximum to longer wavelength
- The absorption spectrum of a drug molecule is due to the particular combination of auxochromes and chromophores present in its structure.

e.g. Auxochrome

e.g. Benzene λ_{max} = 255 nm



Phenol λ_{max} = 270 nm



An auxochrome group

- possess lone pairs of non-bonded electrons that can interact with the π electrons of the chromophore
- allow light of longer wavelength to be absorbed

c. pH of the solution

- pH can change the nature of the substituent group
- Deprotonation of oxygen gives more available n-pairs, lowering transition energy
- protonation of nitrogen eliminates the n-pair, raising transition energy

d. Solvent effect

A solvent for UV-Vis spectroscopy must be:

- Transparent in the region of the spectrum where the solute absorbs
- Solvents can interact with molecules and may alter their absorbance
- Absorption bands are relatively sharper when measured in solvents of low dipole moment
- The nonbonding electrons in compounds usually interact with polar solvents and hence shifts to shorter wavelengths

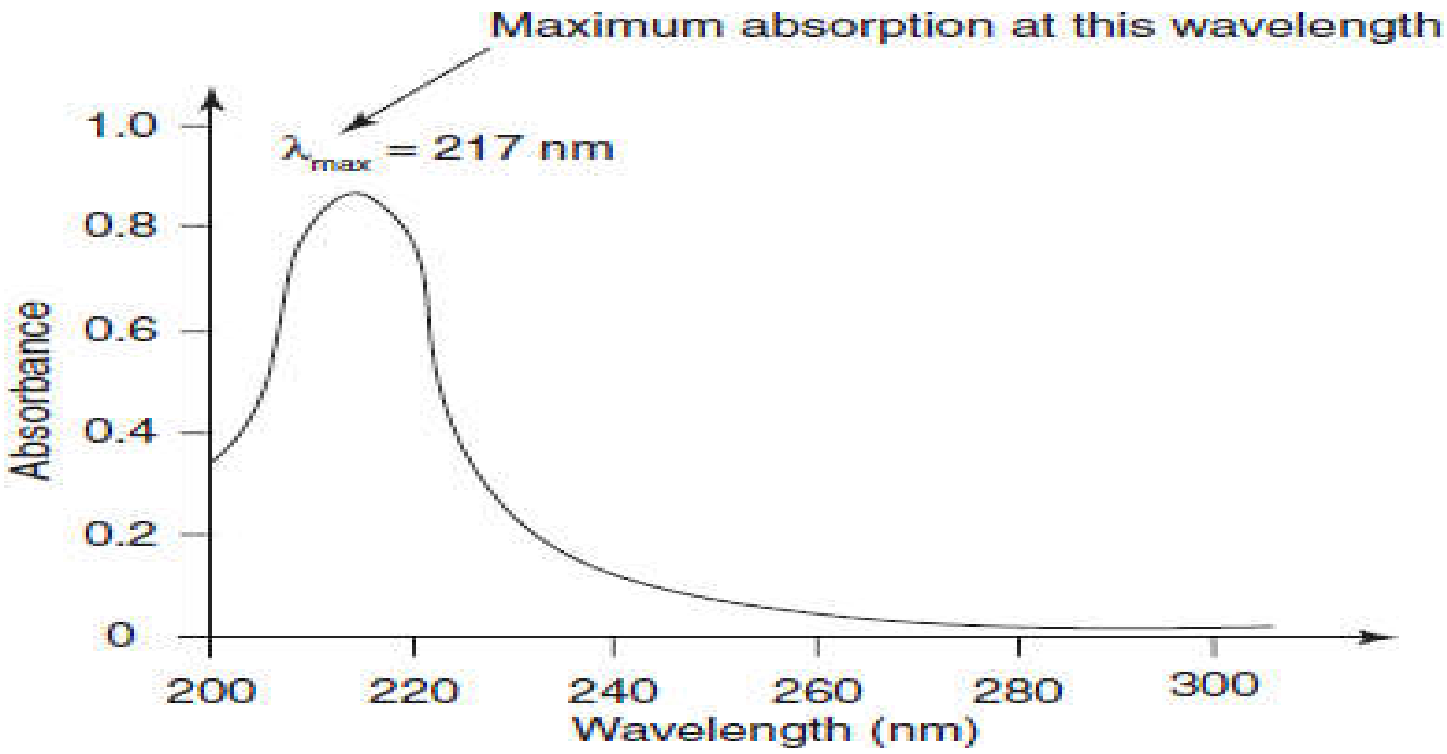
Characteristics of UV-Vis spectrum

- Most drug molecules absorb radiation in the UV region of the spectrum
- some are colored and thus absorb radiation in the visible region
- The amount of the radiation absorbed at each wavelength is measured and plotted against the wavelength
- a typical UV spectrum is a plot of Absorbance versus wavelength

Characteristics of UV-Vis spectrum……

- The UV spectrum is characterized by two major parameters, λmax, and the intensity of the bands (ε).
  - The λmax = The wavelength at which the absorbance (*A) is* highest
  - The intensity (ε) = is indicative of the probability of the transition i.e., whether the transition is allowed or not
    - The probability that light of a given wave length will be absorbed by the chromophores
- λmax ('lambda max') is a characteristic of a particular chromophore
- The λmax of a compound is sometimes used in the BP for identification of drugs and unknown compounds

UV-Vis spectrum……



a simple UV/visible absorption spectrum

- Y axis is Absorbance and the x- axis is the wave length(nm)

1.4. Absorption & Intensity Shifts

- Absorption band can be changed in its intensity or its location, or both effects

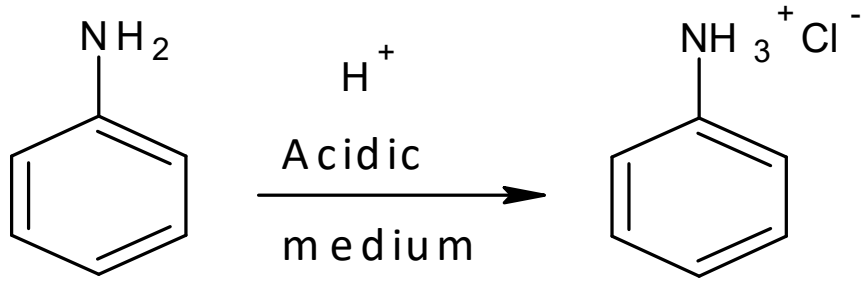
1. Bathochromic Shift

- It is the shift of λ_{max} to a longer wavelength due to:
  - substitution with certain functional groups (e.g. –OH and –NH2, OCH_3)
  - when two or more chromophores are present in conjugation
  - decreasing polarity of solvent
  - presence of an auxochrome

2. Hypsochromic Shift

- shift of λ_{max} to a shorter wavelength due to removal of conjugation or by changing polarity of the solvent
- usually occurs when compounds with a basic auxochrome ionize and the lone pair is no longer able to interact with the electrons of the chromophore
- can also be seen when spectra are run in different solvents

- Aniline shows blue shift in acidic medium, it loses conjugation



Aniline
λ_{max} = 230 nm

aniline hydrochloride
λ_{max} = 203 nm

- When aniline is placed in a solution of pH< 7, λ_{max} returns to 203 nm
- aniline in acidic solution reacts to form the anilinium salt
- The lone pair of electrons on the nitrogen is now involved in bond formation to an H+ ion and can no longer function as an auxochrome

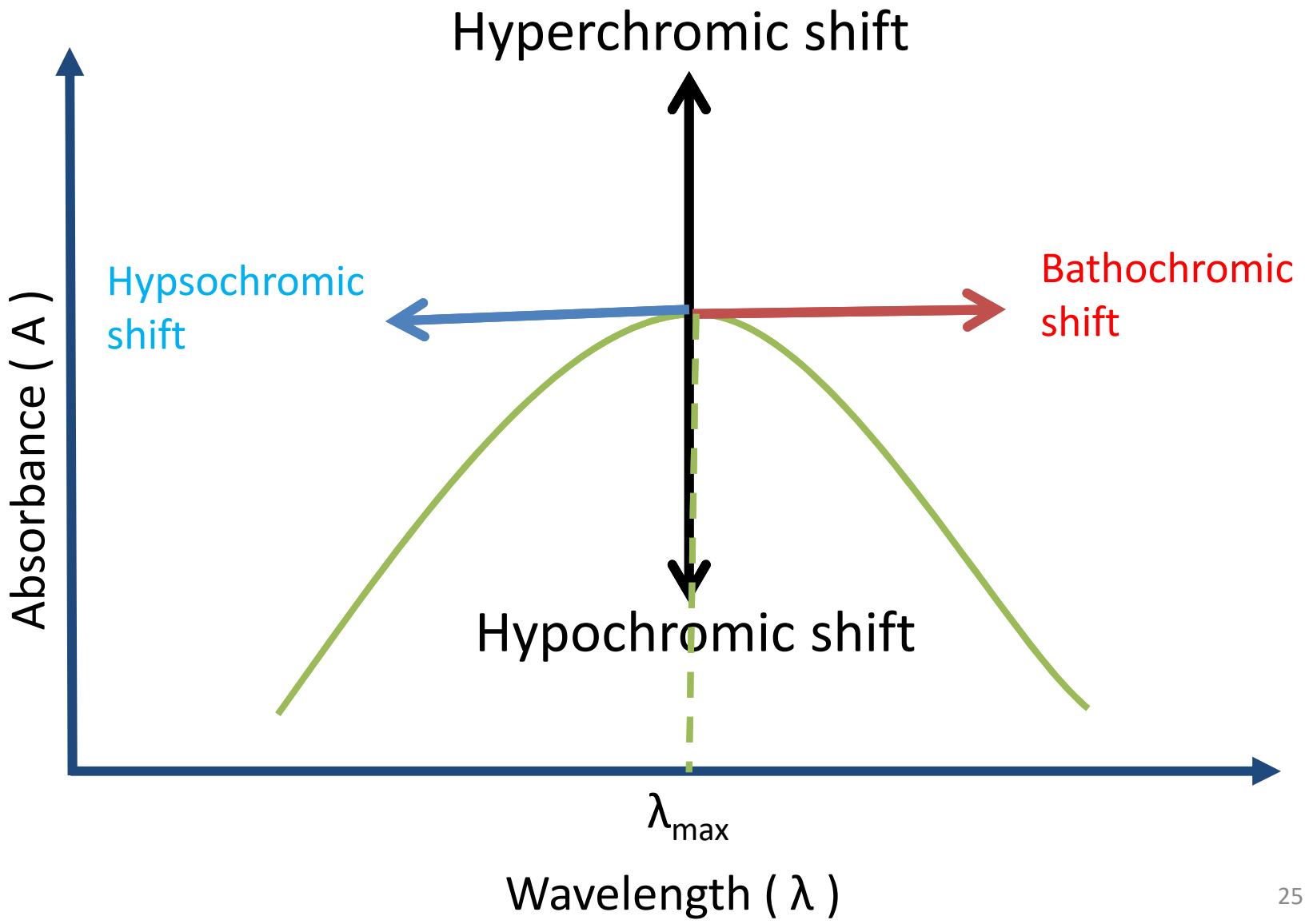
3. Hyperchromic shift

- absorption intensity (ε) of a compound is increased
- If auxochrome introduces to the compound, the intensity of absorption increases

4. Hypochromic shift

- absorption intensity (ε) of a compound is decreased
- Generally, Bathochromic effects are usually associated with increases in the intensity of light absorbed
- While Hypsochromic effects usually occur with decreases in absorbance

Shifts and Effects



1.5. Woodward- Fieser Rules

- The effect of substituent groups can be reliably quantified from empirical observation of known conjugated structures and applied to new systems
- This quantification is referred to as the Woodward- Fieser Rules which we will apply to specific chromophores:
  - Conjugated dienes
  - Conjugated dienones
- Are sets of empirically derived rules which attempt to predict the wave length of the absorption maximum in an UV-Visible spectrum of a given compound

Woodward- Fieser Rules

1. Dienes

- The rules begin with a base value for λ_{max} of the chromophore being observed:

acyclic butadiene = 217 nm (core chromophore)

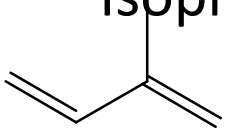
- The incremental contribution of substituents is added to this base value from the group tables:

Group	Increment
Extended conjugation	+30
Each Exocyclic C=C	+5
Alkyl	+5
$-OCOCH_3$	+0
-OR	+6
-SR	+30
-Cl, -Br	+5
$-NR_2$	+60

λ_{max} (calculated)= base (217) + substituent contributions

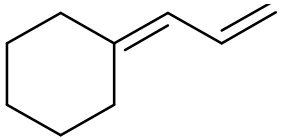
For example:

Isoprene



acyclic butadiene =	217 nm
one alkyl subs.	= + 5 nm
	222 nm
Experimental value	220 nm

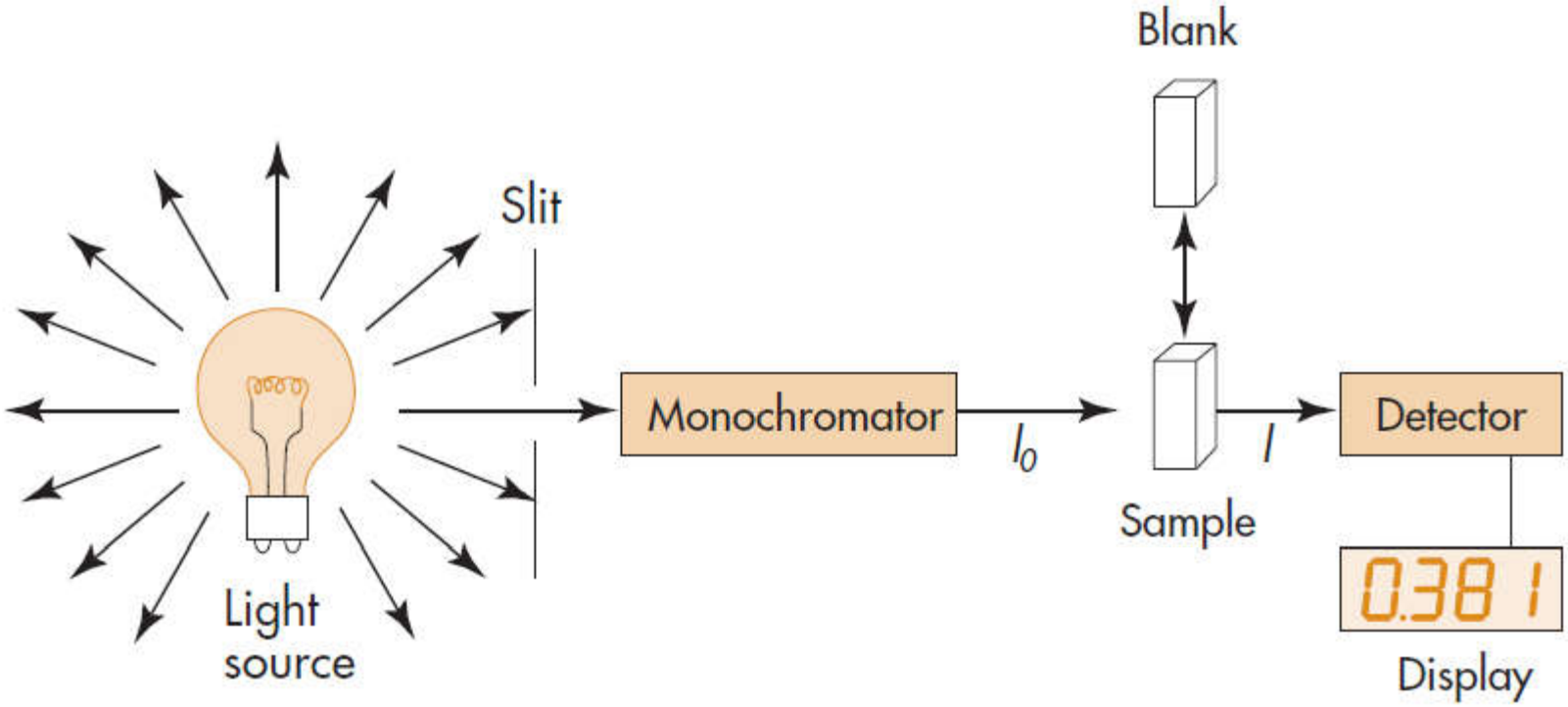
Allylidenecyclohexane



-acyclic butadiene =	217 nm
one exocyclic C=C	+ 5 nm
2 alkyl subs.	+10 nm
	232 nm
Experimental value	**237 nm**

1.5. Instrumentation

- An instrument that measures the intensity of light absorbed by atoms or molecules is called a *spectrophotometer*
- Components of UV Visible spectrophotometer
  - Light Source
  - wave length selector
  - Sample compartment
  - Detector
  - Recorder



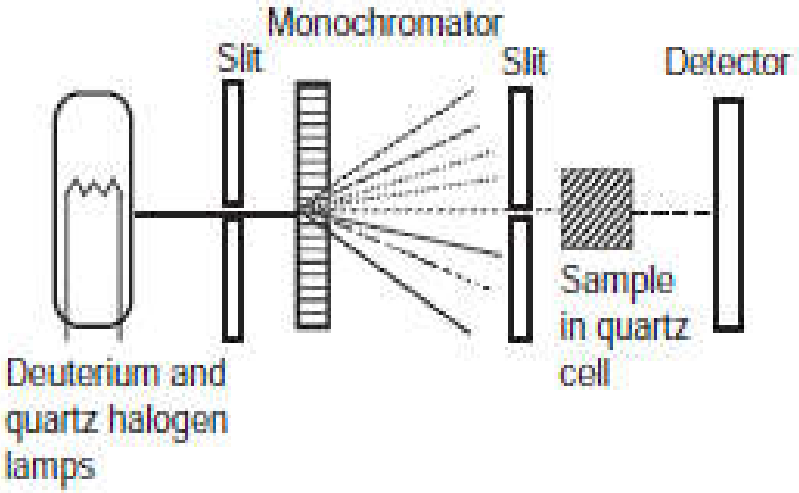
A schematic diagram of a spectrophotometer

The Light Source

- Two lamps are required to cover the visible and UV regions of the EMR
- For compounds that absorb in the UV region of the spectrum
  - a deuterium lamp that emit polychromatic UV radiations is used
  - is a high-energy source that emits continuous radiation in the UV range
  - is used for all spectroscopy in the ultraviolet region of the spectrum
- For visible light
  - a tungsten lamp *is used*
  - *It is* a filament made of the metal tungsten
  - emits light of wavelengths 350–2000 nm and is adequate for Colorimetry
  - The glowing of the filament produces a polychromatic white light

Monochromator

- Is used to disperse the light into its constituent wavelengths
- placed between the radiation source and the sample container
- allows only radiation with a single wavelength to pass through the sample
- For most quantitative measurements, light must be monochromatic, i.e. of one particular wavelength
- achieved by passing the polychromatic light through a Monochromator
- There are two types of monochromator in modern spectrophotometers: prisms or diffraction gratings



Sample holder/compartments

- Tight box in which the container holding the sample solution is placed
- The container in which the sample solution is placed is called cuvette or cells
- The materials making up the cuvette walls must be transparent
- Sample containers of
  - Quartz, glass or plastic can be used

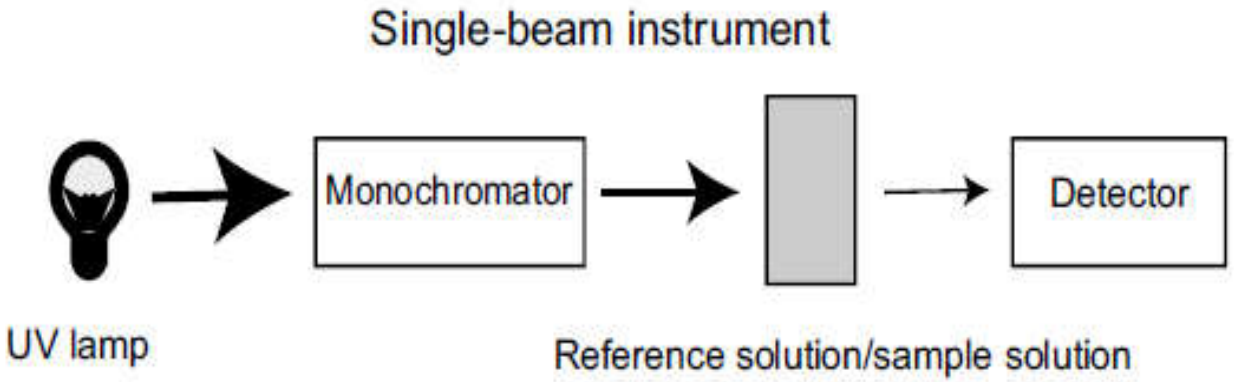
Detector

- After light has passed through the sample, any decrease in intensity, due to absorption, is measured by a detector
- convert the intensity of the beam of light into an electrical signal that can be measured easily
- Includes:
  - photomultiplier tubes
  - photodiodes
- Most modern spectrophotometers are now interfaced to a computer

Two types of instruments can be used

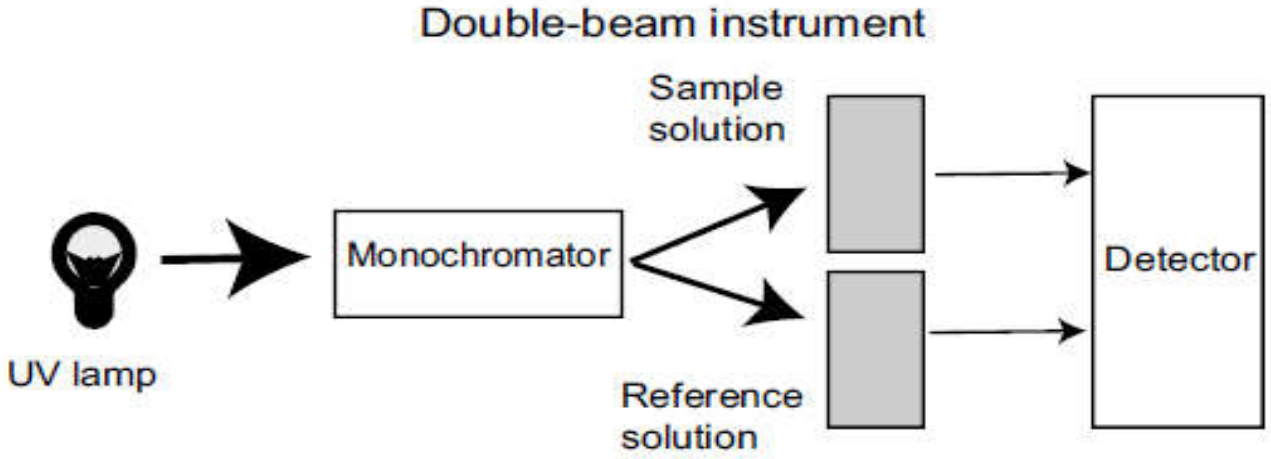
1. Single beam UV-Visible spectrophotometer

- Single-beam instruments have only one sample holder
- First, the solvent must be placed into the sample cell and the absorbance of the solvent must be adjusted to zero
- Then, a cell with the sample solution is inserted and the absorbance is measured
- The absorbance of solvent (blank) and the sample solution cannot be measured simultaneously



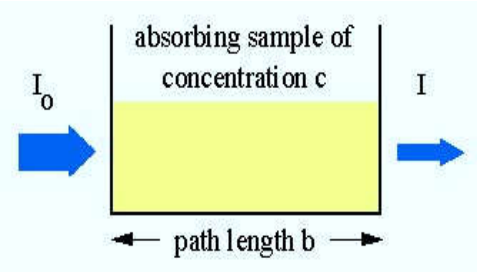
2. Double beam UV-Visible spectrophotometer

- The light from the radiation source is splitted into two parallel beams
- One beam passes through a cell for the solvent (reference cell), while the other beam passes through another cell with the sample solution (sample cell)
- the readout from the instrument is the difference between amounts of the radiations absorbed in the two cells
- The absorbance's of the reference cell and the sample cell are measured simultaneously



Laws of absorption spectrophotometry

- When abeam of light is passed through a transparent cell containing a solution of an absorbing substance, reduction of the intensity of the light may occur due to:
  - Absorption of light by molecules in the solution



The intensity of light absorbed is then given by

$I_{absorbed} = I_0 - I_T$, Where, $I_{absorbed}$= intensity of light absorbed

I_0 = is the original intensity of light

I_T = is intensity of light transmitted from the cell

Transmittance(T) is the ratio of $\mathbf{I_T / I_0}$ and % Transmittance is

$T = [I_T / I_0]$,

$\%\ T = [I_T / I_0] \times 100\% = 100 \times T$

Absorbance (A)=log 1/T= -log T=

ABSORPTION LAWS:

- Lambert’s law
- Beer’s law
- Beer – lambert’s law

Beer- Lambert's law

Beer's law

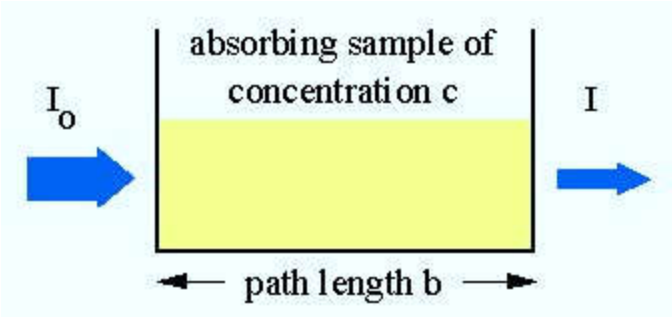
- States that the light absorbed is proportional to the number of absorbing species i.e. the concentrations of absorbing species, **C**

Lambert's law

- States that the amount of light absorbed by a sample is proportional to the path length, b

A combination of the two laws yields the Beer-Lambert's Law

- Amount of light absorbed by a sample is proportional to the concentration of the light absorbing substances **(C)** and path length (b)



A= εbc

- A is known as the **absorbance =** a measure of the amount of light absorbed by the sample;

  ε = is a constant known as **molar absorptivity** = the absorbance of a 1 M solution of the analyte in a l cm cell;
- b is the **path length** of the cell in cm, usually 1 cm
- **c** is the **concentration** of the analyte in moles litre–1

$A = A^{1\%}_{1\ cm}\ b\ c$

$$\varepsilon = \frac{A_1^1 \times \text{relative molecular mass}}{10}$$

- where A is the measured **absorbance**
- A (1%, 1 cm) is a constant known as **specific absorbance** and is the absorbance of a 1% w/v (1 g/100 ml) solution in a 1 cm cell;
- has units of dL g^{-1} cm^{-1}
- b is the **path length** in cm (usually 1 cm)
- c is the concentration of the sample in g/100 ml

BEER – LAMBERT'S LAW:

- On combining the two laws , the beer –Lambert law can be formulated as :
- $\log I_o/I = e.b.c = A = \log 1/T = -\log T$
  - € = molar Absorptivity
  - C = concentration of solution
  - A = absorbance
  - b= path length

Beer's law is applicable

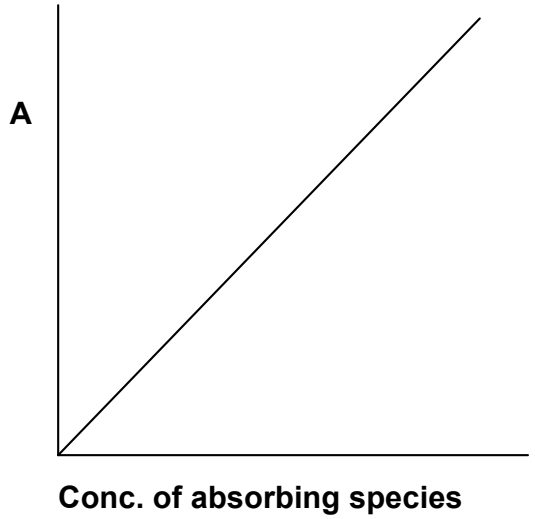
- The light used must be monochromatic i.e. radiation of one wavelength
- The concentration of the solution should be low
- The solutions must not undergo photochemical reactions
- The solute must not form association with the solvent
- The solution must not fluorescence

Deviations from beer's law

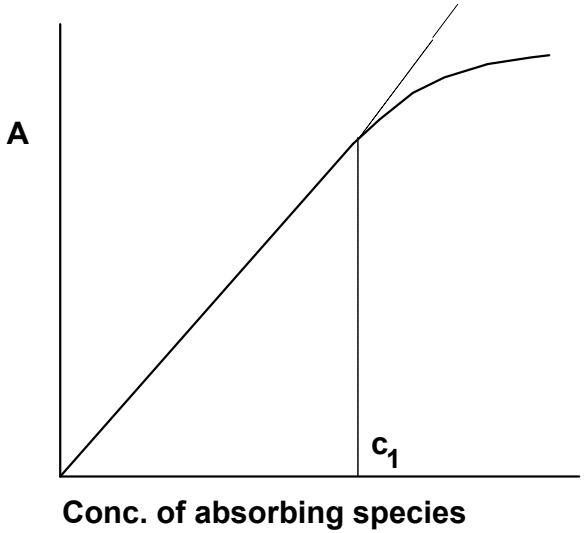
- If monochromatic light is not used
- Concentrated solution
- Instrumental deviations such as stray light, improper slit width, fluctuation in single beam
- Chemical effects such as association, dissociation, complex formation etc

Deviations from Beer's Law

Beer's Law Obeyed



Deviations from Beer's Law



- Generally, the data over a wide range of concentrations will deviate from Beer's law. This indicates that Beer's law is only applicable up to a concentration of c_1

Exercises

1. What are the concentrations of the following solutions of drugs in g/100 ml?
   A. Carbimazole, A (1%, 1 cm) value=557 at 291 nm, measured absorbance=0.557 at 291 nm
   B. Hydrocortisone sodium phosphate, A (1%, 1 cm) value=333 at 248 nm, measured Absorbance =0.666 at 248 nm
   C. Isoprenaline, A (1%, 1 cm) value=100 at 280 nm, measured absorbance= 0.5 at 280 nm

Answers:

A. 0.001 g/100 ml
B. 0.002 g/100 ml
C. 0.005 g/100 ml

Quantitative spectrophotometric assay of drugs

- In quantitative determinations, the amount of absorbed radiation is measured and related to concentration
- Pharmacopoeial methods rely heavily on simple analysis by UV/visible spectrophotometry to determine active ingredients in formulations
- no interference from excipients (preservatives, colorants, etc.) present in formulations and that the sample is free of suspended matter, which would cause light scattering
- The concentration of the absorbing substances is calculated from the measured absorbance using one of three principal methods

a. Use of standard absorptivity value: (A 1%, 1 cm and ε)

- is adopted by official compendia (BP)
- Avoids the use of standard solutions to determine absorptivity
- Advantage when it is difficult or expensive to obtain reference standard
- BP monographs often quote a standard A (1%, 1 cm) value for a drug, which is to be used in its quantitation

Examples

1. Calculate the concentration of a drug in an ethanolic solution of which the absorbance was found to be 0.890 in a 1 cm cell at 241 nm. The ($A^{1\%}_{1\ cm}$) in the B.P Monograph of drug is given as 540 at 241 nm.

   $A = A^{1\%}_{1\ cm}\ b\ c$

   $0.89 = 540 \times 1 \times c$

   $C = 0.00165$ g/100 ml $= 1.65 \times 10^{-5}$ g/ml $= 0.0165$ mg/ml

2. Calculate the concentration of the drug in an aqueous solution in which the absorbance in 4 cm cell at its λ_{max} , 261nm, was found to be 0.87. the A (1%, 1 cm) value of the drug is given as 520 at 261 nm

   $A = A^{1\%}_{1\ cm}\ b\ c$

   $0.870 = 520 \times 4\ cm \times c =$ 0.00668 g/100 ml = 0.0668 mg/ml

Assay examples

1. Assay of Furosemide Tablet

- Tablet powder containing 0.258 g of furosemide is shaken with 300 ml of 0.1 M NaOH to extract the acidic furosemide. The extract is then made up to 500 ml with 0.1 M NaOH. A portion of the extract is filtered and 5 ml of the filtrate is made up to 250 ml with 0.1 M NaOH. The absorbance of the diluted extract is measured at 271 nm and the following results were obtained.
  - Stated content per tablet: 40 mg of furosemide
  - Weight of 20 tablets: 1.656 g
  - Weight of tablet powder taken for assay: 0.53406 g
  - Absorbance reading: 0.596
  - A (1%, 1 cm) value at 271= 580

  a. Calculate the % of stated content in a sample of furosemide tablets:

  b. Calculate the content of furosemide in a tablet of average weight

  c. Does the product comply with pharmacopeial specifications? (BP limit: 95-105%)

Answer

a. Expected content in tablet powder taken: [0.53406 /1.656] x 40 x 20 = 258 mg

Dilution factor: 5 – 250 ml =50

Concentration in diluted tablet: 0.596/ 580=0.001028 g/100 ml= 1.028 mg/100 ml

Concentration in original tablet: 1.028 x 50= 51.40 mg/100 ml

Volume of original extract: 500 ml

Therefore, amount of furosemide in original extract: 51.40 mg x 5 =257.0 mg

Percentage of stated content: [257 mg/258 mg] x 100 = **99.6%**

b. Content of furosemide per tablet in the sample = **39.84 mg**

c. Yes b/c % content of the drug is within the range of pharmacopeial specifications

Self test

Calculate the percentage of stated content of promazine hydrochloride in promazine tablets from the following information:

Tablet powder containing 80 mg of promazine hydrochloride is ground to a paste with 10 ml of 2 M HCl. The paste is then diluted with 200 ml of water, shaken for 15 min and finally made up to 500 ml. A portion of the extract is filtered. Then, 5 ml of the filtrate is taken and diluted to 100 ml with 0.1 M HCl. The absorbance is read at a wavelength of 251 nm:

- A (1%, 1 cm) value of promazine.HCl at 251 nm=935
- Stated content of promazine.HCl per tablet=50 mg
- Weight of 20 tablets=1.667 g
- Weight of tablet powder taken for assay=133.36 mg
- Absorbance reading=0.755

Answer: 99.3%

Self test

1. Determine the concentration of the following injections:

a. Haloperidol injection:

Add 15 ml of 1 M HCl to 5 ml of injection. Extract three times with ether, washing the ether extracts with 10 ml of water. Combine the aqueous layers and dilute to 100 ml. Take 10 ml of the diluted aqueous solution and dilute to 100 ml.

- Absorbance reading at 245 nm=0.873
- A (1%, 1 cm) value at 245 nm=346

b. Isoxsuprine injection is diluted as follows: (i) 10 ml of injection is diluted to 100 ml and then 10 ml of the dilution to 100 ml:

- Absorbance reading at 274 nm= 0.387
- A (1%, 1 cm) value at 274 nm=73

Answers:

haloperidol injection=0.505 % w/v

Isoxsuprine injection= 0.530% w/v

b. Single point standardization

- Involves the measurement of the absorbance of a sample solution and of a standard solution of the drug

$$C_{test} = [A_{test} \times C_{std}] / A_{std}$$

C_{test} and C_{std} - concentrations of sample and standard solutions

A_{test} and A_{std} - absorbance's of the sample and standard solutions

Exercise

In a spectrophotometric determination of a drug in an aqueous solution, the absorbance of 4.5×10^{-5} mg/ml of a standard solution of drug at 273 nm was found to be 0.454 with a path length of 1 cm. The absorbance reading of the sample solution was 0.367 at 273 nm, with the path length of 1 cm. Determine the concentration of a drug in mg/ml?

$$C_{test} = [A_{test} \times C_{std}] / A_{std}$$

$$C = [0.367 \times 4.5 \times 10^{-5} \text{ mg/ml}] / 0.454$$

$$C = 3.64 \times 10^{-5} \text{ mg/ml}$$

$$C = 3.64 \times 10^{-5} \text{ mg/ml}$$

$C = 0.00364$ mg/ml

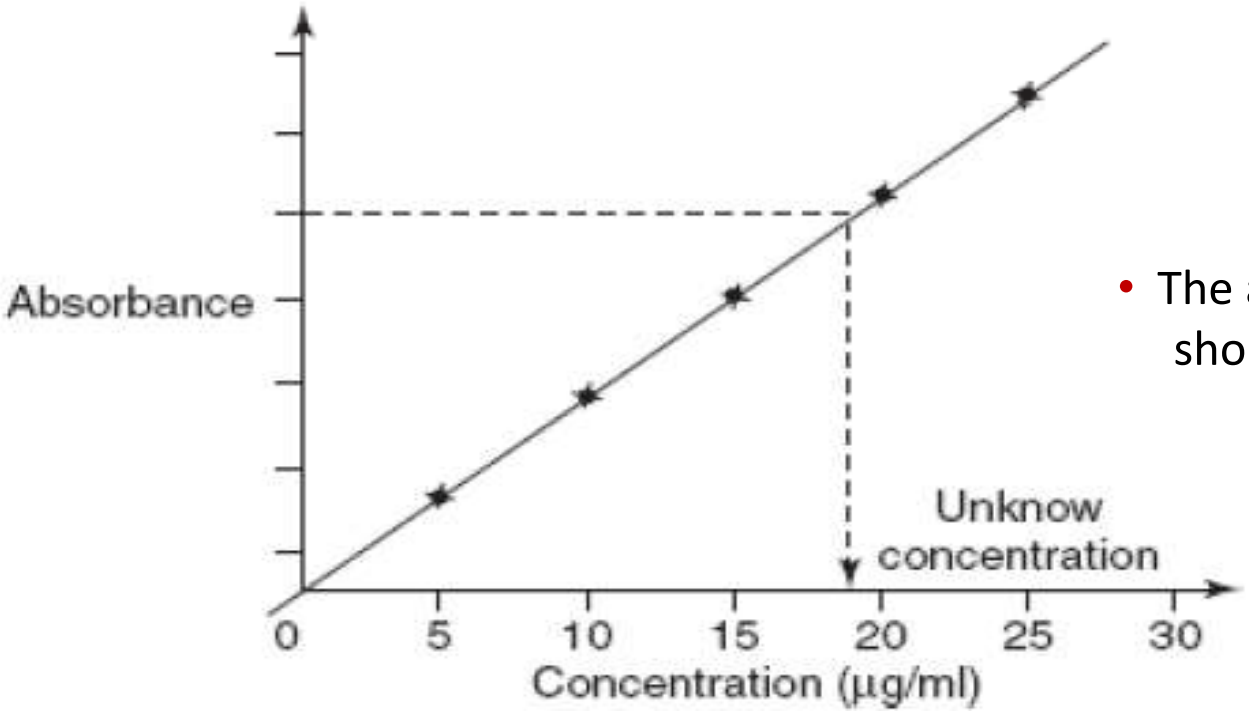
Self exercise

An absorption spectrum of a drug at 400 nm gives absorbance of 0.456 when 32 µg/ml solution is taken. Unknown sample of a drug is treated in identical fashion gives an absorbance of 0.501 assuming identical cell. Determine unknown concentration?

Answer : 35.1578 µg/ml

c. Use of Calibration graph

- Calibration graph is constructed by preparing different concentrations of a reference standard
- The concentration of the analyte can be read from the graph as the concentration corresponding to the absorbance of the solution



- The absorbance readings should be below 0.9

Assay of drugs in multicomponet samples

- The spectrophotometric assay of drugs rarely involves the measurement of samples containing only one absorbing component
- however, interferences may arise due to the presence of impurities, decomposition products and excipients
- Unwanted absorption form these sources is termed irrelevant absorption
- The basis of all the spectrophotometric techniques for multi- component samples is at all wave lengths:
  - The absorbance of a solution is the sum of absorbances of the individual components
  - the measured absorbance is the difference between the total absorbance of the solution in the sample and the reference (blank) cell

- The techniques of spectrophotometric assay of multicomponet samples are

a) Assay as a single component ⟹ Absorption by interference is low

b) Assay using absorbance corrected for interference

c) Assay after solvent extraction of the sample

d) Simultaneous equation method

e) Absorbance ratio method

f) Differential analysis

g) Derivative spectrophotometry

1. Analysis of single component system

- May be determined by a simple spectrophotometric measurement of absorbance
- Can be done using calibration curve
- Absorption of several dilutions of the standard solution will be measured
- Absorbance Vs concentration will be drawn
- The best line could be drawn by the list square method

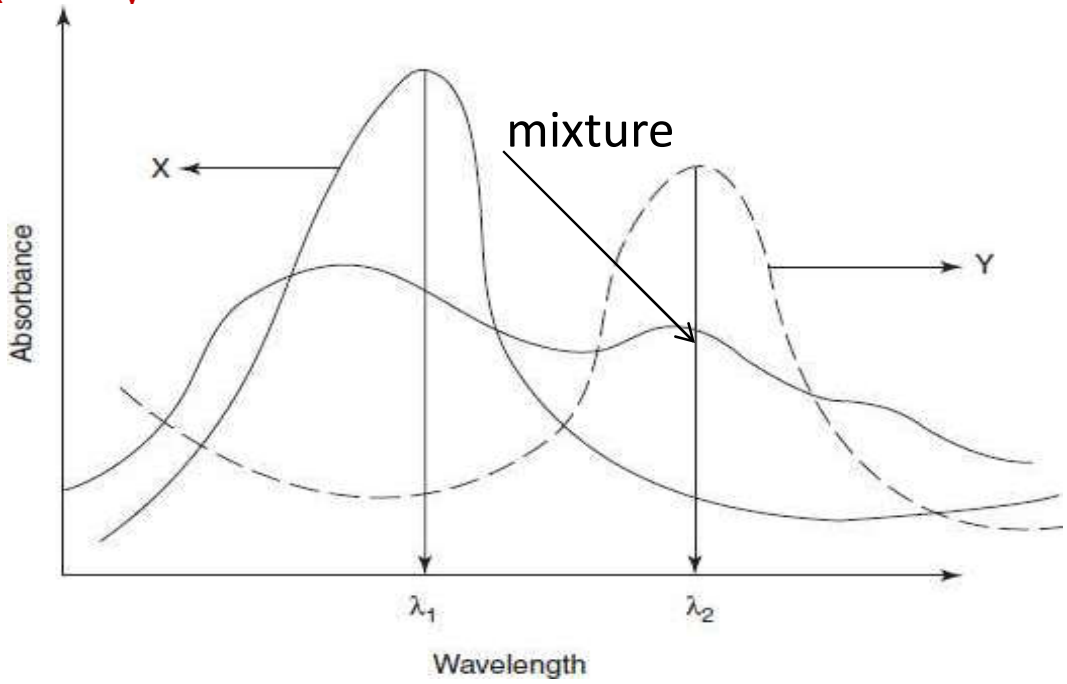
  (regression line Y=a + bx)
- Then, the concentration of sample will be calculated

2. Absorbance corrected for interferences

- If the concentration and absorptivity of the absorbing interference is known at the λ_{max} of the sample, it is possible to calculate their concentration to the total absorbance of a mixture
- The concentration of the absorbing component of interest is then calculated from the corrected absorbance

3. Simultaneous method

- Applicable when a sample contains two absorbable samples X and y
  - Let λ_1 and λ_2 be their absorbing maxima
  - a_{x1} and a_{x2} be absorptivity of x at λ_1 and λ_2 respectively
  - a_{y1} and a_{y2} be absorptivity of y at λ_1 and λ_2 respectively
  - Absorbance of the sample at λ_1 and λ_2 be A_1 and A_2
  - c_x and c_v concentration of x and y



$$\text{At } \lambda_1 \qquad A_1 = a_{x1}\, bc_x + a_{y1}\, bc_y$$

$$\text{At } \lambda_2 \qquad A_2 = a_{x2}\, bc_x + a_{y2}\, bc_y \quad \Rightarrow \quad C_y = \frac{A_2 - a_{x_2} c_y}{ay_2}$$

For measurements in 1 cm cells, b=1

Substituting the c_y value in the first equation gives

$$C_x = \frac{A_2 a_{y_1} - A_1 ay_2}{ax_2 a_{y_1} - a_{x_1} a_{y_2}} \qquad \text{and} \qquad C_y = \frac{A_1 a_{x_2} - A_2 a_{x_1}}{ax_2 a_{y_1} - a_{x_1} a_{y_2}}$$

Simultaneous equations method…

Exercise

The molar absorptivities of drug X and Y were measured with pure samples of each

λ (nm)	X [ε (M^{-1} cm^{-1})]	Y [ε (M^{-1} cm^{-1})]
272 nm	16440	3870
327 nm	3990	6420

A mixture of drug X and Y in a 1.000 cm cell has an absorbance of 0.957 at 272 and 0.559 at 327 nm

Find the concentrations of X and Y in the mixture

At λ_1 $A_1 = a_{x1}\, bc_x + a_{y1}\, bc_y$

At λ_2 $A_2 = a_{x2}\, bc_x + a_{y2}\, bc_y$

Total absorbance at 272 nm, $0.957 = 16440c_x + 3870c_y$

Total absorbance at 327 nm, $0.559 = 3990c_x + 6420c_y$

Reading assignment

- Difference spectrophotometry
- Derivative spectrophotometry
- spectrophotometric titration

2. Colorimetric analysis

- Measures how much light is absorbed by a colored solution
- The amount of light absorbed by a colored solution is directly proportion to the conc. Of substance in the colored solution

a. Direct method

- The drug must be self colored. E.g. pyrantel pamoate

b. Indirect method (chemical derivatization)

- Indirect spectrophotometric assay technique
- Shifting of the λ_{max} of the analyte by chemical reagents to the visible region by forming colored derivative
- The conversion of the analyte to a derivative that has longer λ_{max}
- The drug must react with a reagent to produce a sub, w/c is colored

Chemical nature of substances

- Chromophore functional groups are responsible for light absorption
- Highly conjugated compounds (double bonds, triple bond or aromatic ring) absorb light in longer wavelengths with intense absorption (Visible region)

Colorimetric analysis……..

- The chemical derivatization may be adopted for any of the following reasons

a. If the analyte absorbs weakly in the ultraviolet region

   - Changed to a more sensitive absorbing chromophore

b. The interference from irrelevant absorption may be avoided by converting the analyte to a derivative which absorbs in the visible region, where irrelevant absorption is negligible

c. To improve the selectivity of the assay of an ultraviolet absorbing substance in a sample that contains other ultraviolet absorbing component

d. Less costly when compared to the ultraviolet spectrophotometer

Derivatization techniques

The following are the mainly used methods for chemical derivatization

1. Diazotisation and coupling of primary aromatic amines

- The amine is first diazotised with a aqueous solution of nitrous acid at 0–5 °C

$$Ar - NH_2 + HNO_2 + H^+ \longrightarrow Ar - N^+ \equiv N + 2H_2O$$

- The colorless diazonium salt is very reactive when treated with a suitable coupling agent, e.g. Phenol or aromatic amine produces an azo derivative.

$$Ar - N^+ \equiv N + Ar' - H \longrightarrow Ar - N = N - Ar' + H^+$$

- The azo derivatives are colored and consequently have an absorption maximum in the visible region
- Most widely used coupling reagents are
  - 1- naphthol, 2- naphthol and N-(1- naphthyl-ethane-1,2 diamonium dichloride (Bratton- marshal reagent)
- Chlorothiazide and Chloramphenicol can be assayed spectrophotometrically

2. Condensation reactions:

- These reactions involve the nucleophilic attack by the amine on carbonyl carbon with elimination of H2O
- Substances containing the carbonyl group react with a variety of reagents containing an amino group

- When R" = NH2 (hydrazine) the product is hydrazone
- 2,4 dinitrophenylhydrazine is used in the assay of methyl testosterone

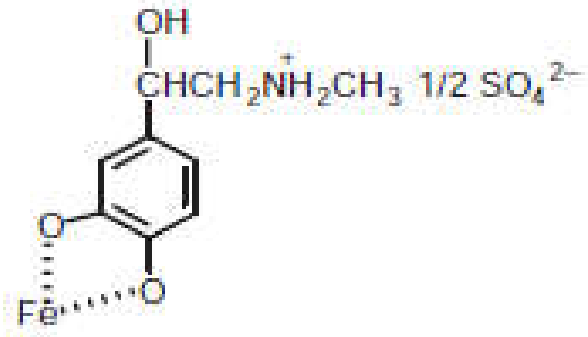
3. Metal ligand complexation

- The complexes formed with metal are often colored

Examples

a) Assay of adrenaline in adrenaline injection

- The selectivity of UV/visible spectroscopy for the analysis of adrenaline can be increased by complex formation, which occurs between iron (II) and molecules containing a Catechol group
- These complexes are purple in colour and absorb at 540 nm
- The adrenaline in the injection is quantified against a standard solution of adrenaline

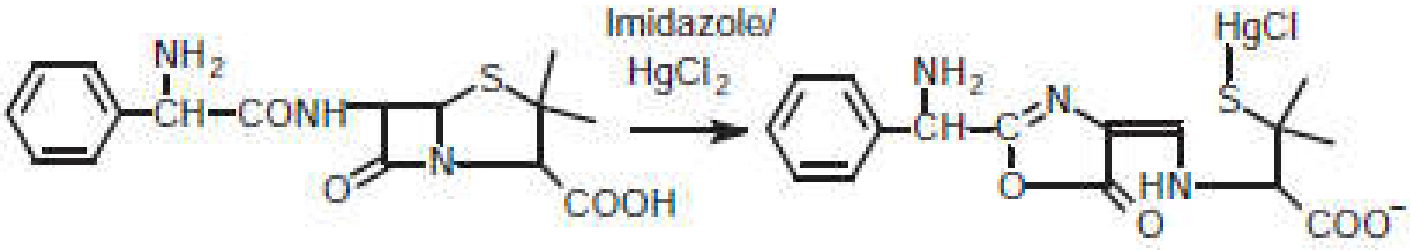


Adrenaline iron (II) complex

The complex formed between adrenaline and iron, which is used to analyse adrenaline at low levels in an injection

b. Assay of penicillins

- The BP utilizes formation of a derivative in order to quantify penicillins in formulations
- The formation of a complex with the mercuric ion in the presence of imidazole as a catalyst, a derivative of the penicillin structure, which has an absorption maximum between 325 and 345 nm is produced
- This assay is used by the BP for analysis of preparations containing Ampicillin, amoxicillin, carbenicillin, cloxacillin, flucloxacillin and phenoxymethylpenicillin



Ampicillin

λ max *ca* 325 nm

Quantification

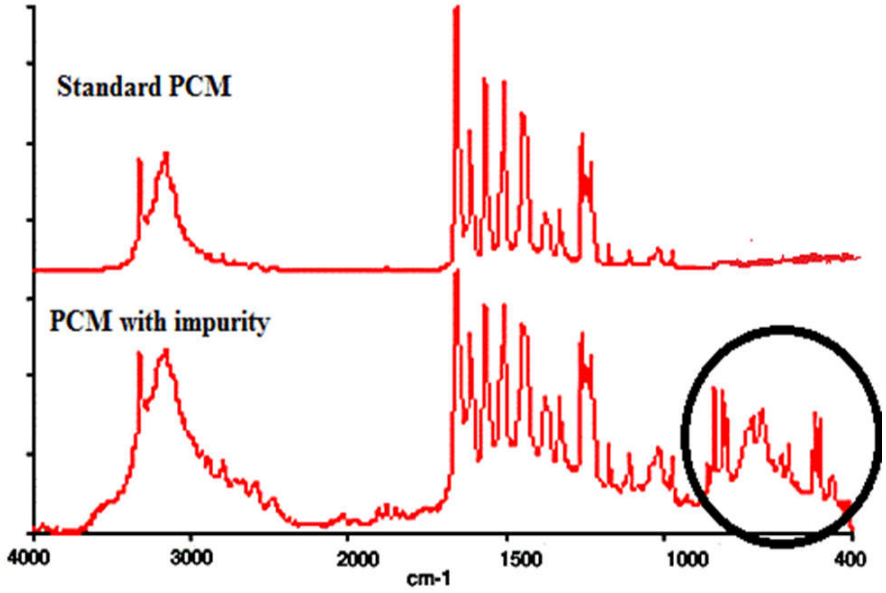
- There is a linear relationship between absorbance and concentration
- Beer lamberts law is applicable
- Calibration curves are used for quantification

Applications of UV-Vis Spectroscopy

1. Detection of Impurities

- UV absorption spectroscopy is one of the best methods for determination of impurities in organic molecules

- Additional peaks can be observed due to impurities in the sample and can be compared with reference standard



2. Structure elucidation of organic compounds

- UV spectroscopy is useful in the structure elucidation of organic molecules, the presence or absence of unsaturation, the presence of hetero atoms
- Provide information about presence and absence of unsaturated functional groups

3. Quantitative analysis of pharmaceutical products

- UV absorption spectroscopy can be used for the quantitative determination of compounds that absorb UV radiation
- This determination is based on Beer's law which is as follows

$$A = \log I_0 / I_t = \log 1/ T = - \log T = abc = \varepsilon bc$$

- Many drugs are either in the form of raw material or in the form of formulation can be assayed by making a suitable solution of the drug in a solvent and measuring the absorbance at specific wavelength

UV-Visible Spectrophotometric method employed for the assay of the following drugs

- amphetamine
- Ampicillin
- Betamethasone
- Carbamazepine
- Carbimazole
- Chloramphenicol
- Digitoxin
- diazepam
- diphenhydramine
- ephedrine
- Ergotamine Tartrate
- Griseofulvin
- Hydrocortisone sodium phosphate
- ibuprofen
- Imipramine HCl Tablets
- Indomethacin Capsules
- Isoprenaline HCl Injection
- Isoprenaline Tablets
- Isoxsuprine HCl
- paracetamol
- Phenylephrine HCl Injection
- procaine
- Rifampicin
- Spironolactone

4. Qualitative analysis

- UV absorption spectroscopy can characterize those types of compounds which absorbs UV radiation
- The identification is based on UV spectra, where the absorbance of the drug is recorded as a function of wavelength
- Different substances may have different UV spectra
- Identification is done by comparing the absorption spectrum with the spectra of known compounds

5. As HPLC detector

- A UV/Vis spectrophotometer may be used as a detector for HPLC