Pharmaceutical Instrumental Analysis PHC 425

Prepared by:

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References


Note:
The handouts are for guidance and studying must be from textbook
SPECTROMETRY
• ULTRAVIOLET AND VISIBLE SPECTROSCOPY
In this topic, we

- Describe the absorption of radiation by molecules and its relation to molecular structure;
- Make quantitative calculations, relating the amount of radiation absorbed to the concentration of an absorbing analyzed;
- Describe the instrumentation required for making measurements.
- Describe a related technique, fluorescence spectrometry, in which a mount of light emitted upon excitation is related to the concentration.
Principles:
Radiation in the wavelength range 200 – 780 nm is passed through a solution of a compound. The electrons in the bonds within the molecule become excited so that they occupy a higher quantum state and in the process absorb some of the energy passing through the solution. The more loosely held the electrons are within the bonds of the molecule the longer the wavelength (lower energy) of the radiation absorbed.
Applications in pharmaceutical analysis

- A robust, workhorse method for the quantification of drugs in formulations where there is no interference from excipients.
- Determination of the pKa values of some drugs.
- Determination of partition coefficients and solubilities of drugs.
- Used to determine the release of drugs from formulations with time, e.g. in dissolution testing.
- Can be used to monitor the reaction kinetics of drug degradation.
- The UV spectrum of a drug is often used as one of a number of pharmacopoeial identity checks.

Strength:

- An easy to use, cheap and robust method offering good precision for making quantitative measurements of drugs in formulations.
- Routine method for determining some of the physico-chemical properties of drugs, which need to be known for the purpose of formulation.
Background

• Spectroscopy:
  – The study of **interaction** of the **light** with substances.
  – Qualitative and Quantitative information.

• **Light**
  – EMR (electromagnetic radiation).
  – Dual nature of light
  – Form of Energy
What Is Electromagnetic Radiation EMR?

Electromagnetic (EM) radiation is a form of energy that is all around us and takes many forms, such as radio waves, microwaves, X-rays and gamma rays. Sunlight is also a form of EM energy, but visible light is only a small portion of the EM spectrum, which contains a broad range of electromagnetic wavelengths.

EM radiation is created when an atomic particle, such as an electron, is accelerated by an electric field, causing it to move. The movement produces oscillating electric and magnetic fields, which travel at right angles to each other in a bundle of light energy called a photon. Photons travel in harmonic waves at the fastest speed possible in the universe: 186,282 miles per second (299,792,458 meters per second) in a vacuum, also known as the speed of light. The waves have certain characteristics, given as frequency, wavelength or energy.
EMR

• Wavelength ($\lambda$):
  A wavelength is the distance between two consecutive peaks of a wave. This distance is given in meters (m) or fractions thereof.

• Wave number ($\delta = 1 / \lambda$) number of waves/ cm

• Frequency ($\nu$) : number of waves (cycles/ second – CPS) or hertz (Hz).

• Speed of light ($c = \lambda \cdot \nu$) = 300,000 Km/s

• Energy: ($E = h \cdot \nu$), $h = $ Max Planck constant = 6.63 x $10^{-27}$ (erg. s)
Spectrum of EMR
Spectrum of EMR
The Electromagnetic Spectrum

Wavelength

- **Meters:**
  - $10^{-13}$ to $10^{-11}$
  - $10^{-9}$
  - $10^{-7}$
  - $10^{-5}$
  - $10^{-3}$
  - $10^{-1}$
  - 10

- **Centimeters:**
  - $10^{-11}$
  - $10^{-9}$
  - $10^{-7}$
  - $10^{-5}$
  - $10^{-3}$
  - 10
  - $10^{3}$

- **Nanometers:**
  - $10^{-10}$
  - $10^{-8}$
  - $10^{-6}$
  - $10^{-4}$
  - $10^{2}$
  - $10^{4}$
  - $10^{6}$
  - $10^{8}$
  - $10^{10}$

Frequency (Hz)

- $10^{21}$
- $10^{19}$
- $10^{17}$
- $10^{15}$
- $10^{13}$
- $10^{11}$
- $10^{9}$
- $10^{7}$

Energy (kcal)

- $10^{8}$
- $10^{6}$
- $10^{4}$
- $10^{2}$
- $10^{0}$
- $10^{-2}$
- $10^{-4}$
- $10^{-6}$

\[ \nu = \frac{c}{\lambda} \]

- \( \nu \) = frequency, \( \lambda \) = wavelength, \( c \) = velocity of light (\( c = 3 \times 10^{10} \text{ cm/sec} \))

\[ \Delta E = h \nu \]

- \( \Delta E \) = energy, \( \nu \) = frequency, \( h \) = Planck’s constant (\( h = 6.6 \times 10^{-27} \text{ erg sec} \))
The wavelength of electromagnetic radiation varies from a few angstroms to several meters. The unit used to describe the wavelength are as follows:

\[ \text{Å} = \text{Angstrom} = 10^{-10} \text{ meter} = 10^{-8} \text{ centimeter} = 10^{-4} \text{ micrometer} \]
\[ \text{nm} = \text{nanometer} = 10^{-9} \text{ meter} = 10 \text{ angstroms} = 10^{-3} \text{ micrometer} \]
\[ \mu\text{m} = \text{micrometer} = 10^{-6} \text{ meter} = 10^4 \text{ angstroms} \]
Dual nature of light

• **Wave-particle duality** by Albert Einstein in the early 1900s (Nobel Prize in 1921).

• The theory states that everything has both a **particle nature** and a **wave nature**.

• Light exists in tiny "packets" called **photons**, exhibits properties of both waves and particles.

• **Photon** is the basic unit of light and carries energy.
Interaction of light with a substance

• Gain of energy (Photons) by a molecule.

1. Raising electrons to a higher energy level (**Transitional energy**)
   • When molecule absorb in visible and UV region (200 – 780 nm).

2. Raising the vibration of constituent nuclei (**Vibrational energy**)
   • When the molecule absorb in IR region (Mid-IR, 2.5-15 µm).

3. Increasing rotation of the molecule around its axis (**Rotational energy**)
   • When the molecule absorb in far-infrared region (FIR) or microwave.

➢ Relative energy levels in the order:

**Transitional > Vibrational > Rotational**
Excitation and relaxation

Excited state $E_s$ \[ \Delta E = E_s - E_g = h \nu \]

Ground state $E_g$

- $\Delta E =$ Transitional Energy = absorbed light
- $\nu$ is the frequency of EMR absorbed
- $\Delta E$ determines the light wavelength absorbed
Electronic transition

Diagram showing electronic transitions between ground state and excited states, with labels for UV, VIS, and IR transitions.
Types of electronic transitions

• **Sigma (σ) electrons:**
  – The bonding electrons of covalent bonds.
  – Lowest energy - most stable.
  – Saturated hydrocarbons - absorbs < 170 nm (far UV).
  – Transparent in the near UV (200 – 300 nm) - solvents for spectro.

• **Pi (π) electrons:**
  – The bonding electrons constituting the pi bonds (double bonds).
  – Higher energy than sigma electrons.

• **Non-bonding (n) electrons:**
  – Electrons of atomic orbital of hetero atoms (N,O, or S).
  – Don’t participate in bonding
  – They usually occupy the highest energy level of ground state.
Types of electronic energy levels and different types of transition

- The $\sigma - \sigma^*$ and $n - \sigma^*$ transitions require relatively high energy and are therefore associated with shorter wavelength radiation (UV).

- Lower energy $n - \pi^*$ and $\pi - \pi^*$ are UV or Vis induced transitions.
Effect of molecular structure

• In order for a molecule to absorb light:

• **Chromophore:**
  – Unsaturated group which is responsible for electronic absorption

• **Auxochrome:**
  – A saturated group which alters both the $\lambda$ and intensity of absorption maximum e.g -OH, - NH$_2$, - Cl.
Absorption spectrum

- A plot of Absorbance (A) as a function of wavelength (\( \lambda \)).

- Shows the \( \lambda \) of maximum absorbance (\( \lambda_{\text{max}} \)).

- It is characteristic for each molecule according to its structure:
  - Number and arrangement of electrons
  - Type of transitions

- Used for:
  - **Identification** of a chemical substance (qualitative analysis).
  - **Quantitative measurement** at \( \lambda_{\text{max}} \)
Absorption Spectrum

Is a plot of absorption intensity versus the wavelength of the absorbed light.

Absorption band spectrum for some molecules

Absorption spectrum: it is characteristic to substance, and the wavelength at which the maximum absorption is recorded and used to trace the substance strength to enhance the sensitivity.
Absorption spectrum

Methyl Red

[Chemical Structure]

Absorbance

Wavelength (nm)

- Acid
- Base
- Neutral

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Methyl orange
Shifting of $\lambda_{\text{max}}$

- **Bathochromic shift (or red shift)**
  - Shift of $\lambda_{\text{max}}$ to a longer wavelength
    - Substitution with certain functional groups (e.g. $-\text{OH}$ and $-\text{NH}_2$)
    - Effect of the medium pH / solvent on conjugation.

- **Hypsochromic shift (or blue shift)**
  - Shift of $\lambda_{\text{max}}$ to a shorter wavelength
    - Removal of conjugation by changing pH.
Shifting of $\lambda_{\text{max}}$

- **Hyperchromic effect**
  - An increase in the intensity of absorption.
    - Introduction of an auxochrome
    - Change in medium conditions

- **Hypochromic effect**
  - It involves a decrease in the intensity of absorption
    - Change in medium conditions.
Shifting of $\lambda_{\text{max}}$
Factors affecting absorption spectrum

1-Effect of pH

The spectra of compounds containing acidic (phenolic -OH) or basic (-NH$_2$) groups are dependent on the pH of the medium, e.g. phenol & aniline.

**Phenol**

Benzenoid form

\[
\text{Phenol} \xrightleftharpoons[OH^- \text{or } H^+]{\text{OH}} \xrightleftharpoons[OH^- \text{or } H^+]{H^+} \text{Quinonoid form (bathochromic shift and hyperchromic effect in alkaline medium)}
\]
**Aniline**

Aniline behaves like phenol, i.e. its spectrum exhibits bathochromic shift and hyperchromic effect in alkaline medium due to its conversion to the quinonoid species, while in acid medium its spectrum exhibit hypsochromic shift and hypochromic effect due to its conversion to the benzenoid species.
**2-Effect of redox reaction.**

Oxidation of diphenylamine will convert the benzenoid spectrum of diphenylamine to the quinonoid spectrum of the oxidised form, as represented by the following equation.

\[
\begin{align*}
2 \text{N-H} & \xrightarrow{\text{ox.}} \text{N-H} \quad \text{Reduced Form} \\
\text{N-H} + 2\text{H}^+ + 2\text{e} & \xrightarrow{\text{red.}} \text{Oxidized Form}
\end{align*}
\]
3-Effect of conjugation

Increase in conjugation, increase absorbance of light to higher $\lambda$ (bathochromic shift) as electrons become more energetic and need less energy; higher $\lambda$; to be excited. The next figure represents naphthalene, anthracene and tetracycline, respectively.
**Isosbestic point**

- On running U.V spectrum of known concentration of phenol as a function of pH (i.e at different pH).
- The spectrum will be shifted to different $\lambda_{max}$ by changing the pH, but all spectra intersect at certain $\lambda$ which is known as isosbestic point.
• At isosbestic point, the same absorbance is given for the same concentration at different pH, i.e. absorbance is not pH dependent but concentration dependent.
• Isosbestic point can be defined as the wavelength at which all the spectra at different pH of a certain concentration of a certain compound intersect.
• Therefore, if the $\lambda_{\text{max}}$ of the tested solution is affected by pH and we want to measure the absorbance at certain constant wavelength which is not affected by the variation of pH so the solution of the substance must be buffered at specific pH or measurements should be carried out at the isosbestic point not at its $\lambda_{\text{max}}$. 
Laws of light absorption

Concentration of a Solution Using Beer’s Law

White light → violet (400nm) – red (800nm)

“visible spectrum”

sample (light absorbed)

Light incident → light transmitted
Laws of light absorption

Basically, we can detect the amount of light absorbed by the sample by monitoring the amount of light before and after the sample.

\[ I_o = \text{intensity of incoming light (incident light)} \]

\[ I_T = \text{intensity of outgoing light} \]

Amount of light absorbed is proportional to the concentration of the sample (at proper \( \lambda \)):

\[ A = \alpha c \]

absorbance (unitless) \quad \text{concentration}
Laws of light absorption
Laws of light absorption

The amount of radiation absorbed may be measured in a number of ways:

Transmittance, \( T = \frac{I}{I_o} \) \hspace{5cm} (1)

\% Transmittance, \( \%T = 100 \times T \) \hspace{5cm} (2)

Absorbance,

\( A = \log_{10} \frac{I_o}{I} \) \hspace{5cm} (3)

Combine 1 & 3

\( A = \log_{10} \frac{1}{T} \) \hspace{5cm} (4)

Combine 2 & 4

\( A = \log_{10} \frac{100}{\%T} \)

\( A = 2 - \log_{10} \%T \)
Beer-Lambert Law

**Bouguer – lambert’s law**

\[ A \propto b \]

**Beer’s Law:**

\[ A \propto c \]

**Beer-Lambert Law**

\[ A \propto b \cdot c \]

\[ A = a \cdot b \cdot c \]

- \( a = \text{constant = absorptivity} \)
- \( b = \text{cell thickness ;usually 1 cm} \)
- \( C = \text{concentration of species} \)
- \( A = \text{absorbance ,unitless} \)

**Note:**

Absorbance is dependent on the concentration of solution and also on the quantity of solution(meaning sample size);this relationship can also be written as:
Beer’s - Lambert’s Law:

\[ A = a \times b \times C \]

### Expressions of \( a \) (Molar absorptivity)

<table>
<thead>
<tr>
<th>( a )</th>
<th>absorptivity, if concentration (( c )) expressed as gram / Liter.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varepsilon )</td>
<td>(Epsilon), Molar absorptivity, if concentration (( c )) expressed as molar solution (( M )).</td>
</tr>
<tr>
<td>( A_{1%}^{1cm} )</td>
<td>A one percent one centimeter, if ( c ) is expressed in g/100 mL</td>
</tr>
</tbody>
</table>
The relation between absorptivity \( (a) \), \( (\varepsilon) \) & \( A \) (1%, 1cm)

\[
a = \frac{\varepsilon}{MW} = A (1\%, 1\text{cm}) \times 10
\]

\[
\varepsilon = A (1\%, 1\text{cm}) \times MW \times 10
\]

\[
A (1\% - 1\text{cm}) = \frac{\varepsilon}{10 \times MW}
\]
Example:
A solution containing 1.0 mg paracetamol in 100 ml was observed to transmit 70.0% of the incident light. What is the absorbance of the solution at this wavelength?
Solution
\[ A = \log_{10} \frac{1}{T} = \log_{10} \frac{1}{0.700} = \log_{10} 1.43 = 0.155 \]

Example:
An unknown solution of cloxacillin absorb at 350 nm (\( \varepsilon = 1.25 \times 10^4 \text{ cm}^{-1} \text{ mol}^{-1} \text{ L} \)), exhibit an absorbance of 0.368 in a 1-cm cell. What is the concentration of this solution?
Solution
\[ A = \varepsilon b c \]
\[ 0.368 = 1.25 \times 10^4 \text{ cm}^{-1} \text{ mol}^{-1} \text{ L} \times 1.00 \text{ cm} \times c \]
\[ c = 3.75 \times 10^{-3} \text{ mol L}^{-1} \]
Note:

- Both $\varepsilon$ and $A_{(1\%, 1\text{cm})}$ are **characteristic** for each substance.
- Used for **qualitative** & **quantitative** purposes.
- Absorptivity = slope of the curve produced on plotting $A$ vs $C$ at fixed (b) — **calibration curve**.
Finding concentration by plotting a calibration curve

**Approach:**

1. Determine the wavelength $\lambda_{\text{max}}$

2. Do make up a number of solutions of known concentration (standard solution)

3. For each solution you measure the absorbance

4. Plot a graph of that absorbance against concentration

5. You would expect a straight line ($R^2$ value $\approx 1$)

6. Measure the absorbance of the solution with the unknown concentration at the same wavelength, read the corresponding concentration from the graph by locating the absorbance of the unknown on the vertical axis of the graph, the corresponding concentration can be found on the horizontal axis. The concentration of the unknown can also be found using the slope of the Beer’s - Lambert’s Law curve (linear equation).
Since linear relationship, we can make a calibration plot of A vs. conc. for known standards
Since linear relationship, we can make a calibration plot of $A$ vs. conc. for known standards

Linear equation

$$Y = mx + b$$

$$Y_A = m_{\text{slope}} \times \text{conc} + b_{\text{intercept}}$$

i.e. Unknown has an absorbance of 0.400

$$\frac{A - y_{\text{inter}}}{m} = \text{conc}$$

$$\frac{0.400 - 0.0274}{0.015} = 24.8 \text{ mg/mL} \text{ Co(NO}_3\text{)_2}$$
Standards: 50 mL per group 0.200 M Co(NO$_3$)$_2$

Make standards:

<table>
<thead>
<tr>
<th>Vol.</th>
<th>0.200 M Co(NO$_3$)$_2$</th>
<th>Vol. H$_2$O</th>
<th>M Co(NO$_3$)$_2$</th>
<th>mg/mL Co(NO$_3$)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 mL (blank)</td>
<td>10.00 mL</td>
<td>0.00 M</td>
<td>0.00 mg/mL</td>
<td></td>
</tr>
<tr>
<td>1.00 mL</td>
<td>9.00 mL</td>
<td>0.0200 M</td>
<td>3.66 mg/mL</td>
<td></td>
</tr>
<tr>
<td>3.00 mL</td>
<td>7.00 mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00 mL</td>
<td>5.00 mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.00 mL</td>
<td>3.00 mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.00 mL</td>
<td>1.00 mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.00 mL</td>
<td>0.00 mL</td>
<td>0.200 M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M: \[ C_b V_b = C_d V_d \] dilution with total volume 10 mL each time
bulk diluted

\[(0.200 \text{ M Co(NO}_3\text{)}_2)(1.00 \text{ mL}) = (C_d)(10.00 \text{ mL solution})\]

\[(0.200 \text{ M Co(NO}_3\text{)}_2)(1.00 \text{ mL}) = 0.0200 \text{ M Co(NO}_3\text{)}_2 = C_d \]

10.00 mL solution

Standards: 50 mL per group 0.200 M Co(NO$_3$)$_2$

Make standards:

<table>
<thead>
<tr>
<th>Vol.</th>
<th>0.200 M Co(NO$_3$)$_2$</th>
<th>Vol. H$_2$O</th>
<th>M Co(NO$_3$)$_2$</th>
<th>mg/mL Co(NO$_3$)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 mL</td>
<td>9.00 mL</td>
<td>0.0200 M</td>
<td>3.66 mg/mL</td>
<td></td>
</tr>
</tbody>
</table>

Convert mol/L to mg/mL:

\[
\left(\frac{0.0200 \text{ mol Co(NO}_3\text{)}_2}{L}\right) \left(\frac{182.95 \text{ g Co(NO}_3\text{)}_2}{\text{mol Co(NO}_3\text{)}_2}\right) \left(\frac{1000 \text{ mg}}{1 \text{ g}}\right) \left(\frac{1 \text{ L}}{1000 \text{ mL}}\right) = 3.66 \text{ mg mL}^{-1} \text{ Co(NO}_3\text{)}_2
\]

\[
\left(\frac{0.0200 \text{ mmol Co(NO}_3\text{)}_2}{\text{ml}}\right) \left(\frac{182.95 \text{ mg Co(NO}_3\text{)}_2}{\text{mmol Co(NO}_3\text{)}_2}\right) = 3.66 \text{ mg mL}^{-1} \text{ Co(NO}_3\text{)}_2
\]
Standards: 50 mL per group 0.200 M Co(NO₃)₂
Make standards:
Vol.
0.200 M Co(NO₃)₂  Vol H₂O  M Co(NO₃)₂  mg/mL Co(NO₃)₂
1.00 mL  9.00 mL  0.0200 M  3.66 mg/mL

Convert mol/L to mg / mL:
Convert through molar mass of Co(NO₃)₂ (182.95g/mol) and units

\[
\left( \frac{0.0200 \text{ mol Co(NO₃)₂}}{L} \right) \left( \frac{182.95 \text{ g Co(NO₃)₂}}{\text{mol Co(NO₃)₂}} \right) \left( \frac{1000 \text{ mg}}{1 \text{ g}} \right) \left( \frac{1 \text{ L}}{1000 \text{ mL}} \right) = 3.66 \frac{\text{mg}}{\text{mL}} \text{Co(NO₃)₂}
\]

\[
\left( \frac{0.0200 \text{ mmol Co(NO₃)₂}}{\text{mL}} \right) \left( \frac{182.95 \text{ mg Co(NO₃)₂}}{\text{mmol Co(NO₃)₂}} \right) = 3.66 \frac{\text{mg}}{\text{mL}} \text{Co(NO₃)₂}
\]
Case study: Determination of MR concentration

<table>
<thead>
<tr>
<th>C (µg/mL)</th>
<th>C (mole/L)</th>
<th>A (428.5 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>0.348</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>0.500</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>0.680</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0.815</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>0.964</td>
<td></td>
</tr>
</tbody>
</table>

\[ C_{15}H_{15}N_3O_2 = 269.3 \text{ g mol}^{-1} \]

Absorbance of unknown solution = 0.61

Concentration of unknown solution =

\[ \varepsilon \& A (1\% - 1\text{cm}) = \]
Conc of unknown = 0.61 - 0.1973 / 0.1547 = 2.67 x 10^{-6} M

A = \varepsilon b c

\varepsilon = 0.61 / 2.67 x 10^{-6} M = 0.2285 x 10^6 \text{ mol}^{-1} \text{ L}

A (1\% - 1\text{cm}) = \varepsilon / 10 \times MW = 0.2285 \times 10^6 / 10 \times 269.3 = 85 \text{ ml} / \text{g}


**Colorimetry**

In colorimetry we are measuring $A$ of visible radiation by **coloured sample**.

**Requirements for substances to be measured colorimetrically:**

1. **Substance must be coloured** e.g. CuSO$_4$, organic dyes,...etc.

2. If the substance to be analysed is colourless, it must react with certain reagent (known as chromogen) to produce equivalent coloured product. e.g orthophenanthroline which reacts with ferrous (Fe$^{2+}$) in buffered medium (acidic pH) to produce intense red colour.

![Chemical reaction](image)
3- If the substance to be analysed is colourless and there is no suitable chromogen, it must be converted to a certain derivative which has a suitable chromogen. e.g esters, which is first converted to hydroxamic acid derivative through the reaction with hydroxylamine. Hydroxamic acid derivative gives purple colour on addition of ferric (Fe\(^{3+}\)) due to the formation of iron chelate.

**Requirements for ideal chromogen**

1. Should be colourless or easily separated.
2. Should be selective.
3. Its reaction to produce coloured product, should be of known mechanism and proceed stoichiometrically.
4. The full development of colour must be rapid.
5. Produce only one colour of specified \(\lambda_{\text{max}}\).
Requirements for coloured product

1. Should be of intense colour, to increase the sensitivity.

2. Should be unaffected by pH or the pH must be specified and maintained by suitable buffer or the measurement is carried out at \( \lambda \) of isosbestic.

3. Should be stable with time.

4. The reaction of its formation must be rapid and quantitative.

5. The coloured product, should obey Beer-lambert’s law, i.e on plotting A versus C at fixed b, we obtain straight line passing through the origin.
Applications of UV/visible spectroscopy to pharmaceutical quantitative analysis

Assay examples

Furosemide (frusemide) in tablet form:
(i) Tablet powder containing 0.25 g of furosemide is shaken with 300 ml of 0.1M NaOH to extract the acidic furosemide
(ii) the extract is then made up to 500 ml with 0.1M NaOH .
(iii) A portion of the extract is filtrated and 5 ml of the filtrate is made up to 250 ml with 0.1M NaOH.
(iv) the absorbance of the diluted extract is measured at 271 nm .
(v) The A (1 %,1cm) value at 271 nm is 580 in basic solution .

From the data below calculate the % of stated content in a sample of furosemide tablets :
1- Stated content per tablet: 40 mg of furosemide
2- Weight of 20 tablets : 1.656 g
3- Weight of tablet powder taken for assay : 0.5195 g
4- Absorbance reading : 0.596
spectrophotometer
What is a spectrophotometer?

Any instrument which deal with spectrum of LIGHT (Electromagnetic Spectrum) is called spectrophotometer.
Basic components of spectrophotometer

- **Light source**: Vis - Tungsten lamp / UV - Deutrium lamp.
- **Monochromator**: Filter / Prism / Grating.
- **Sample compartment**: Cuvette
- **Detector**: Photocell / photomultiplier tube
- **Recorder**
- **Optics**
How Simple UV-Visible Spectrophotometer Work
Monochromators

• It converts polychromatic light to monochromatic light, i.e. of definite range or \( \lambda \).

\textbf{a) Filters}

• Filters act by selective absorption of unwanted \( \lambda \) and transmit the complementary colour, which is needed to be absorbed by the sample to be analysed.

\textbf{b) Prisms}

• Act by refraction of light. In visible range we use glass prism. In U.V range we use prism made of quartz or fused silica.

\textbf{C) Grating} (act by diffraction and interference)

• Grating consists of a large number of parallel lines ruled very close to each other on a highly polished surface e.g. aluminum or aluminized glass (600 line/mm).
Sample compartment (Cuvette)

- It is made of glass for visible range and quartz or fused silica for U.V range. Its standard path length is 1cm (10mm) some times it is ½ cm.
Detector

a) **Photocell (Photovoltaic cell) e.g Barrier layer cell**

- EMR falling upon a semiconductor surface, where electrons are excited and produce EMF (current) proportional to the intensity of incident light.

- *There is no magnification for the electric signal.*
b) Phototube

There is mild magnification for the electric signal.

-Photo emissive tube
-Photomultiplier tube
Stronger magnification power than photo emissive tube.
Deviation from Beer-Lambert's law

1) Real deviations

- Concentrated solution - molecular interaction.

- Association, charge distribution, complexation or hydration, leading to non-linear response when A is plotted against C.

- This deviation decreases or disappear in very dilute solution.
Deviation from Beer-Lambert's law

2) Instrumental deviations

a. **Irregular deviations:**

- The use of unmatched cuvette (due to industrial defect)
- Unclean handling (e.g. finger print on the cuvette)
- Unclean optics (lenses, mirrors or lamp)

b. **Regular deviations**

- Error in $\lambda$ scale or slit width control.
- Stray light, any light reaches the detector without passing through the sample.
- Non-linear response of detector.
- Radio & TV waves.
Deviation from Beer-Lambert's law

3) Chemical deviations

- Effect of pH (which leads to shifting of $\lambda_{\text{max}}$.)
- Temperature effect
- Time factor - colour stability.
Applications

1- Qualitative analysis

• \( \lambda_{\text{max.}} \), Absorptivity (\( \varepsilon \) or \( A(1\% \text{ - 1cm}) \)), UV and visible absorption spectrum usually give fingerprint of the sample to be analysed.
2- Quantitative analysis

A- Quantitative analysis of a single component.

• Detect $\lambda_{\text{max}}$ of the substance to be analyzed after dissolving in a suitable solvent.

• Construct a calibration curve by plotting $A$ against $C$ at fixed $b$ using standard series of the same chemical present in the sample, at the characteristic $\lambda_{\text{max}}$.

• The Absorbance of the sample to be analysed is determined under the conditions adopted during construction of the calibration curve.

• The concentration of the sample can be determined from the calibration curve.
**B. Quantitative analysis of multicomponent mixture**

Consider a binary mixture of X and Y. For quantitative analysis of this mixture, the following requirements must be fulfilled:

1. The absorption spectrum of X and Y should not show severe overlap.
2. Beer-Lambert’s law must be obeyed for X and Y at their characteristic $\lambda_{\text{max}}$.
3. X and Y must be chemically inert to each other.

\[
C_x = \frac{a_1^y A_2 - a_2^y A_1}{a_1^x a_2^y - a_2^y a_2^x}
\]

\[
C_y = \frac{a_1^x A_2 - a_2^x A_1}{a_1^x a_2^y - a_2^y a_2^x}
\]
Environmental Applications

• UV-Vis spectroscopy can be used for quantitative analysis of metals in water and wastewater.

• One advantage to these spectroscopic methods is that they are easily adapted to the field analysis of samples using a filter photometer.

• One ligand used in the analysis of several metals like diphenylthiocarbazone, also known as dithizone.
Dithizone
Dithizone is a sulfur-containing organic compound. It is a good ligand, and forms complexes with many metals such as lead and mercury.
• Dithizone is insoluble in water, but when a solution of dithizone in chloroform is shaken with an aqueous solution containing an appropriate metal ion, a colored metal–dithizonate complex forms that is soluble in chloroform.

• The selectivity of dithizone is controlled by adjusting the pH of the aqueous sample. For example, cadmium is extracted from solutions that are made strongly basic with NaOH, lead from solutions that are made basic with an ammoniacal buffer, and mercury from solutions that are slightly acidic.
Determination of chlorine in water

- When chlorine is added to water that portion available for disinfection is called the chlorine residual.
- Two forms of the chlorine residual are recognized. The free chlorine residual includes Cl₂, HOCl, and OCl⁻.
- The combined chlorine residual, which forms from the reaction of NH₃ with HOCl, consists of monochloroamine, NH₂Cl, dichlororamine, NHCl₂, and trichloroamine, NCl₃.
• Many analytical methods have been developed to determine the concentration of both forms of residual chlorine.
• One such method is the leuco crystal violet method. Free residual chlorine is determined by adding leuco crystal violet to the sample, which instantaneously oxidizes giving a bluish color that is monitored at 592 nm.
• Completing the analysis in less than 5 min prevents a possible interference from the combined chlorine residual.
• The total chlorine residual (free + combined) is determined by reacting a separate sample with iodide, which reacts with both chlorine residuals to form HOI.

• When the reaction is complete, leuco crystal violet is added and oxidized by HOI, giving the same bluish colored product. The combined chlorine residual is determined by difference.
• The concentration of fluoride in drinking water may be determined indirectly by its ability to form a complex with zirconium. Solutions of zirconium form a reddish colored compound, called a “lake,” that absorbs at 570 nm.
Clinical Applications

• UV/Vis molecular absorption is one of the most commonly employed techniques for the analysis of clinical samples.

• The analysis of clinical samples is often complicated by the complexity of the sample matrix, which may contribute significant background absorption at the desired wavelength.
• The determination of serum barbiturates provides one example of how this problem is overcome. The barbiturates are extracted from a sample of serum with CHCl₃, and extracted from the CHCl₃ into 0.45 M NaOH (pH13). The absorbance of the aqueous extract is measured at 260 nm and includes contributions from the barbiturates and other components present in the serum sample.

• The pH of the sample is then lowered to approximately 10 by adding NH₄Cl, and the absorbance remeasured. Since the barbiturates do not absorb at this pH, the absorbance at pH 10 is used to correct the absorbance at pH 13
Examples for clinical applications

• Total serum protein can be determined by reaction with NaOH, and Cu^{2+} produces blue-violet complex and measure at 540nm.

• Serum cholesterol can be determined by reaction with Fe^{3+} in presence of isopropanol, acetic acid, and H_2SO_4 produces blue-violet complex and measure at 540nm.

• Uric acid can be determined by reaction with phosphotungstic acid produces tungsten blue and measure at 710nm.

• Glucose can be determined by reaction with o-toludine at 100°C produces blue-green complex measure at 630nm.
MASS SPECTROMETRY
(MS)
MS

• Analytical technique that measures the mass-to-charge \( (m/z) \) ratio of ions from a sample.

• The mass spectrum:
  – A pattern representing the distribution of components in a sample.
  – Relative abundance
  – Parent peak
  – Base peak
Mass spectrometry

• For small organic molecules the MW can be determined to within 5 ppm or 0.0005%.

• For large biomolecules the MW can be determined within an accuracy of 0.01%.

• 1 dalton = 1 atomic mass unit (1 amu)
Components of Mass spectrometer

• **Ion Source**
  – Production of Ions from a sample
    • Ions are easier to manipulate than neutral molecules

• **Analyzer**
  – Separation of ions with different masses...
    • mass (m) -to-charge (z) ratios (m/z)

• **Detector**
  – Detects the number of ions of each mass produced and m/z ratios are stored...
    • this signal is sent to a data system
How does a mass spectrometer work?

1. Create ions
2. Separate ions
3. Detector

Ionization source

Mass analyzer
- Triple Quadrupole

Mass spectrum
Fragmentation pattern
Database analysis

Ionisation: $M \rightarrow M^+ + e^-$
Fragmentation: $M^+ \rightarrow m_1^+ + m_2^-$

molecule molecular ion fragment ion
Mass spectrometer

Diagrammatic representation

Sample

Ionizer

Mass Analyzer

Detector
Mass spectrum

Base peak

Parent peak

Base peak

Parent peak
Mass spectrum

151.17 g/mol

Base peak
Parent peak
Analysing results

- **Biomolecules:**
  - molecular masses measured accurately within **0.01%** of the total of the sample
  - This is sufficient to allow minor mass changes to be detected

- **Organic molecules:**
  - molecular mass measured accurately within **5 ppm** or less
  - sufficient to confirm the molecular formula of a compound

- **Qualitative results:** “*What is in the Sample?*”
- **Quantitative results:** “*How much of it is in the sample?*”
Applications

- **Biotechnology**
  - Analysis Proteins, peptides, oligonucleotides

- **Pharmaceutical**
  - drug discovery
  - Pharmacokinetics
  - drug metabolism

- **Clinical**
  - neonatal screening
  - hemoglobin analysis
  - drug testing

- **Geological**
  - oil composition
Applications

• Environmental
  ▫ PAH - Polycyclic aromatic hydrocarbons
  ▫ water quality
  ▫ food contamination

• Forensic analysis
  ▫ Identification of Crime Scene Materials
  ▫ Analysis of body fluids and hair for drugs
  ▫ Detection of hidden explosives in luggage and mail
  ▫ Examination of evidential materials
Peptide Mass Fingerprinting (PMF)
INFRARED SPECTROSCOPY (IR)
Spectrum of EMR
✓ Infrared radiation lies between the visible and microwave portions of the electromagnetic spectrum.

✓ Infrared waves have wavelengths longer than visible and shorter than microwaves, and have frequencies which are lower than visible and higher than microwaves.

✓ The Infrared region is divided into: near, mid and far-infrared according to its position from visible region.
Near-infrared refers to the part of the infrared spectrum that is closest to visible light and far-infrared refers to the part that is closer to the microwave region.

Mid-infrared is the region between these two.

IR absorption positions are generally presented as wavenumber ($\nu'$).

Wavenumber defines the number of waves per unit length (cm$^{-1}$).

Wave number = 4000 to 600 cm$^{-1}$
✓ Molecules absorb IR radiations leading to change in the vibrational energy of the compound.

✓ IR absorption leads to change in bond length and bond angle.

✓ Infrared spectroscopy can be used to find out about **covalent bonds** in molecules.

✓ The change in bond length occurs due **stretching vibrations** on the other hand, change in bond angle occurs due to **bending vibrations**.

✓ The bonds between atoms in the molecule stretch and bend, absorbing infrared energy and creating the infrared spectrum.

✓ IR spectrum is a plot between of % transmission vs. frequency (wavenumbers)
Types of Molecular Vibration in IR Spectroscopy
Principles:

Electromagnetic radiation ranging between 400 cm$^{-1}$ and 4000 cm$^{-1}$ is passed through a sample and is absorbed by the bonds of the molecules in the sample causing them to stretch or bend. The wavelength of the radiation absorbed is characteristic of the bond absorbing it.
General uses

- All types of organic and many types of inorganic compounds
- Identification of functional groups in organic materials
- Fingerprinting for polymers, films, coatings, packing plastics and complex compounds.
- Quantitative determination of compounds in mixtures
- Determination of molecular conformation (structural isomers) and stereochemistry (geometrical isomers).
Advantages

• One of the most common spectroscopic techniques used by organic and inorganic chemists.

• Important and popular tool for structural elucidation and compound identification.

• Non destructive method.

• Accept a wide range of sample types such as gases, liquids, and solids.
**Instrumentation**

There are two types of instruments commonly used for obtaining IR spectra:

(i) Dispersive instruments, use a monochromator
(ii) Fourier transform instrument, use an interferometer
The most important parts are:
1- Source of IR radiation
2- Sample and blank compartments
3- Detectors
3- Readout meter.

Source of IR radiation
An inert solid is electrically heated to a temperature in the range 1500-2000 K. The heated material will then emit infra red radiation.

Detectors
There are three categories of detector;
✓ Thermal
✓ Pyroelectric
✓ Photoconducting
✓ **Thermocouples** consist of a pair of junctions of different metals; for example, two pieces of bismuth fused to either end of a piece of antimony. The potential difference (voltage) between the junctions changes according to the difference in temperature between the junctions.

✓ **Pyroelectric detectors** are made of a pyroelectric material, such as triglycerine sulphate. The properties of a pyroelectric material are such that when an electric field is applied across it, electric polarization occurs. In a pyroelectric material, when the field is removed, the polarization persists. The degree of polarization is temperature dependant.
✓ Photoelectric detectors

Absorption of IR promotes nonconducting valence electrons to a higher, conducting, state. The electrical resistance of the semiconductor decreases where the resistance is highly affected by temperature changes. These detectors have better response characteristics than pyroelectric detectors.
Instrument calibration

To ensure that instruments conform with BP specifications, the wavelength scale of the instrument is checked by obtaining an IR spectrum of polystyrene film (shown in figure below).

Some of bands used to check the accuracy of the wavelength scale of an IR spectrophotometer are shown in figure above.

The permitted tolerances for variation in the wavelengths of absorptions are mainly ±0.3
Sample preparation

There are three traditionally modes of sample preparation:

(i) Run as a film sandwiched between two NaCl or KCl disc. For this method sample must be liquid or must be ground to a paste in a liquid, usually liquid paraffin.

(ii) The sample is ground to a powder with KBr or KCl. KBr is usually used unless a hydrochloride salt is being analysed, in which KCl is used to avoid halogen exchange. On weight for weight basis, the weight of the sample used is about 1% of the weight of KBr used. Finely ground powder compressed into a disc under vacuum by subjecting it to a pressure of 800 kpa.

(iii) IR spectra of liquids or solutions in an organic solvent, commonly chloroform, may be obtained by putting the liquid into a short pathlength cell with a width of ca 1 mm. Cell are constructed from sodium or potassium chloride and obviously aqueous samples can not be used.
Preparation of sample as disc and mull

**Preparation as mull**

Sandwich between NaCl discs

Grind sample in liquid paraffin

**Preparation as KBr disc**

KBr disc containing dispersed sample

Compress powder at 800 KPa under vacuum

Grind sample in KBr and transfer to die
IR spectrum

4000-1000 cm\(^{-1}\)  < 1000 cm\(^{-1}\)

Functional group region

Fingerprint region

C-H

C=O
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GOOD LUCK
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