Lecture 2

Clinical biochemistry Laboratory Training

By:

Ahmed Danbous Obayes AL-Hussainy

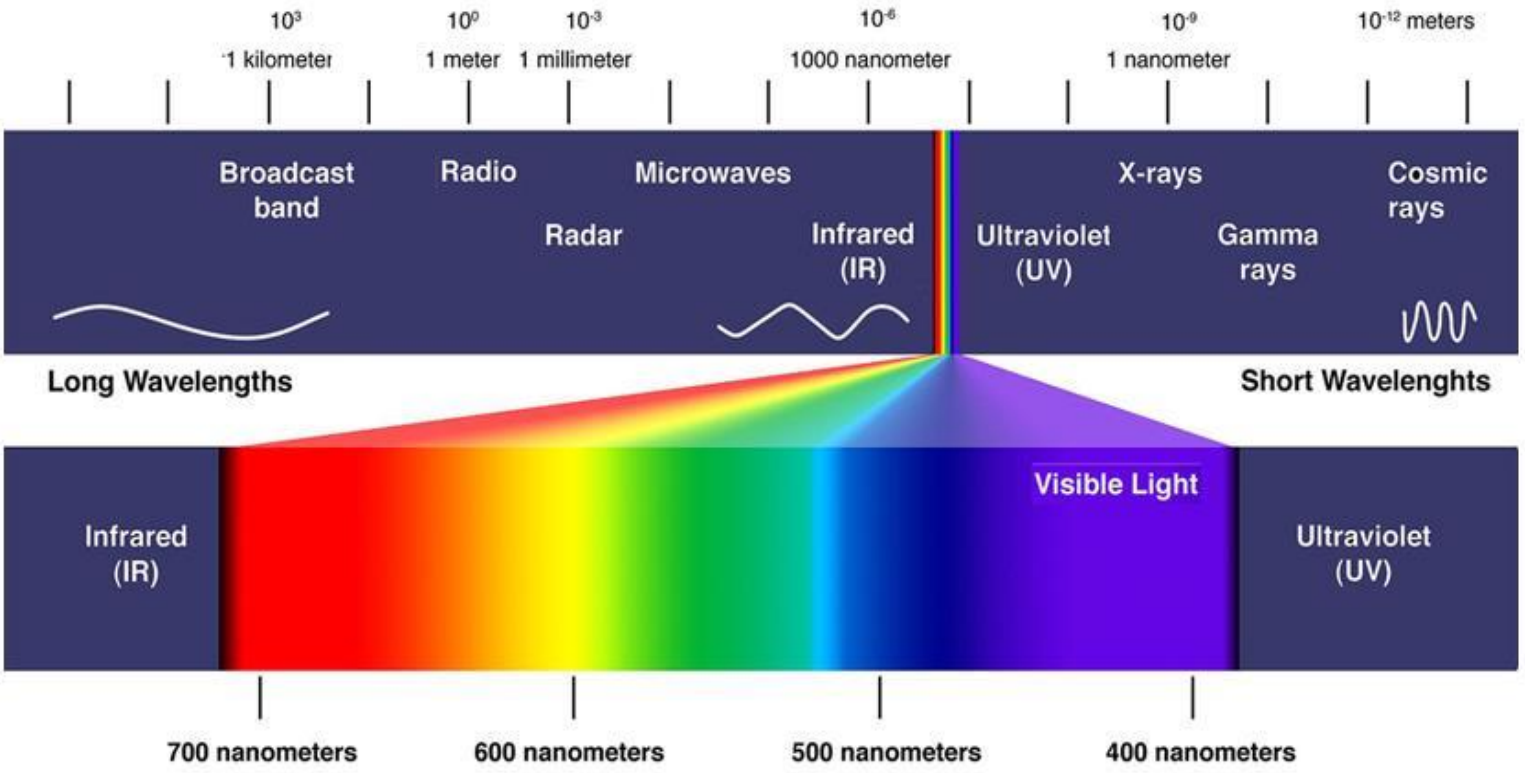
Spectral Technique

A spectrum is defined as the characteristic wavelengths of electromagnetic radiation that is emitted or absorbed by an object or substance, atom, or molecule, and by the spectral technique can be estimation that different in the wavelength.

Areas of the electromagnetic spectrum in laboratories

1-UV radiation (10 -350) nm
2-Visible light (390 -780) nm

Spectrum



Spectral techniques

1- *Spectrophotometer*

2- *Atomic spectroscopy*

Spectrophotometer

Definition:

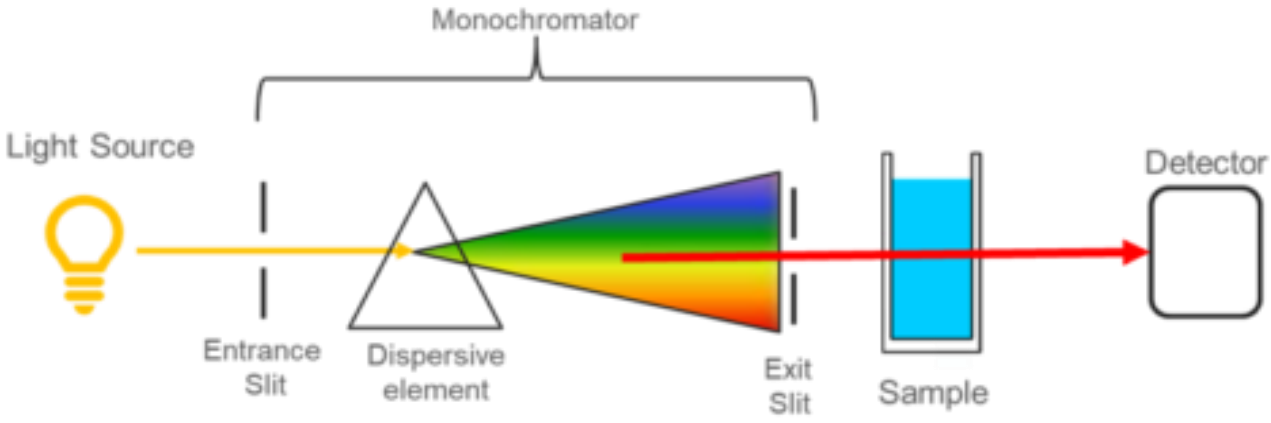
Is an instrument that measure the amount of light absorbed or transmitted by the sample.

Principle:

Spectrophotometer consists of two:

1- **Spectro-meter : to produce light for any selected wave length**
2- **Photo-meter: to measure the intensity of light and the analyte put between them. Spectrophotometer is a procedure for determining how much light is reflected by a chemical material by measuring the strength of light as a light beam travels through the sample solution .The fundamental theory is that light is absorbed or emitted over a certain wavelength spectrum by each compound.**

Components of Spectrophotometer:



1- Light source

<u>For ultraviolet absorption use:</u>

a- H_2 Halogen lamp its wave length ranges from 190-380 nm

b- D_2 Deuterium lamp its wave length ranges from 185-400 nm

We prefer D_2 lamp because of its higher stability and it emits continuous radiation.

<u>For visible absorption use:</u>

a-Tungsten lamp 350-2200 nm

b- Tungsten halogen lamp 240-2500 nm

We prefer tungsten halogen lamp because it has longer life can be used at lower wave length.

2-Prism:

Dispersion devices causes a different wave length of light to be dispersion at different angle

3-Slit:

Monochromators used for selecting one wavelength.

4-Cell (cuvette):

The cuvette or absorption cells, must made from material that is transparent to radiation in the spectral region of interest.

a- glass 320-950 nm
b- quartz < 320 nm

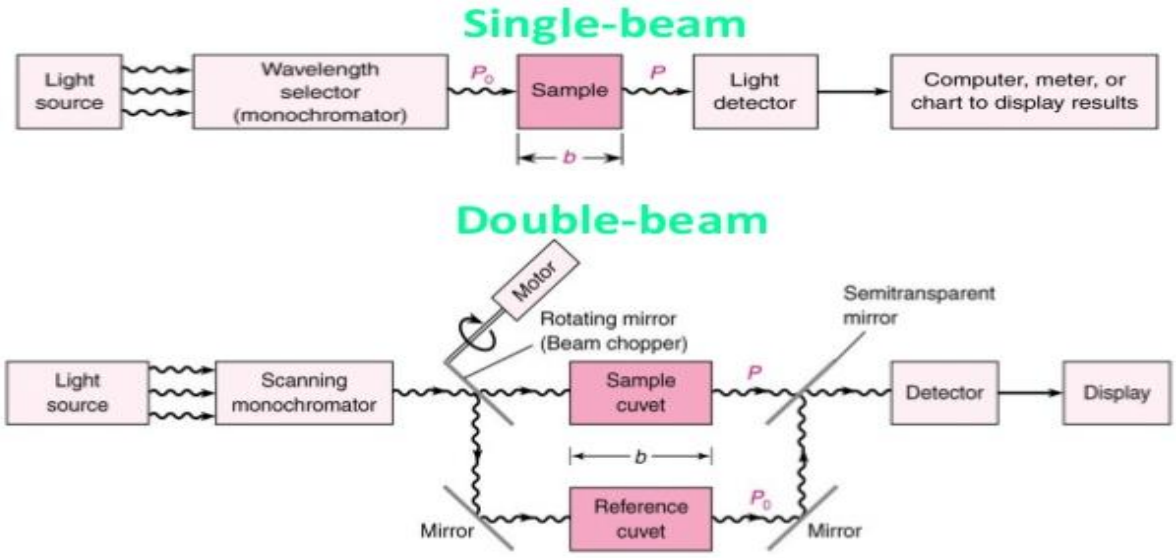
5-Detector (Photometer):

A device used to convert the radiant energy to electrical signal.

6-Read out device:

The data from the detector are displayed by a readout device, such as an analog meter, digital display.

Design of Spectrophotometer



Applications in our life
-Clinical
-Food and drink
-industrial / pharmaceutical

Beer-Lambert law:

The concentration is directly proportional to the absorbed radiation

$$A=E\ b\ c$$

A= Absorbance
E = Molar absorptivity coefficient (L/mole.cm).
c = Concentration (mole/L).
b = Path length (cm).
Instrument which measures an amount of light that a sample absorbs .The spectrophotometer works by passing a light beam through a sample to measure the light intensity of a sample.
The intensity of light is symbolized as I_0 measure the number of photons per second. When the light is passed through the blank solution, it does not absorb light and is symbolized as I.
Other important factors are Absorbance A and Transmittance T.
$T = I / I_0$
$A = - \log T$
Here we need to measure the intensity of light that passes a blank solution, and later measures the intensity of light passing a sample. Calculate the transmittance and the absorbance.
A number of protons transmitted and absorbed depended on the length of the cuvette and the concentration of the sample.

The transmittance and absorption relation is:
Absorbance (A) = log 1/T

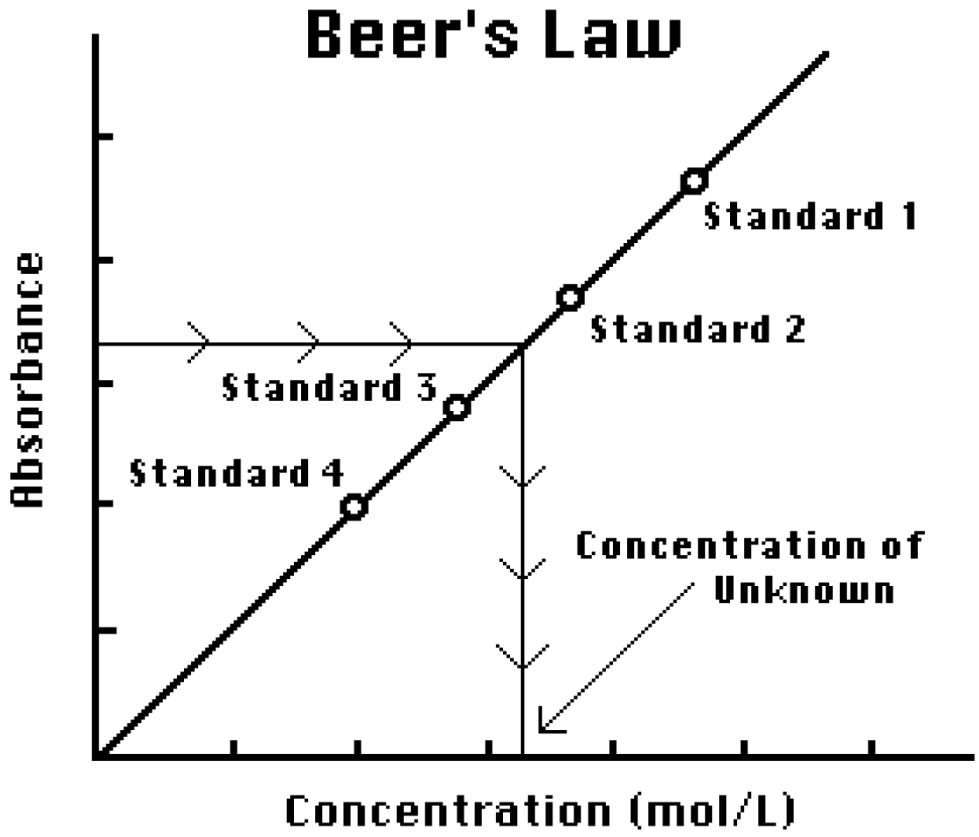
$$A = \log 1 - \log T$$
$$A = -\log T$$
$$T = I_t / I_0$$
$$A = -\log (I_t / I_0)$$

The transmittance of an unknown sample can be calculated using the formula given below:
Transmittance (T) = I_t/I_0

Here,

I_t = Light intensity after passing via cuvette.

I_0= Light intensity before passing via cuvette.



Concentration unknown/concentration standard =Absorbance unknown / Absorbance standard

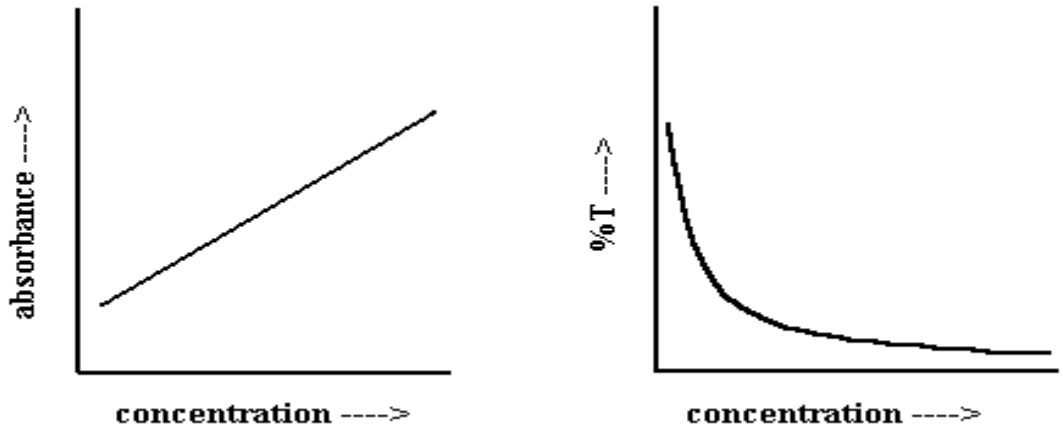
C unk. = (A unk / st unk) * c st

Blank or reference solution

Reference solutions are those with a known measured amount of the target element. A Blank solution is one without any known amount of target element.

Colorimetric analysis

Chromatic analyzes in which the concentration depends on the color.



Borders of Beer-Lambert Law

1- Solution should be clear
2- Solution should be low concentration
3- Solution should be colored
4- Monochromatic light

Atomic spectroscopy

1- Atomic absorption spectroscopy
2- Atomic emission spectroscopy

Atomic spectroscopy is the determination of metal elemental composition by its electromagnetic or mass spectrum.

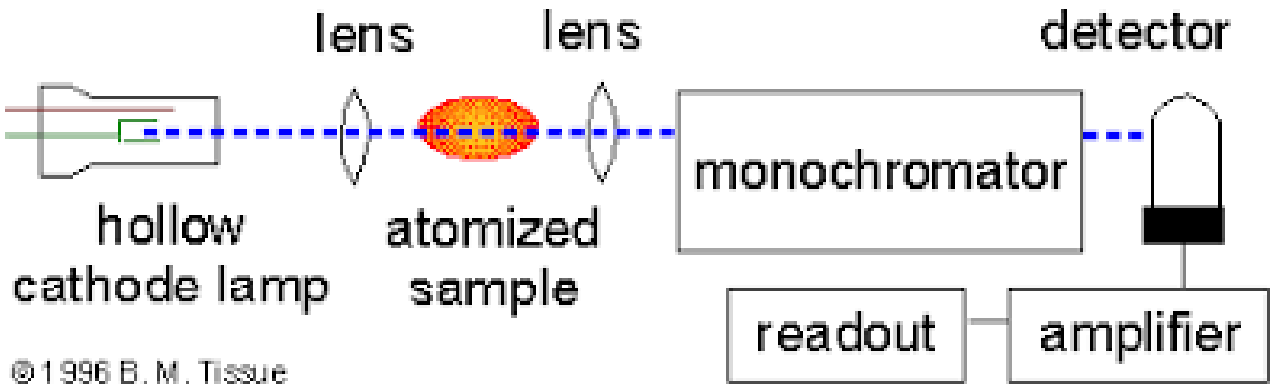
Principle:
Each element absorb its own unique wavelength.
Estimation of the sample as it's qualitative and quantitative.

Application:
1- Quantative analysis by calibration curve method

2-Used to estimation very low amount for metal reach ppm (part per million)

3-Used to estimation of the metal toxic in food (Cu, Ni, Zn ….)

4-Used to estimation of the Na ,k in blood



Component of Atomic absorption spectroscopy

1- Flame

Insert the dissolved sample that contains a metal into the flame. There will be two processes, the first process is vaporized, the solvent will evaporate, and the second process will turn into atoms.

2-Hollow cathode lamp

This part absorbs the energy from turning the sample into atoms when the sample is inserted into the flame and emits it in the form of light, but not all the atoms are excited by the flame and these atoms will absorb part of the energy produced by the hollow cathode lamp and passed light through monochromator

3-monochromator

Selected wavelength

4-Detector

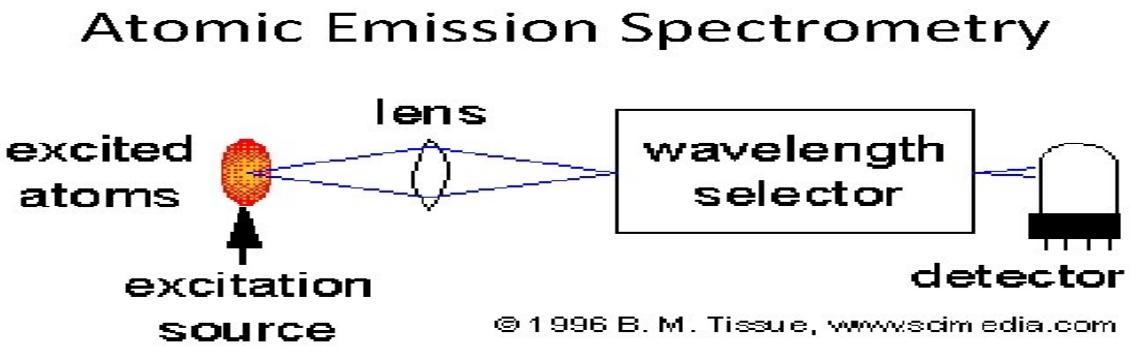
Estimation the absorbance

2-Atomic emission spectroscopy (flame photometry)

Principle:

Each metal emits its own unique wavelength.

Estimation of the sample as it's qualitative and quantitative.



Inserting the sample and subjecting it to high pressure with oxygen, the sample will turn into aerosol and be of a high energy level and then exposed to a flame and these high energy atoms will return to the normal energy level and emit energy in the form of photons.

Estimation qualitatively & quantative by emission spectrum

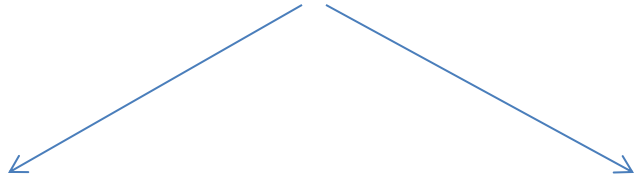
Example:

Na: yellow

Atomic emission spectroscopy	**Atomic absorption spectroscopy**
1-Atoms -thermal energy-excited-ground state	Atoms unexcited
2-Estimation of emitted light	Estimation of absorbed light
3-Emitted light depend on atom excited	absorbed light depend on atom unexcited
4-Does not require additional radiation source	Required hollow cathode lamp
5-does not follow Beer s law	Follow Beer s law

The principle of chemical analysis

chemical analysis



1-colorimetric methods 2- U.V methods

colorimetric methods: A technique used to measure the concentration of a compound in a solution.

Analyte + chromogen ⟶ product (colored)

400 – 750 nm visible light

Analyte : a substance or material determined by a chemical analysis.

Chromogen : a substance which can be readily converted into a dye or other compound.

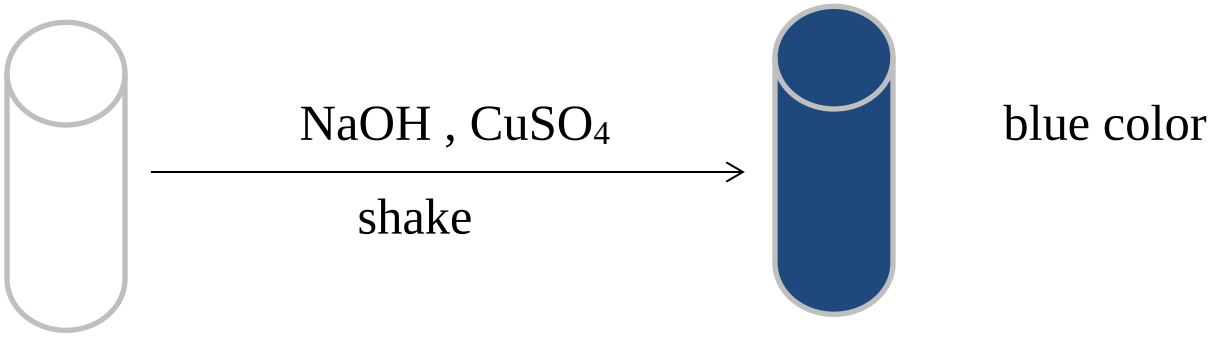
Colorimetric methods

Single step assay **coupled steps assay**

Examples

Single step assay : Biruet reaction

test tube contain protein



blue color

Coupled steps assay:

Enzymatic methods (enzymatic determination of cholesterol)

Cholesterol + oxygen $\xrightarrow{\text{cholesterol oxidase}}$ **cholestenone + H_2O_2**

H_2O_2 + chromogen + phenol $\xrightarrow{\text{peroxidase}}$ **product (quinoneimine)**

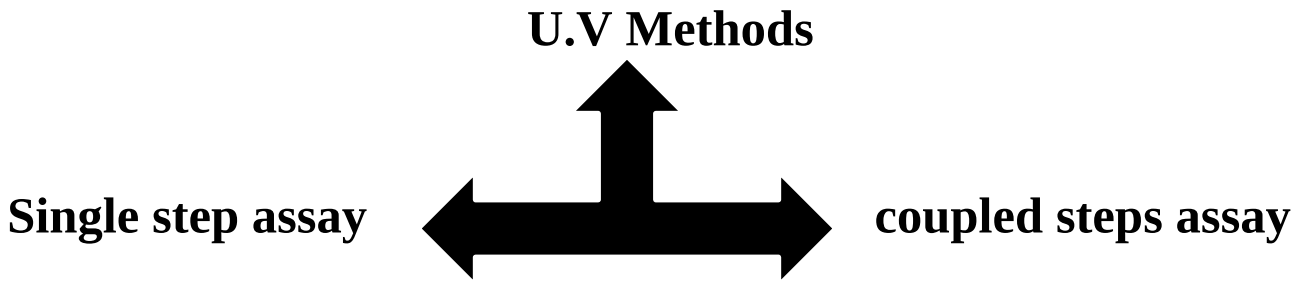
U.V Methods

No visible color change

(10 - 400)

Difference in absorbance

Oxidation & Reduction



Example

Single step assay

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} NAD^{+} + \text{lactate}$$

340 nm

Coupled steps assay

$$\text{Alanine} + \alpha\text{-ketoglutarte} \xrightarrow{\text{ALT}} \text{glutamate} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} NAD^{+} + \text{lactate}$$

Kinetic assay (colormetric & UV)

Depend on the rate or speed of reaction

Analyte $\xrightarrow{\text{enzyme}}$ product

A ——— B

In kinetic assay estimation of different in absorbance according to time .

Absorbance 1 after 1 minute

Absorbance 2 after 2 minute

etc

ΔAbsorbance = (A2 – A1)+(A2-A3) /number of absorbance

Example

UV kinetic assay (LDH ,AST ,ALT ,Amylase ,Ck)

Single step:

Creatin phosphate + ADP $\xrightarrow{CK}$ Creatinine +ATP

Two step :

Glucose + ATP $\xrightarrow{HK}$ ADP + Glucose-6-phosphate

G-6-p + $NADP^+$ $\xrightarrow{G6PD}$ 6-phosphogluconate + $NADPH+H^+$

Colorimetric kinetic assay

Example

Creatine $\xrightarrow{\text{picrate alkaline}}$ color complex (yellow)

492 nm

End point assay

Allow to reaction complete

End point (UV & Colorimetric)

Example

End point UV:

Urea + $2H_2O \xrightarrow{\text{urease}} 2NH_4^+ + CO_2$

End point colorimetric:

Cholesterol-oxidase-peroxidase.