Bioassay

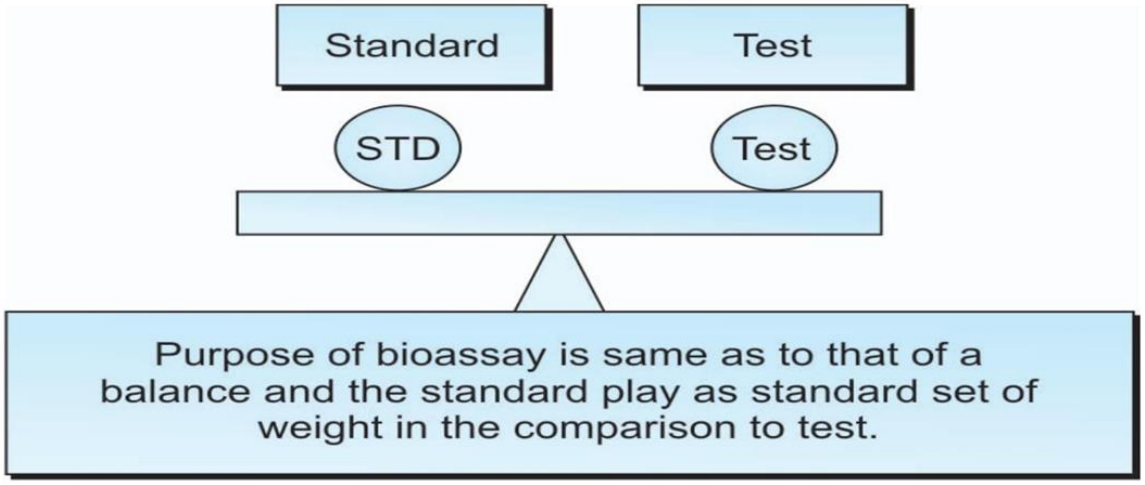
Presenter: Dr Amresh kumar (PGJR)
Moderator: PROF Dr VINAY GUPTA
DEPARTMENT OF PHARMACOLOGY
UPUMS

Outline:

- Introduction
- Indications of bioassay
- Principles of bioassay
- Advantages of bioassay
- Types of bioassay
- Organ bath
- Physiological salt solution
- Methodology
- Conclusion
- References

Introduction

- Bioassay is defined as estimation of relative potency of active principle in the test solution by comparing with standard solution on living tissue (Intact -animal/isolated tissue)



- It was started in the late 18th century, when standardization of diphtheria antitoxin was done by **Paul Ehrlich**

Bioassay in History

Canary in coal Mine?

To provide advance warning of dangerous levels of methane in the air, miners would take methane-sensitive canaries into coal mines. If the canary died due to a build-up of methane, the miners would leave the area as quickly as possible.

Indications of Bioassay

- Active principle has some pharmacological action but chemical structure is not known

  e.g: long acting thyroid stimulants

- Estimation of biological activity of substance which are obtained from natural source

  e.g: penicillin G

- Screening of new compound for biological activity

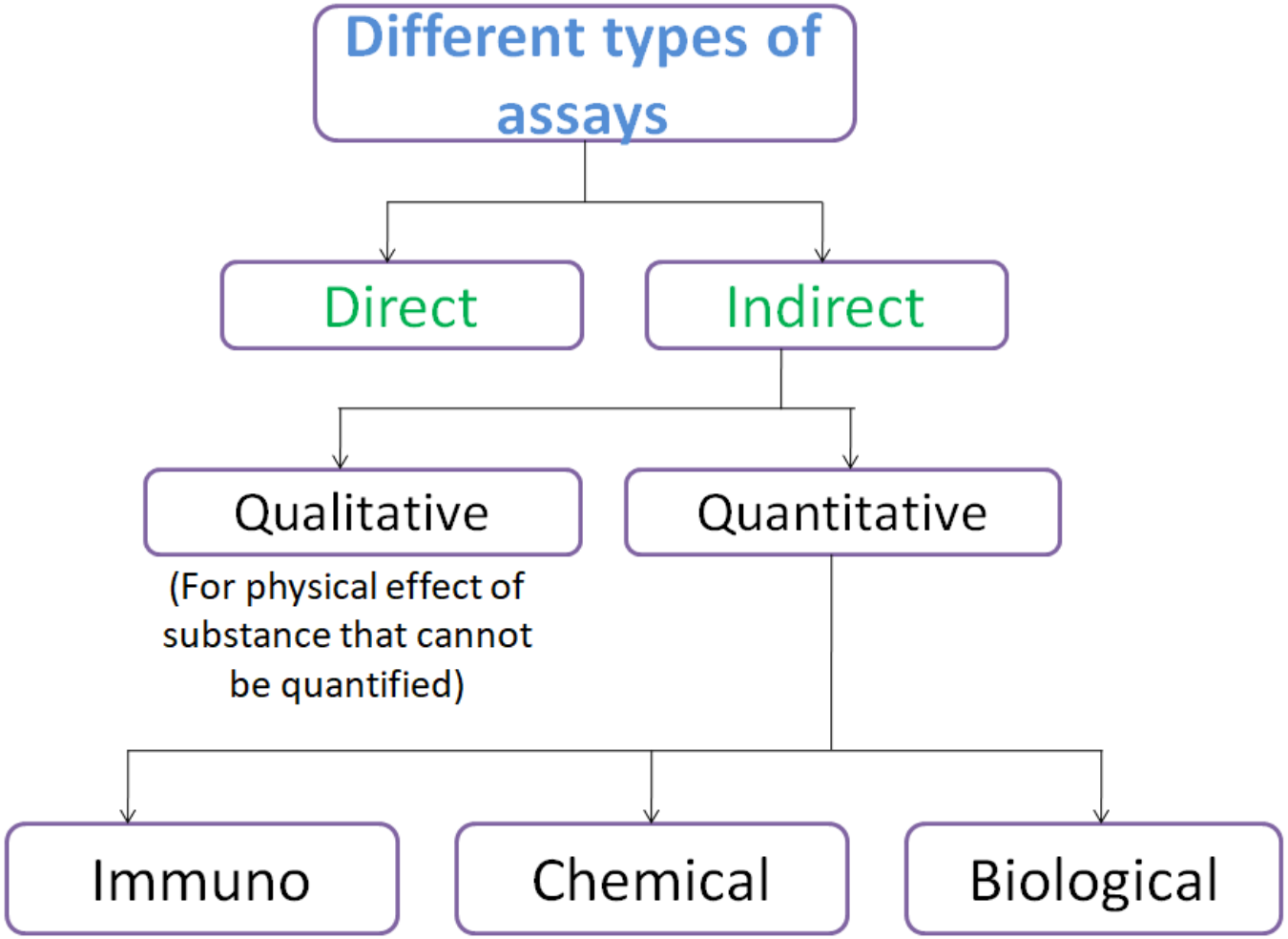
  e.g: new drug development

- Study of LD50/ED50

Contd..

- Biological standardization of drugs from natural sources which cannot be obtained in pure form

  e.g: oxytocin, heparin, insulin
- Compound with similar structure but different biological activity
- Standardization of vaccines, biologics, antisera, disinfectants and antiseptics


Different types of assays
Direct
Indirect
Qualitative
Quantitative
(For physical effect of substance that cannot be quantified)
Immuno
Chemical
Biological

Principles of Bioassay

- Biological response produced by the active principle has to be same in all animals of same species
- Should be sensitive i.e quantity of response produced by particular dose should be same in same animal when tested at different times or all animals of same species at same time
- Standard solution should have same activity as the test solution
- Activity assayed should be of intrest

Contd..

- Should be specific
- Methods should be reproduced and stable over a period of time
- Individual variation should be considered and minimized

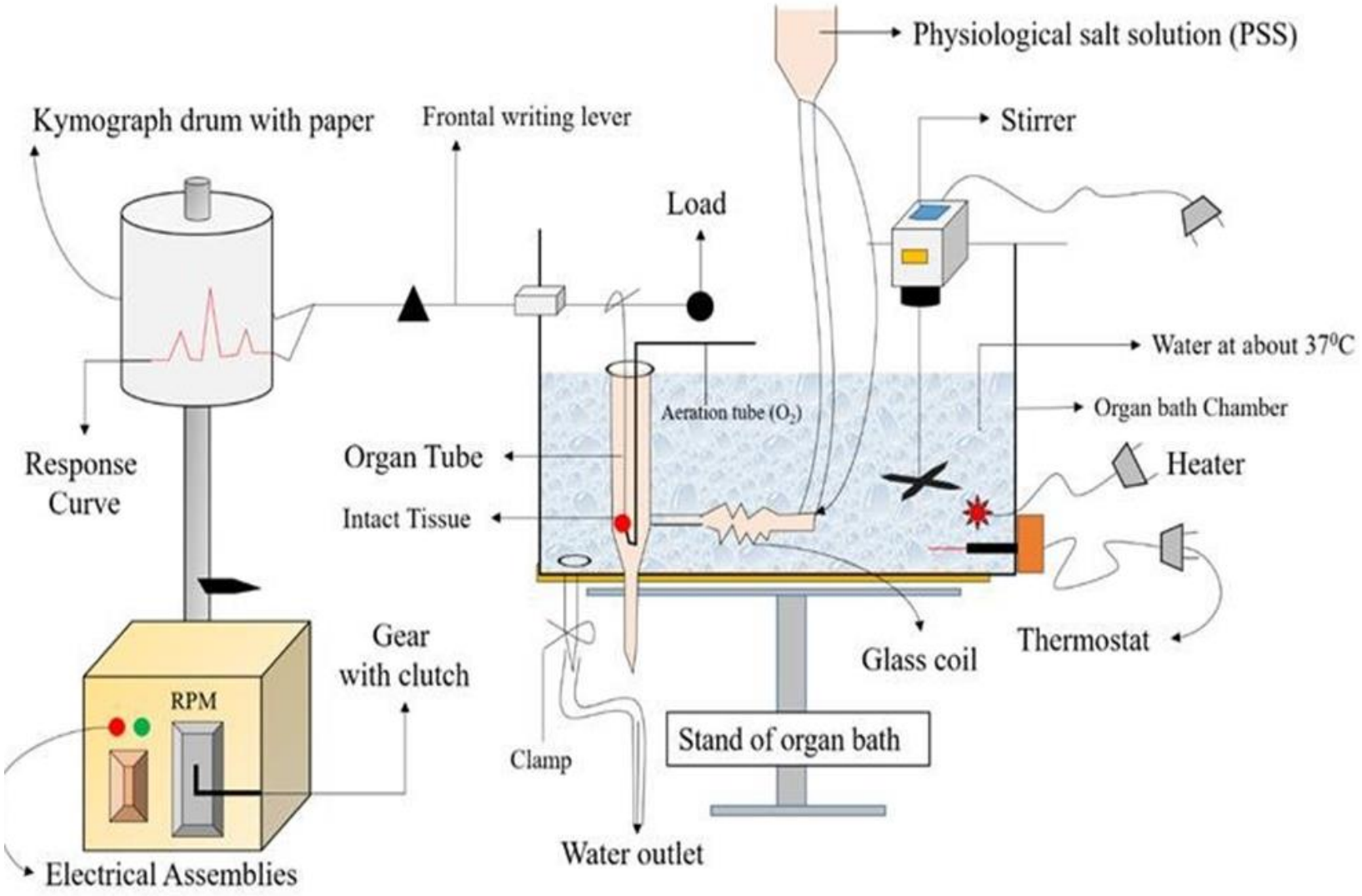
Advantages of Bioassay

- Highly sensitive, specific, reproducible, and stable
- Simple and fast procedure
- Can be possible with less amount of test solution

Disadvantages:-

- Loss of tissue sensitivity over a period of time
- Biological and methodological errors
- Time consuming
- Dose ranging study cannot be possible

ORGAN BATH


Physiological salt solution (PSS)
Kymograph drum with paper
Frontal writing lever
Stirrer
Load
Water at about 37^{0}C
Aeration tube (O_2)
Organ bath Chamber
Response Curve
Organ Tube
Heater
Intact Tissue
Gear with clutch
Glass coil
Thermostat
RPM
Clamp
Stand of organ bath
Water outlet
Electrical Assemblies

Sherrington recording drum with Kymograph

- Adjustable speed according to tissue
- **Slow contracting**-low speed, **fast contracting-** high speed
- Standard speed 1 revolution/96 minute (0.014 rpm)
- Kymograph to record the response of the tissue/muscle against known/ unknown conc. of the drug
- Glossy/ smooth side faced outside
- Rough side is stuck to drum during mounting
- Stylus as a writer on smoked/ink kymograph

LEVER AND MAGNIFICATION

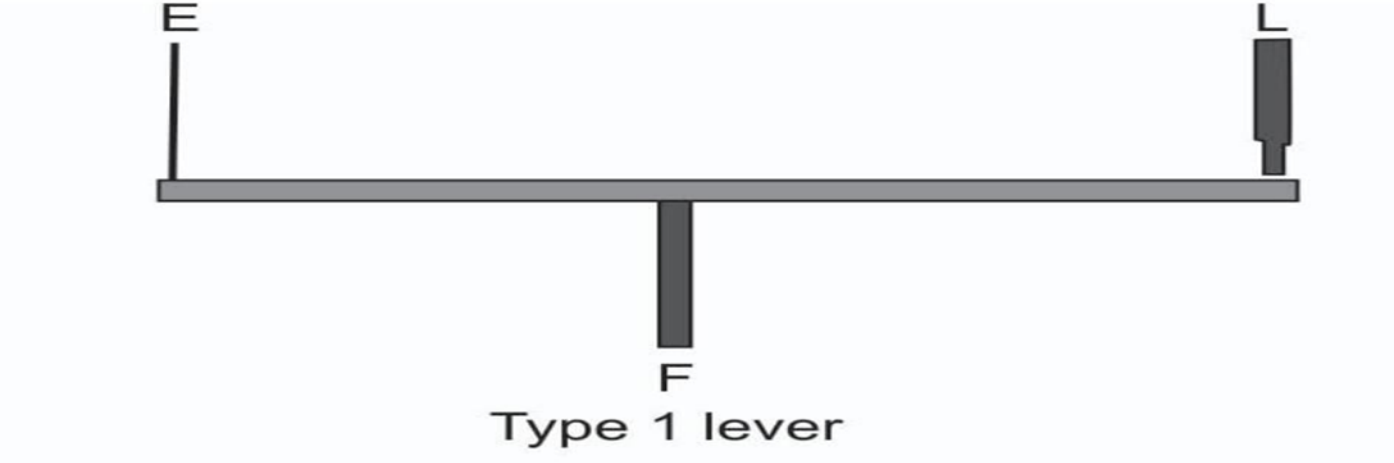
- Working of the lever is denoted by Newton's law of motion and statics:

Load force × length of load arm = Effort force × length of effort arm."

- **Lever has four basic parts:**

1. Effort arm(E): Tissue attachment
2. Load arm(L): Where response is recorded
3. Fulcrum: Point from which distance is calculated for load arm and effort arm
4. Stylus: Writing point on the lever

- Type 1 lever is used in the in vitro bioassays



Type 1 lever

- Lever should be light weight, fine and rigid to avoid bending while recording response

- **Magnification (Mx):-** It is the ratio of distance between fulcrum and writing point to the distance between fulcrum and tissue attachment
- **Mx = A/B**

  A = distance between fulcrum and writing point

  B =distance between fulcrum and tissue attachment
- **The standard rule is that**

- If tissue preparation is slow contracting, magnification is kept high (10-15 times)
- But when tissue is fast contracting then magnification is kept low (5-10 times)

Different types of lever

Simple lever: Made of stainless steel, aluminum or wood with stylus* attached to it.
Attachment of simple lever should be ***tangential*** to the smoked drum

Frontal writing lever (FWL): Made of stainless steel, and aluminum with stylus* (two arm) attached to it. Attachment of FWL should be ***perpendicular*** to the smoked drum. It magnified a small contraction of tissue/muscle on the kymograph

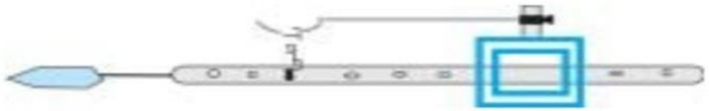
Gimbal lever (GL): Made of stainless steel, or aluminum with stylus* attached to it. A roller is fitted in between for free movement by the gravity force. Attachment of GL should be ***tangential*** to the smoked drum.

Sprung lever: Made of stainless steel, or aluminum with stylus* attached to it with help of return spring. Tension of the lever is adjusted with the screw attached at the opposite end of stylus. Attachment of simple lever should be ***tangential*** to the smoked drum

Auxotonic lever (AL): Made of stainless steel, aluminum or wood with stylus* attached to it.
Attachment of AL should be ***perpendicular*** to the smoked drum.

$\frac{A}{B}$ $\frac{20\text{ cm}}{4\text{ cm}}$ 5

Torsion lever (TL): Made of stainless steel, aluminum or wood with stylus* attached to it.
Attachment of TL should be ***tangential*** to the smoked drum



Starling heart lever (SHL): Made of stainless steel or aluminum with detachable stylus attached to it, Attachment of SHL is perpendicular to the smoked drum.

Physiological Salt Solution (PSS)

- It is necessary to keep tissue viable outside the body
- It provides ionic and nutritional supply to the tissue
- There are different types of PSS and selection of them varies with the tissue
- Distilled or deionized water is used to prepare it
- Most commonly used PSS:-

- Frog Ringer
- Tyrode
- De-Jalon
- Krebs solution

Main components of PSS

1. Sodium (Na+)
2. Chloride (Cl–)
3. Potassium (K+)
4. Magnesium (Mg+)
5. Calcium(Ca+)
6. Glucose

Role of Each Ingredient

1 **Sodium (Na+):** One of the major extracellular cation

It makes solution isotonic by maintaining the osmolarity

2 **Potassium (K+):** It is the major intracellular cation

Its role is remarkable in maintaining heart rate and rhythm

3 **Calcium chloride (CaCl2):**

It controls excitability of Contraction-relaxation coupling

4 **Magnesium chloride (MgCl2):**

Second most common intracellular cation

Reduce the spontaneous activity of tissue

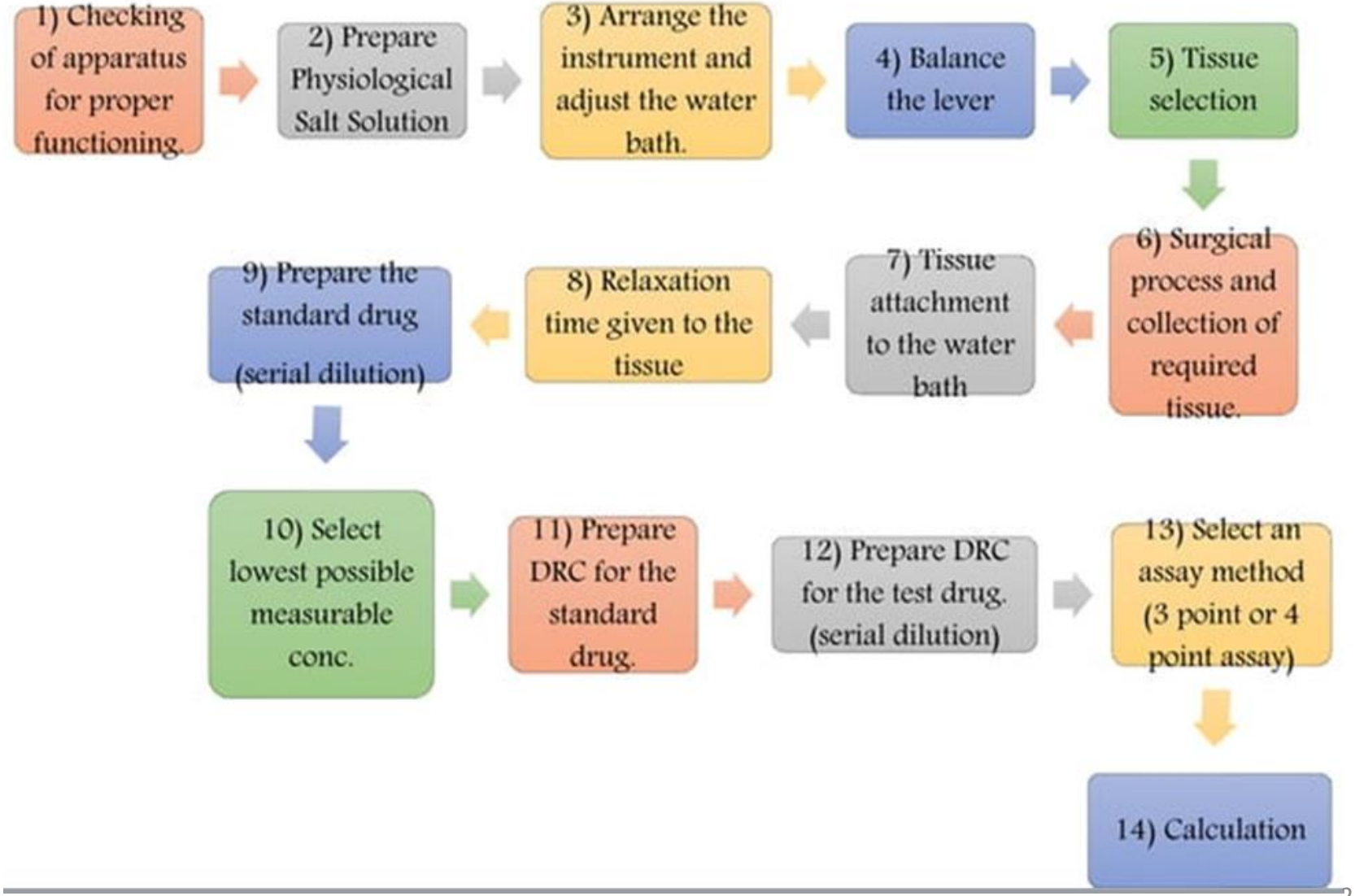
5 **Bicarbonate (HCO3 –) and Sodium hydrogen phosphate (NaHPo4):** Acts as a buffer

6 **Glucose**: Major nutrient

Composition of different types of PSS (Salts in g/10 liters)

Ingredients	*Tyrode*	*Krebs*	*De Jalon*	*Frog Ringer*	*Ringer Locke*	*McEwens*
NaCl	80.0	69	90.0	65.0	90.0	76.0
$NaHCO_3$	10.0	21	5.0	2.0	2.0	21.0
D Glucose	10.0	20	5.0	20.0	10.0	20.0
KH_2PO_4	–	1.6	–	–	–	–
NaH_2PO_4	0.50	–	–	–	–	1.44
KCl	2.0	3.6	4.2	1.4	4.2	4.2
$MgSO_4.7H_2O$	–	2.90	–	–	–	–
$MgCl_2$	1.0	–	–	–	–	–
Sucrose	–	–	–	–	–	5
$CaCl_2$	2.64	3.70	0.8	1.58	3.2	3.0
Aeration	Air	95%O_2 + 5% CO_2	95%O_2 + 5% CO_2	Air	O_2	95%O_2 + 5% CO_2

Methodology

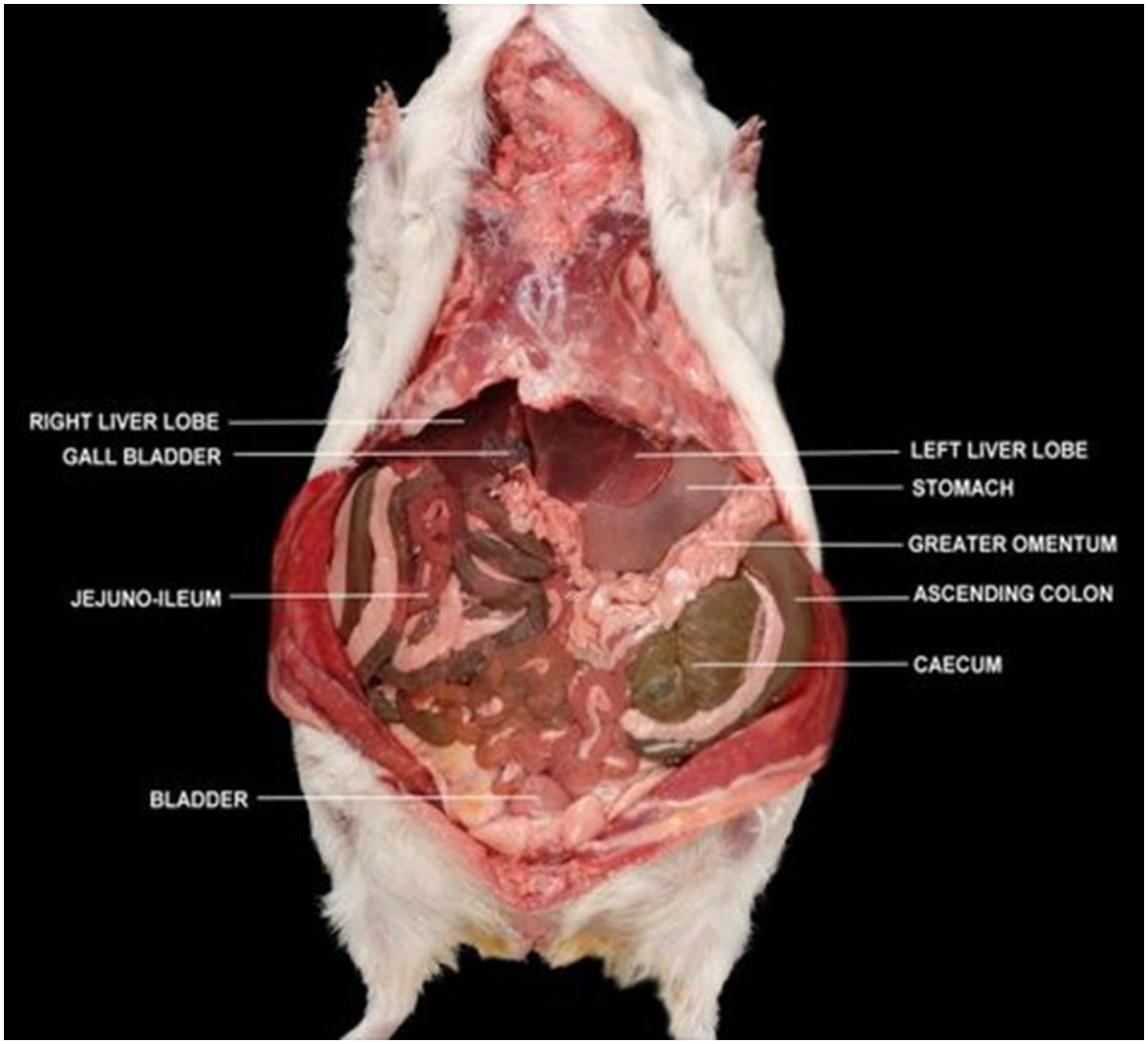


Surgical process and collection of tissue

- Keep the animals Fasting for at least 24-48 hr, water is given ad libitum
- Sacrifice the guinea pig by stunning (a strong blow) on the head
- keep the animal on the dissecting board (DB) & Fix by tying its legs with thread
- Vertical midline incision after a small horizontal cut to expose the abdominal contents
- Identification of the ileum is done by-

  (1) identify the cecum then come back at least of 10 cm

  (2) identify stomach, then go forward to identify the ileum 10 cm before cecum

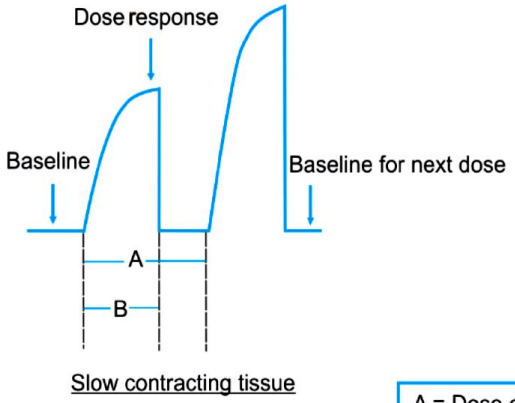

RIGHT LIVER LOBE
GALL BLADDER
JEJUNO-ILEUM
BLADDER
LEFT LIVER LOBE
STOMACH
GREATER OMENTUM
ASCENDING COLON
CAECUM

Tissue Handling

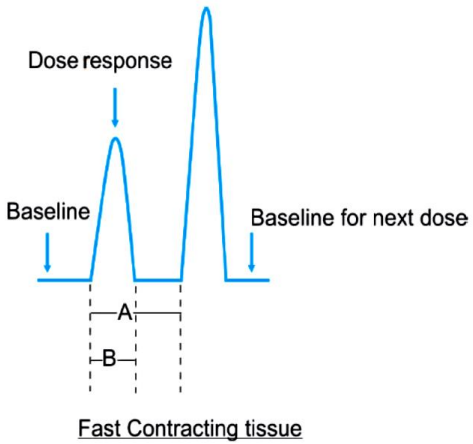
- Proper relaxation of the tissue ensured for 30-45 minutes
- Proper baseline estimation before actual dosing
- After each contact time, washing of the tissue is ensured
- Minimum tissue handling
- Dosing cycle should be maintained
- Avoid contact of very high dose of the drug; may loss sensitivity due to “tachyphylaxis”

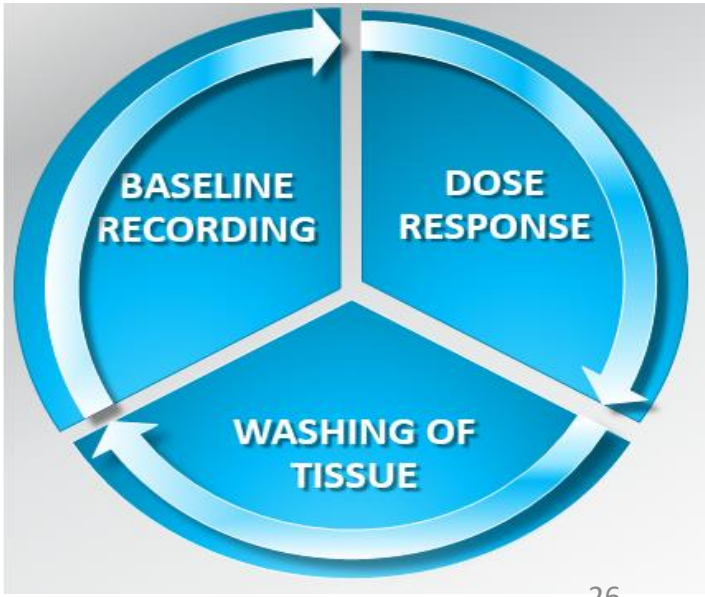
Dose cycle

- ➢ The time gap between drug dose addition
- 3 mins-fast contracting tissues/5 mins-slow contracting tissues
- Includes at least two tissue wash
- ➢ Contact time is defined as time duration at which tissue come in contact with drug

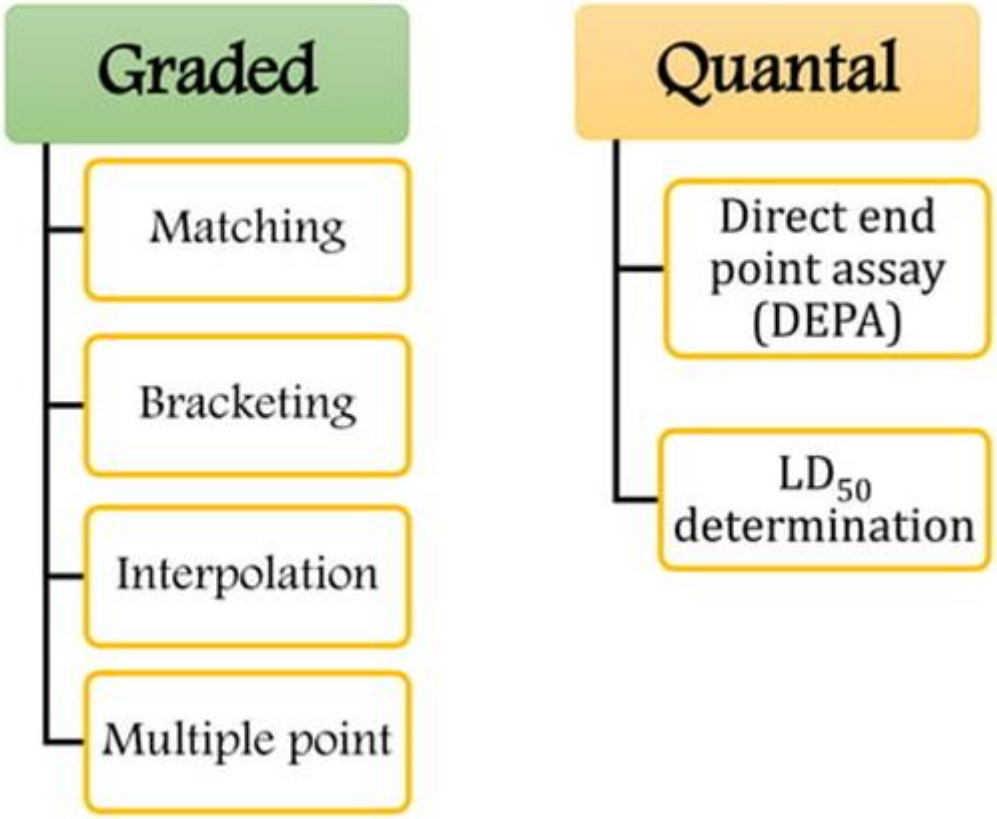


A = Dose cycle
B = Contact time





Types of Bioassay



Direct Endpoint/Quantal Assay

- Elicit an 'all or none' response in different animals e.g. digitalis induced cardiac arrest in guinea pigs,

  hypoglycemic convulsions in mice

- In this method threshold dose, i.e. dose required for obtaining the predetermined response is calculated for standard and test drug

$$\text{Concentration of test} = \frac{\text{TDS}}{\text{TDT}} \times \text{CSD}$$

Where,

TDS = Threshold dose of standard

TDT = Threshold dose of test

CSD = Concentration of standard drug

Contd… Quantal Assay

➢ Advantages:-

- Drug effects appear rapidly and are easily recognized
- Drug effect is directly proportional to drug dose
- Rapid end-point detection

➢ Disadvantages:-

- Only toxicity study or high dose study is possible
- Dose ranging study cannot be done

Graded Response Assay (GRA)

- Most graded reactions are consistent with the sigmoid curve
- The potency of a test agonist is determined by comparing its mean response to standard mean response
- This process is known as 'analytical dilution assay' (Serial dilution of standard/test drug)
- This assay simply depends on the several graded responses by exponential increase in the test dose and which is compared with the standard graded dose response

- GRA is simplest way of determining potency of a test drug because it does not require statistical analysis

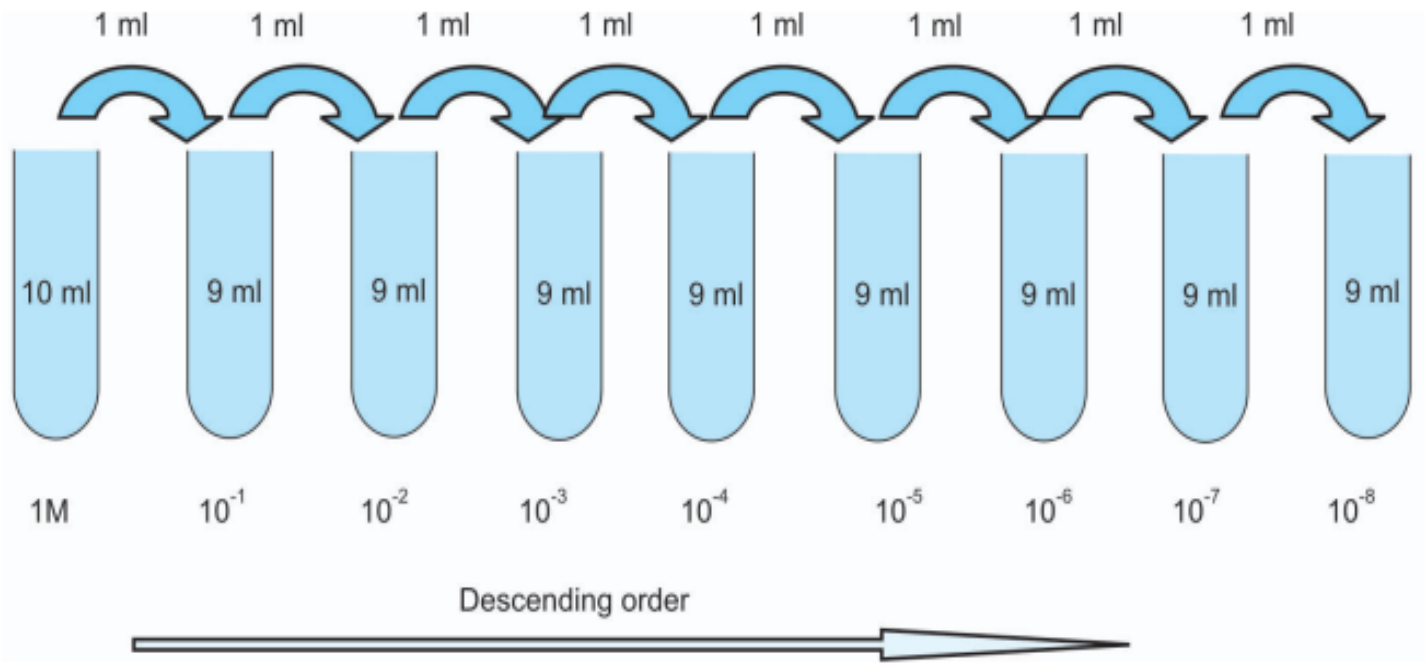
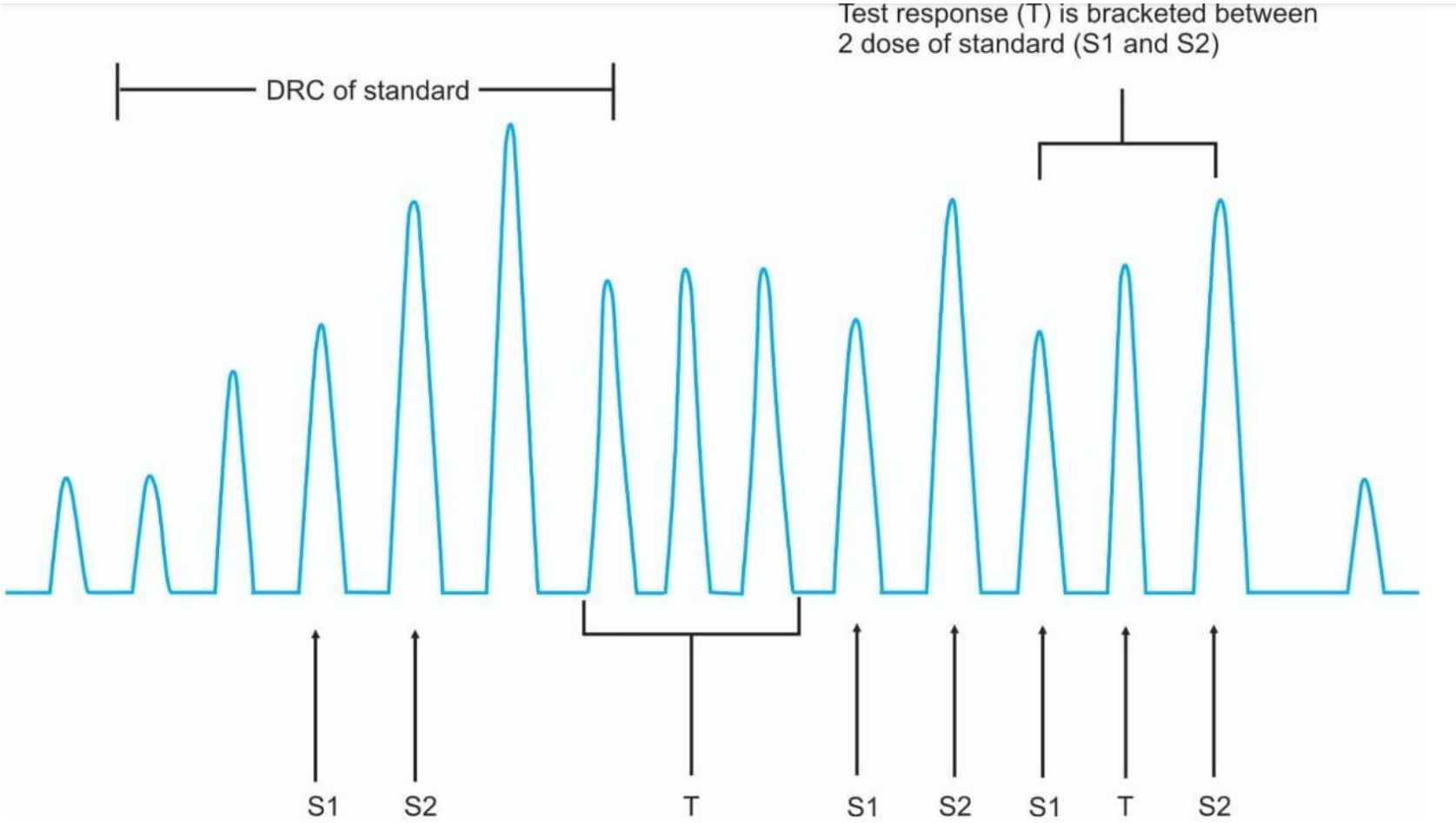


Fig. 2.15: Serial dilution (descending order)

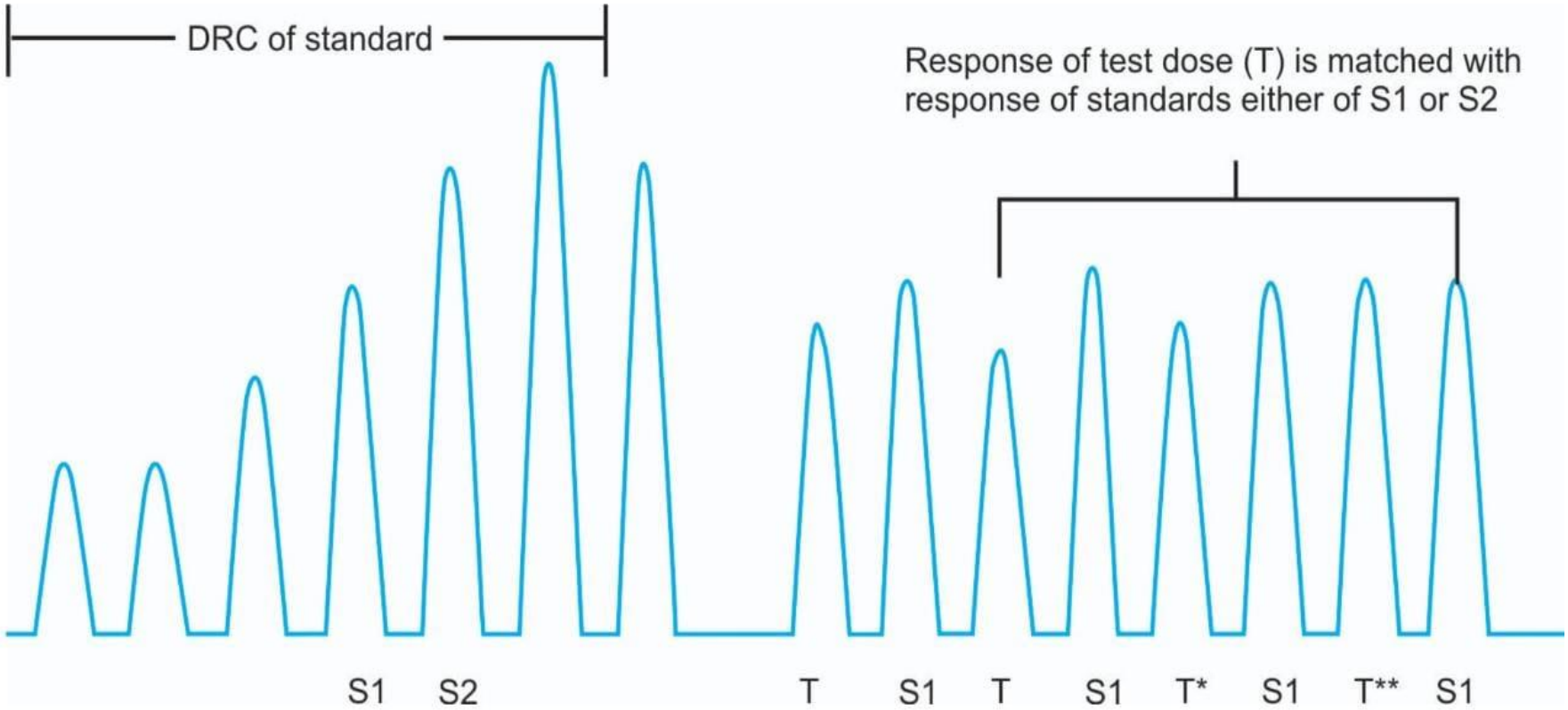
Bracketing Assay

- Preferred when test sample volume is too small
- Single or few response (s) is taken by using any test drug concentration
- This response is bracketed between two responses (one higher and one lower) of the standard drug
- The potency of the test drug is directly calculated from concentration of standard drug or by interpolation through dose response curve
- Limitation:
  - poor precision
  - poor reliability
  - unable to calculate error


Test response (T) is bracketed between
2 dose of standard (S1 and S2)
DRC of standard
S1
S2
T
S1
S2
S1
T
S2

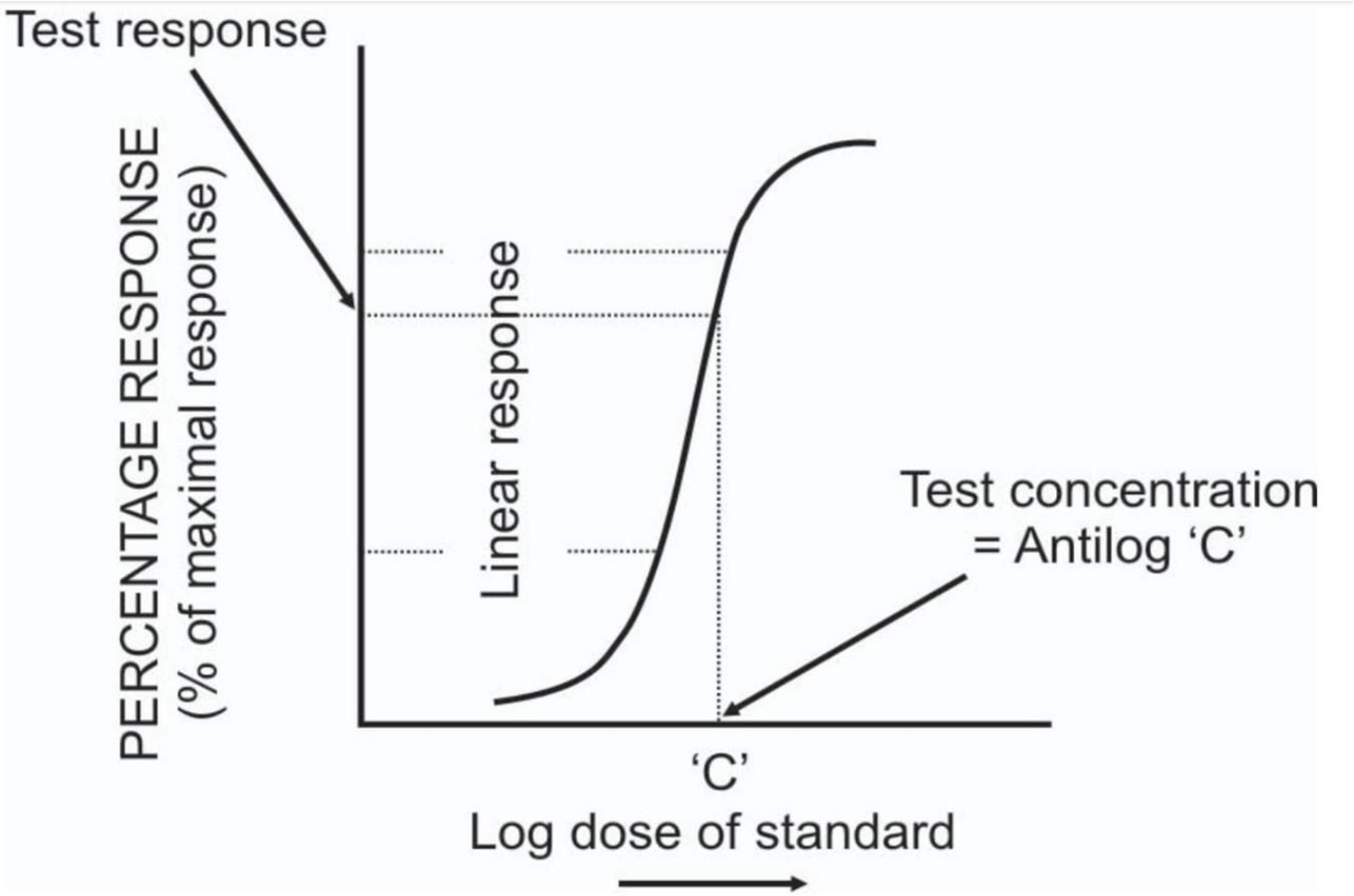
MATCHING ASSAY

- Comparison of potency between the unknown and standard drug is done by trial and error method
- Requires very small sample volume
- Simple and less time consuming
- **Disadvantages:**
  - Purely subjective, experimental error is not excluded out
  - No sign of parallelism as it lacks dose response relationship
  - Requires most sensitive tissue, so selection of tissue is the most important aspect in this assay


DRC of standard
Response of test dose (T) is matched with response of standards either of S1 or S2
S1
S2
T
S1
T
S1
T*
S1
T**
S1

Interpolation Assay

- This method depends on the assumption of dose response curve
- Concentration of unknown is interpolated from the dose response curve graph
- At the first step DRC of the standard drug is plotted then single or few responses of the test drug are plotted
- The dose of the test drug which comes at the linear log dose-response relationship is interpolated from the dose response plot


Test response
PERCENTAGE RESPONSE
(% of maximal response)
Linear response
Test concentration
= Antilog 'C'
'C'
Log dose of standard

Multiple Point Assay

- The selection of 1 or more dose responses of test compound and these responses are compared with 2 or more responses of standards

- Repeated response recording in graded response assays minimize the tissue sensitivity error and improve the methodological errors

Calculation of 3-point assay & 4-point assay

Latin square randomization

S1	S2	T
S2	T	S1
T	S1	S2

Calculation for 3′point assay:

$$\text{Relative potency (M)} = \frac{T - S1}{S2 - S1} \log \frac{s_2}{s_1}$$

Latin square randomization

S1	S2	T1	T2
S2	T1	T2	S1
T1	T2	S1	S2
T2	S1	S2	T1

$$M = \frac{(S2 - T2) + (S1 - T1)}{(S2 - S1) + (T2 - T1)} \times \log\left(\frac{s_2}{s_1}\right)$$

Three-point assay

- 2 response from standard and 1 response from test
- Consecutive 9 response of Latin square randomization

Percentage error (%)= $\frac{ACT - OCT}{ACT} \times 100$

ACT = Actual concentration of test

OCT = Observed concentration of test

Four-point assay

- 2 response from standard and 2 response from test
- Consecutive 16 response of Latin square randomization
- More sensitive than 3-point assay
- Reduces the error or variability

6-point and 8-point Assay

- These methods of bioassays are generally not adopted for the experiment purpose because of the time consuming lengthy procedure
- The responses obtained for the 6-point is ‘36’ and ‘64’ for 8-point
- The advantage being reduced error ,variability and greater specificity

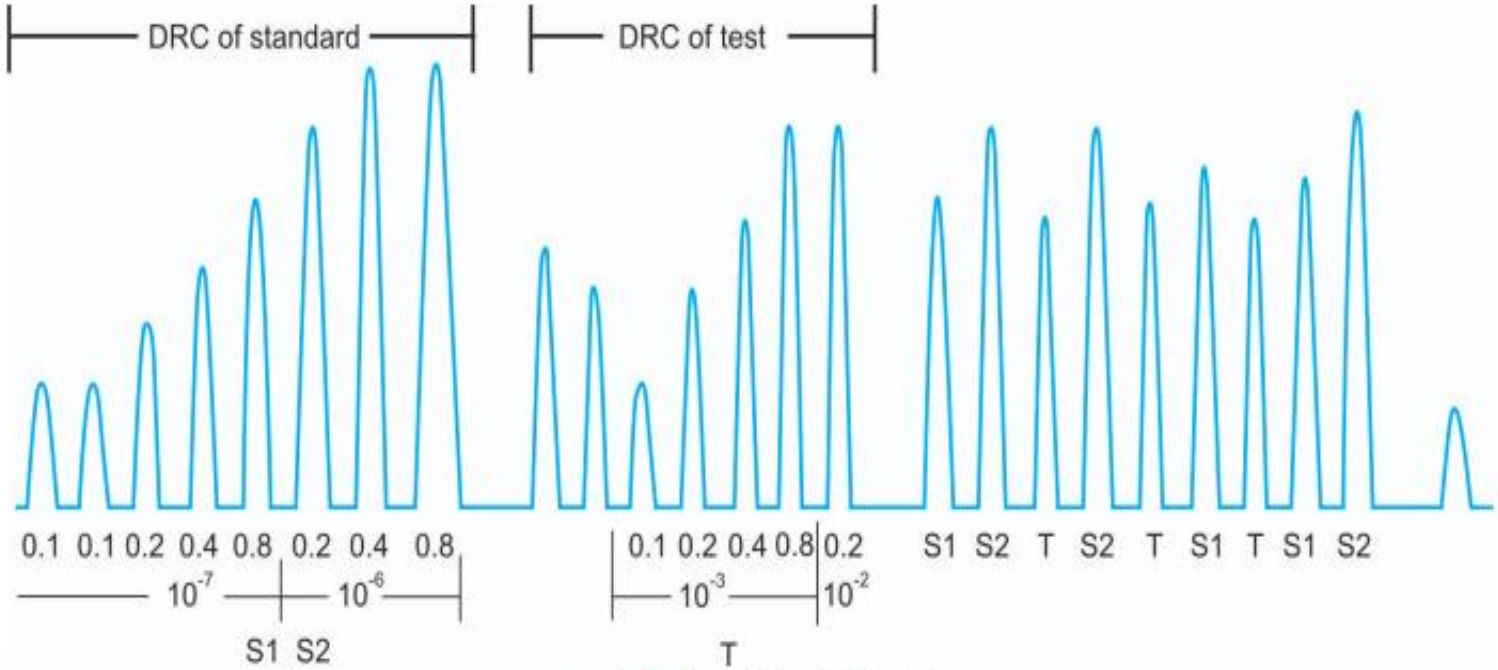


Fig. 2.20: Three-point assay

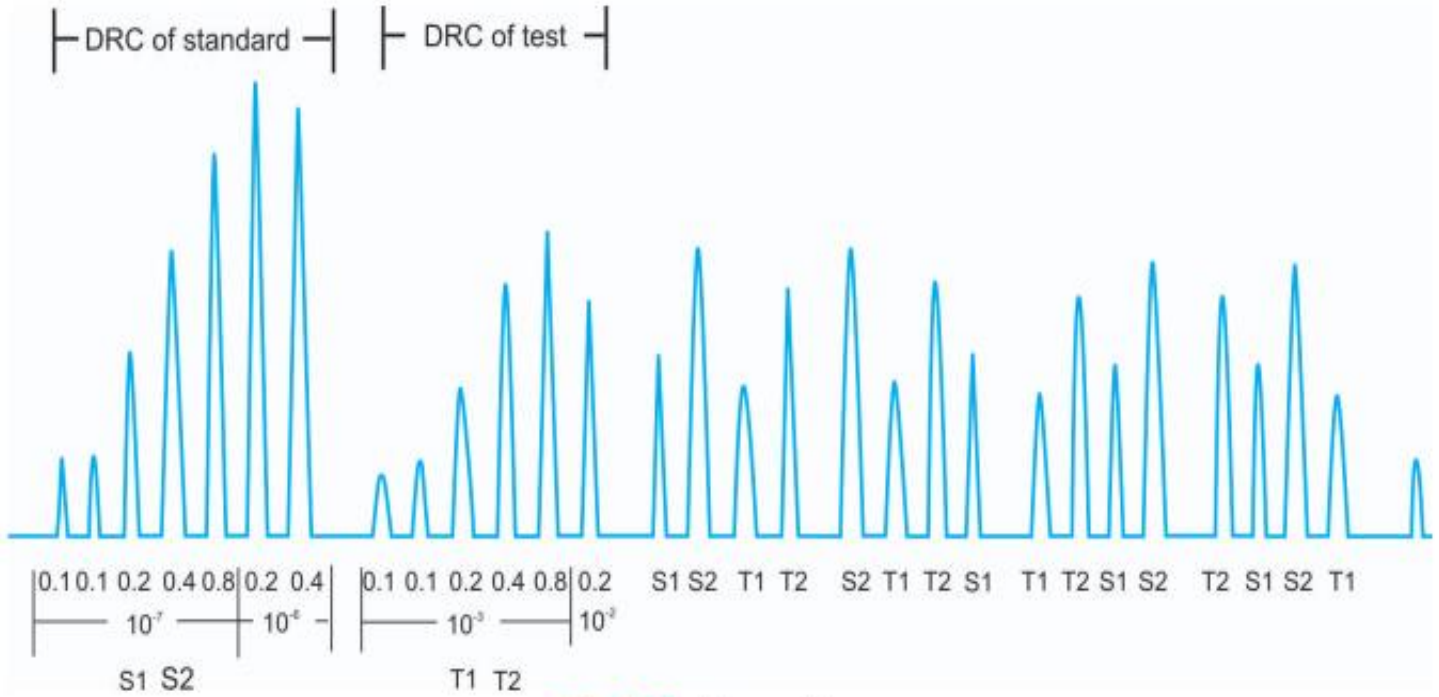


Fig. 2.21: Four-point assay

EXAMPLE OF PERFORMING A SET OF BIOASSAY

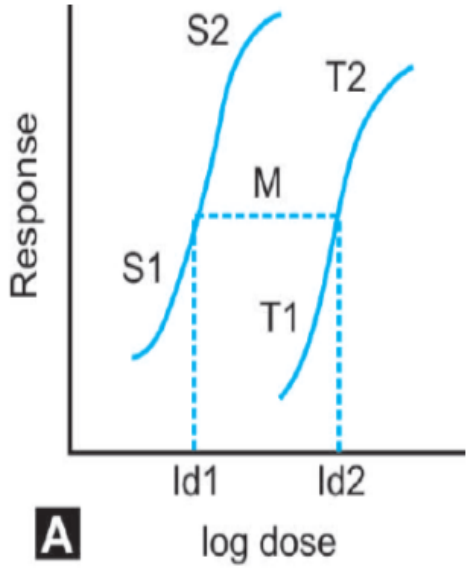
Question: 1

Write the ***Protocol*** and undertake the ***4-point bioassay*** experiment to determine the concentration of ***histamine*** in the given sample in an *in vitro* tissue of your preference.

The response taken here is approx value (not to scale; made for understanding purpose)

	Responses (mm)				Mean
S1	34	38	33	32	34.25
S2	52	57	49	53	52.75
T1	26	29	23	27	26.25
T2	45	43	44	43	43.75

$s1 = 0.2 \times 10^{-7}$
$s2 = 0.4 \times 10^{-7}$
$t1 = 0.2 \times 10^{-3}$
$t2 = 0.4 \times 10^{-3}$



A

Relative Potency (M) $= \frac{(S2-T2)+(S1-T1)}{(S2-S1)+(T2-T1)} \log \frac{s2}{s1}$

$M = \frac{(52.75-43.75)+(34.25-26.25)}{(52.75-34.25)+(43.75-26.25)} \log \frac{0.4}{0.2}$

$= \frac{9+8}{18.5+17.5} \log 2$

$= \frac{17}{36} \times 0.30103 = 0.142153$

Now, **conc. of unknown** $= \frac{s1}{t1} \times$ antilog M

$= \frac{0.2 \times 10^{-7}}{0.2 \times 10^{-3}} \times$ antilog 0 .1421

$= 10^{-4} \times 1.39 = 10^{-3} \times 0.139$ mg/ml

$= 0.139$ mg/ml $= 139$ microgram/ml

Conclusion

- Bioassay is successful tool in estimation & discovery of biologically active substances
- Being sensitive & Specific – important tool in pharmacology
- GRA is simplest way of determining potency of a test drug because it does not require statistical analysis
- 4-point assay is more sensitive than 3-point assay and reduces the error or variability
- 6-point and 8-point assay are generally not adopted for the experiment purpose because of the time consuming lengthy procedure

References

- Principles of pharmacology – sharma & sharma 3rd edition
- Practical Manual of Experimental and Clinical Pharmacology- Bikash Medhi & Ajay Prakash 2nd edition
- Louisse J, Mulder PPJ, Gerssen A, Stoopen G, Rijkers D, van de Schans MGM, Peijnenburg AACM. Bioassay-directed analysis-based identification of relevant pyrrolizidine alkaloids. Arch Toxicol. 2022 Aug;96(8):2299-2317. doi: 10.1007/s00204-022-03308-z. Epub 2022 May 24. PMID: 35610518; PMCID: PMC9217854

Thank you