



A Practical Book of Pharmacology IIIrd

JV'n Asheesh Kumar Gupta

JAYOTI VIDYAPEETH WOMEN'S UNIVERSITY, JAIPUR

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A Practical Book of

Pharmacology-III

**(Strictly As Per Syllabus Prescribed for B. Pharmacy
Semester 6th by P.C.I, New Delhi)**

By:

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Dedicated to
God, Parents,
Family & Friends

Preface

These manual documents the lesson plans of the fifteen exercises in practical pharmacology scheduled for the Sixth semester of the B.Pharmacy course under the PCI curriculum. In planning these experiments, we have tried to fulfill the objectives stated by the PCI in their document. Hence, throughout this manual, attempt has been made to involve the learner in the development of intellectual skills such as planning an experiment, critical analysis of scientific documents, use of statistics for analyzing and interpreting data and scientific methods of documentation.

We have made every effort to avoid printing errors. However, despite best efforts some might have crept in involuntarily. We shall be obliged if these are brought to our notice.

Users are welcome to share their views, experiences and innovations with us. As observed earlier, this manual will be constantly modified and regularly updated. Therefore, your ideas may find a place in the next edition. We hope that this manual will serve as a matrix for other departments to develop their own exercises to suit the facilities, staff – student ratio, availability of animals, equipment and last but not least a desire to change.

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Experiments

- 1.** To study dose calculation in Pharmacological experiments.
- 2.** To study Anti-allergic activity by mast cell stabilization assay.
- 3.** To study anti-ulcer activity of a drug using pylorus ligand (SHAY) rat model and NSAIDS induced ulcer model.
- 4.** To study the effect of drugs on gastrointestinal motility.
- 5.** To study the effect of agonist and antagonists on Guinea pig ileum.
- 6.** To study the estimation of serum biochemical parameters by using semi-autoanalyser.
- 7.** To Study the effects of saline purgative on frog intestine.
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- 10.** To determine acute oral toxicity (LD_{50}) of a drug from given data.
- 11.** Determination of acute skin irritation/corrosion of a test substance.
- 12.** Determination of acute eye irritation/corrosion of a test substance.
- 13.** To calculate pharmacokinetic parameters from a given data.
- 14.** Biostatics methods in experimental pharmacology (Student's t test, ANOVA).
- 15.** Biostatics methods in experimental pharmacology (Chi square test, Wilcoxon Signed Rank test).

Experiment No. 1

Aim: To Study dose calculations in pharmacological experiments.

Introduction

The dose is the amount or quantity of drug taken at any one time or the total quantity administered during a specific period.

Factors affecting dose a drug:

- a) Species difference
- b) Age
- c) Body Weight
- d) Sex
- e) Pharmacokinetic parameters
- f) Genetic factors
- g) Dietary factors
- h) Circadian rhythm
- i) Routes of administration
- j) Frequency of administration

Pharmacological experiments are performed in animals, tissue, organ or microorganism. In any experiment of pharmacology most important step is dose calculation. *In-vivo* pharmacological experiments animals are used for discovery of development of new drug.

Dose Calculation for Experiment Animal:

Often the drug to be tested for any activity comes in a very small quantity. It is in milligram. It is very important to know the right way to calculate the dose of any chemical or drug before starting the experiment. This will help to save the time and money, more importantly, will be using the accurate dosage and thereby will get accurate result.

Some parameters should be considered for calculating the dose

- Doses are administered in mg/kg or $\mu\text{g/kg}$.
- How many animals have to be treated?
- How many treatments have to be given?
- Desired injection volume of the drug.

- Body weight of the animals.

Unit conversion for dilution preparation

$$1\text{gm} = 1000\text{mg} \text{ or } 1 \times 10^3$$

$$1\text{mg} = 0.001\text{gm} \text{ or } 1 \times 10^{-3}$$

$$1\text{mg} = 1000\mu\text{g} \text{ or } 1 \times 10^3$$

$$1\mu\text{g} = 0.001\text{mg} \text{ or } 1 \times 10^{-3}$$

$$1\mu\text{g} = 1000\text{ng} \text{ or } 1 \times 10^3$$

$$1\text{ng} = 0.001\mu\text{g} \text{ or } 1 \times 10^{-3}$$

$$1\text{gm} = 1 \times 10^6 \mu\text{g}$$

$$1\text{gm} = 1 \times 10^9 \text{ ng}$$

Dilution preparation

Q:1. How to prepare a final dilution of $10\mu\text{g/ml}$ from a stock solution of 1mg/ml .

Ans. Stock solution = 1mg/ml

Required concⁿ. = $10\mu\text{g/ml}$

$$1\text{mg} = 1000\mu\text{g} \text{ or } 1 \times 10^3$$

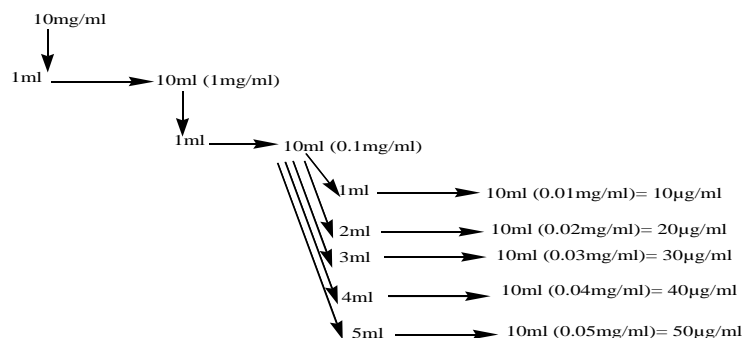
$$Diltion = \frac{1000\mu\text{g}}{10 \mu\text{g}} = 100$$

$1000\mu\text{g}$ drug is available in 1ml thus take 1 ml of this stock solution in a volumetric flask and make up the volume up to 100ml with solvent. The final solⁿ. will contain $10\mu\text{g}$ drug in 1ml solⁿ.

Q:2. How to prepare a serial dilution of $10,20,30,40,50\mu\text{g/ml}$ from a stock solution of 10mg/ml . if max. available glassware capacity is 10 ml .

Ans: Stock solution = 10mg/ml

Required concⁿ. = $10,20,30,40,50\mu\text{g/ml}$

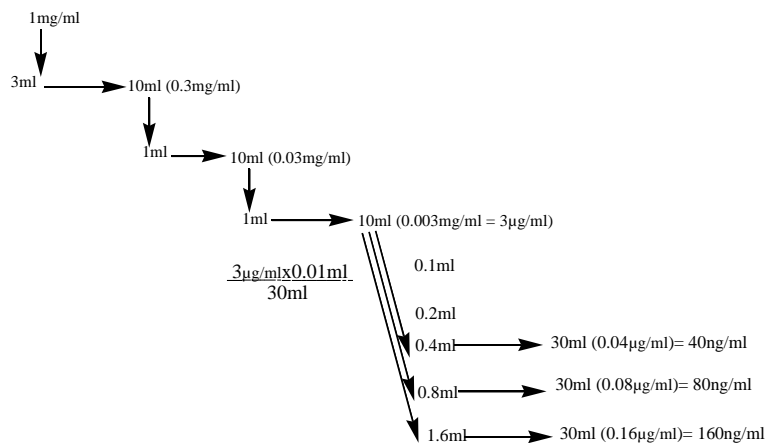


All concentrations can be prepared from second dilution only.

Q:3. How to prepare a serial dilution of 10,20,40,80,160ng/ml from a stock solution of 1mg/ml.
if max. available glassware capacity is 10 ml. and final dilution has to be prepared in 30ml.

Ans: Stock solution = 10mg/ml

Required concⁿ. = 10,20,40,80,160µg/ml



All concentrations can be prepared from second dilution only.

Dose calculation

Q:4. What will be the dose of a drug to a animal weighing 250gm if prescribed dose is 100mg/kg.

Ans: if 1000gm animal receives 100mg drug

$$\text{Then 1gm animal will receive} = \frac{100\text{mg}}{1000}$$

$$\text{Then 250gm animal will receive} = \frac{100\text{mg}}{1000} \times 250 = 25\text{mg}$$

Conclusion: 25mg drug can be administered to this animal.

Q:5. What will be the dose of a drug to a animal weighing 280gm if prescribed dose is 50mg/kg. Available dosage form is tablet whose label claims each tab. contains 75mg drug & total weight of tablet is 150mg.

Ans: if 1000gm animal receives 50mg drug

$$\text{Then 1gm animal will receive} = \frac{50\text{mg}}{1000}$$

$$\text{Then 280gm animal will receive} = \frac{50\text{mg}}{1000} \times 280 = 14\text{mg}$$

Conclusion: 14mg drug can be administered to this animal but drug is not pure so tablet has to be crush powdered.

150mg weighing tab contains 75mg drug that is 2mg tab powder will contain 1mg drug.

Thus 14mg drug will be available in 28mg tab powder that can be weight administer.

Q:6. What will be the dose of a drug to a animal weighing 450gm if prescribed dose is 150mg/kg. Available dosage form is inj. whose label claims each ml contains 100mg drug.

Ans: if 1000gm animal receives 150mg drug

$$\text{Then 1gm animal will receive} = \frac{150\text{mg}}{1000}$$

$$\text{Then 450gm animal will receive} = \frac{150mg}{1000} \times 450 = 67.5mg$$

Conclusion: 67.5mg drug can be administered to this animal but drug is not pure so inj. Has to be calculated. Since 1ml inj. contains 100mg drug-

That is $\frac{1}{100} \times 67.5 = 0.675ml$, this will contain 67.5mg drug that can be administer.

Experiment No. 2

Aim: To Study Anti-allergic activity by mast cell stabilization assay.

Introduction

Hypersensitivity refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. They usually referred to as an over- reaction of the immune system and these reactions may be damaging, uncomfortable, or occasionally fatal.

Signs and symptoms of Hypersensitivity: Swelling, itching, irritation, redness, increase in nasal and lachrymal secretions, fever, chills, muscle or joint pain, headaches, cough, chronic bronchitis, shortness of breath, anorexia & fatigue etc.

Types of Hypersensitivity reactions: 4 types

Type 1–Immediate Hypersensitivity (Anaphylactic Reaction)

Type II- Cytotoxic Reaction (Antibody dependent)

Type III- Immune Complex Reaction

Type IV- Cell Mediated (Delayed Hypersensitivity)

Principle

In allergic disease mast cells plays an important role by defending the antigens. IgE antibodies formed in response to antigen antibody complexes attaches to the surface receptors of mast cells and rises calcium influx leading to degranulation of mast cells which releases some pro-inflammatory mediators (also known as local hormones) such as histamine and eicosanoids.

Requirements

Animals: Guinea pigs of 400-600g of either sex,

Albino rats of 175-200g of either sex,

Drugs: Histamine dihydrochloride aerosol (0.2% w/v), Chlorpheniramine maleate (2mg/kg,s.c.), Disodium chromoglycate(50mg/kg.i.p.)

Reagents: Saline solution(0.9%)

RPMI 1640 buffer medium (pH 7.2-7.4)

Egg albumin (100µg/ml)

Toluidine blue solution (1%)

Instruments: Microscope with 10X magnification lens.

Procedure

1 Mast cell stabilization activity

Albino rats of either sex are divided into two groups consisting of 3 animals in each. Group-1 receives normal saline, Group-2 receives Disodium chromoglycate (50mg/kg i.p.) for 3 days. Inject 10ml/kg of 0.9% saline into peritoneal cavity on 4th day to each animal. Massage the peritoneal region of the animal gently for 5min, then collect the peritoneal fluid and transfer to the test tube which is carrying 7-10ml of RPMI buffer. Centrifuge the fluid for 400-500 RPM. Discard the supernatant and wash the pellets of mast cells twice with the same biffer by centrifugation. Add egg albumin to the above cell suspension and incubate at 37°C fot 10min. Later the suspension has to stain with 1% toluidine blue solution and observe the slide under microscope for calculating number of granulated and degranulated mast cells in each group(total 100 cells are has to be counted from different visual areas)

Table 1: Observational table

GROUP	Total number of cells (n=100)	
	GRANULATED	DEGRANULATED
Group- 1 (saline)		
Group- 2 (Disodium chromoglycate 50mg/kg I.P.)		

Conclusion

Pretreatment of animals with standard drug stabilizes mast cell membrane and generates nitric oxide as defensive mechanism that inhibits the release of chemokines, which are responsible for vasoconstriction.

2 Clonidine induced Mast cell degranulation

Rationale: Both clonidine and compound 48/80 act through the dynamic expulsion of granules without damaging cell wall. Drugs stabilizing mast cell will inhibit Mast cell degranulation.

Requirements:

RPMI-1640 buffer medium (pH 7.2-7.4)

Clonidine (80µg/ml) and water bath.

Procedure

Initially 4ml of normal saline was injected into the peritoneal cavity of mice. After a gentle massage, the peritoneal fluid was collected and transferred into the test tube containing 3-4 ml of RPMI-1640 buffer medium(pH:7.2-7.4)

Mast cells were then washed by centrifugation at a 400-500 rpm. Supernatant was discarded and the pellets of mast cells were suspended in the medium.

The mast cells were treated with 1-2 drops of clonidine (80µg/ml) and incubated at 37 °C in a water bath for 10 minutes. They were stained with Toluidine blue and observed under microscope.

100 mast cells were observed and mast cells showing degranulation were counted. The percentage of protection against degranulation was calculated.

The animals were treated with vehicle or disodium cromoglycate (10 mg/kg, i.p.) for 4 days.

The last dose was administered 30 minutes before collection of mast cells.

Statistical analysis: Student's t- test.

Table 2: Observation table**Effect of disodium cromoglycate on clonidine induced mast cell degranulation**

Sr. No.	Treatment	Percentage protection of mast cells (Mean \pm SEM)
1	Vehicle	
2	Disodium cromoglycate (10mg/kg)	

3 Evaluation of bronchoconstriction in Guinea pig using histamine aerosol**Principle:**

When guinea pig exposed to aerosol containing histamine it causes bronchoconstriction leading to asphyxia and death. Antihistaminic drug prevent or prolong the time of onset of attack by histamine aerosol.

The death is preceded by dyspnoea and convulsion. The animal can be saved if the aerosol is promptly removed and stopped.

The instrument consists of transparent box of Perspex divided by a perforated wall into two. There is a small hole to spray aerosol. The spray is done using a nebulizer which is connected to sphygmomanometer and the compressor. One side of the box has a movable partition is to remove the aerosol.

Animal: Guinea pig (400-600gm),

Drugs: 1. Histamine dihydrochloride aerosol (0.2% w/v)

2. Chlorpheniramine maleate (2mg/kg, s. c.).

Procedure:

1. Selected guinea pigs are divided into two groups consisting of 3 animals in each. Animals have to be fasted overnight.

2. Group-1 receives normal saline. Group-2 animal receive chlorpheniramine maleate (2mg/kg, s. c.). (Before the drug treatment animals should be exposed to histamine aerosol (0.2%) in histamine chamber).
3. Then determine the end point i.e. pre-convulsion dyspnoea (PCD) (is the time of exposure of histamine aerosol to onset of dyspnoea that leads to convulsion).
4. As early as PCD is observed the animal should be removed from chamber and placed in fresh air and time of onset of PCD is to be noted day zero.
5. Then animals have to treat with drug after 24 hours. After 1 hour of drug administration once again animal are exposed to histamine aerosol and PCD is determine. Percentage (%) of protection offered by the drug can be calculated by the bellow formula.

$$\text{Percentage protection} = (1 - T1/T2) * 100$$

Where,

T1 = Value of PCD before drug administration,

T2 = Value of PCD after drug administration.

Table 3: Observation table

Sr. No.	Treatment	Percentage protection
1	Vehicle	
2	Chlorpheniramine maleate (2mg/kg s/c)	

Experiment No.3

Aim: To study the antiulcer activity of a drug using pylorus ligand (SHAY) Rat Model and NSAIDS induced Ulcer Model.

Introduction

Gastro- intestinal disorders are one of the severe classes of human ailments causing maximum discomfort and morbidity.

Peptic Ulcer is one such GIT disorder. It is a chronic inflammatory condition involving a group of disorders characterized by ulceration in regions of upper gastrointestinal tract where parietal cells secrete pepsin and hydrochloric acid. There are several causes including stress, alcohol consumption, cigarette smoking, H.Pylori infection, ingestion of certain drugs and chemicals etc.

Pylorus Ligand Rat Model

Principle: Peptic ulcers constitute a major disease that affect human gastrointestinal tract. The common clinical features of peptic ulcers are hyper acid secretion and ulcer formation in the stomach and duodenal part of the intestine. Acute gastric ulcers can be produced in laboratory animals by the use of corrosives substances such as alcohol, aspirin or indomethacin, or by surgical manipulation like pyloric ligation, or by subjecting the animals to acute stressful conditions. The most commonly used technique is the pyloric ligation-induced acid secretion and ulcer formation. The procedure is simple and produces reliable number of gastric ulcers. Drugs like H₂ histamine antagonists (cimetidine) inhibits pyloric ligation-induced peptic ulcers.

Requirements

Animals: Rats (150-200g, overnight fasted)

Drugs: Anaesthetic ether. Cimetidine (Dose 10mg/kg, ip; Prepare a stock solution containing 2mg/ml of the drug and inject 0.5ml/100g body weight of the animal).

NaOH (0.01N), Topfer's reagent, collodion.

Equipments: Dissecting microscope, 10× magnification, pH meter, burette, surgical equipment.

Procedure

1. Anaesthetize the overnight fasted rat with anaesthetic ether.
2. Secure the rat on the operating table. Give an incision of 1cm long in the abdomen just below the sternum. Expose the stomach. Pass a thread around the pyloric sphincter and apply a tight knot. While putting the knot care should be taken so that no blood vessel is tied along the knot. Close the abdomen wall by putting the sutures. Clean the skin from any blood spots and bleeding. Apply collodion over the wound. Keep the rat in a separate cage and allow it to recover.
3. To another rat inject cimetidine (10mg /kg , ip).After 15 min perform pyloric ligation as described in step 2.
4. After 4 hour of pyloric ligation sacrifice both the animals by decapitation. Open the abdomen and tie the oesophageal end(cardiac end) of the stomach .Cut and remove the entire stomach from the body of the animal.
5. Give a small cut to the pyloric region just above the knot and collect the contents of the stomach in a graduated centrifuge tube.
6. Open the stomach along the greater curvature and wash it slowly under the running tap water .Put it on the slide glass and observe under 10×magnification for ulcers. Score the ulcers as below :

0 = Normal coloured stomach

0.5 = Red colouration

1 = Spot ulcers

1.5 = Haemorrhagic streaks

2 = Ulcers ≥ 3 but ≤ 5

3 = Ulcers > 5

Mean ulcer score for each animal is expressed as ulcer index.
7. Centrifuge the gastric content at 1,000rpm for 10 min. Note the volume. Pipette out 1ml of supernatant liquid and dilute it to 10 ml with distilled water. Note the pH of this solution with the help of pH meter. Titrate the solution against 0.01N sodium hydroxide using Topfer's reagent as indicator.(It is dimethyl-amino-azo-benzene with phenolphthalein and used for detection and estimation of hydrochloric acid and total acidity in gastric fluids).Titrate to the end point when the solution turns to orange colour.

Note the volume of NaOH which corresponds to the free acidity. Titrate further till the solution regains pink colour .Note the total volume of NaOH which corresponds to the total acidity. Acidity (mEq/l/100g) can be expressed as:

$$\text{Acidity} = \frac{\text{Vol. of NaOH} \times \text{Normality} \times 100}{0.1} \text{mEq/l/100g}$$

8. Compare the gastric volume, acidity and ulcer index of control animal and the animal treated with cimetidine.

Inference: Cimetidine reduces gastric secretion and inhibits ulcer formation in pyloric ligated animals.

Observation

S.No.	Treatment	Dose(mg/kg, ip)	Volume ml.	pH	Acidity (mEq/l/100g)	Ulcer index
1.	Control*					
2.	Cimetidine*	10				

NSAIDS induce ulcer model

Purpose and Rational

Nonsteroidal anti-inflammatory agents like indomethacin and acetyl salicylic acid, induced gastric lesions in man and in experimental animals by inhibition of gastric cyclo-oxygenase resulting in less formation of prostacyclin, the prominent prostanoid produced in the gastric mucosa.

Procedure

1. Group of 8-12 wistar rats weighing 150-220g are used.
2. The test drugs are administered orally in 0.1% Tween 80 solution 10 minutes prior to oral indomethacin in a dose of 20mg/kg. (4 mg/ml dissolved in 0.1 % Tween 80 solution)

3. Six hour later, the rats are sacrificed in CO₂ anesthesia and their stomachs are removed.
4. Formal Saline (2% v/v) is then injected into the totally ligated stomach and removed.
5. The next day, the stomach is opened along the greater curvature, then washed in warm water and examined under a 3 fold magnifier.
6. The lengths of the longest diameter of the lesion are measured and summated to give a total lesion score (in mm) for each animal, the mean count for each group being calculated.

Evaluation

The mean score in control rats is about 25 (range 20-28). Inhibition of the lesion production is expressed as percentage value.

Experiment No.4

Aim: To Study of effect of drugs on gastrointestinal motility.

Introduction

Intestinal motility is regulated by the enteric nervous system of the gut (Auerbachs & meissners plexuses) & the activity of this system can be modified by autonomic nervous system. Hence effect of sympatomimetic and parasympatomimetic drugs on intestinal motility can be studied by using isolated piece of intestine. Parasympathomimetic drugs stimulate enteric neurons to release acetylcholine at neuromuscular junction and enhance muscle tone & rhythmicity of intestine. Sympathomimetic drugs act on alpha & beta receptors and release adrenaline which in turns prevent release of acetylcholine & inhibits muscle tone and rhythmicity. Many animal models can be employed to study intestinal motility of sympathetic & parasympathetic drugs. Guinea pig ileum is advantageous for assay purposes as it produces steady. Baseline for studying effect of drugs. Rabbit intestine (ileum, deudenum, jejunum) usually jejunum is used for effects of pendular movements. In the present study rabbit ileum is selected for estimating the effects of selected drugs on intestinal motility.

Requirements

Animals: Medium sized rabbits

Drugs: Adrenaline/Acetylcholine-10 μ g/ml, Atropine sulphate-100 μ g/ml, Isoproterenol/isoprenaline-10 μ g/ml, propanolol-1 μ g/ml, phenylephrine-10 μ g/ml, phentolamine-0.1 μ g/ml

Solutions: Tyrode solution

Apparatus: Kymograph, Dissecting board, Dissecting instruments, Scissors, Petri plates, Syringe, Frontal writer lever, Water bath with temperature controlling unit, Organ bath with aeration tube.

Procedure

The procedure adopted for the study is the modified Finkleman method developed by walker & scott. Select a medium sized rabbit for the study. Fast the animal for 24 hours prior to experiment as food in gut results in messy dissection & flushing of gut contents may damage the intestine.

Before sacrificing the rabbit, prepare Tyrodes ringer solution & place about 250ml of this solution in an ice cold flask. Sacrifice the animal by cervical decapitation without use of anesthetic as it may affect gut motility. Shave the abdomen of the animal & vacuum the surface to remove adhered fur. Make a midline incision through the skin & abdominal muscles. Locate ileum and part of ileum was taken 10cm away from ileocaecal valve. An optimal length of the tissue (5-6cm) is cut carefully & tie the thread to antimesenteric border on both sides & place them in the Tyrode solution (extra pieces of ileum can be stored in ice cold Tyrode solution so that they are viable for hours. In ice cold solution the motility will cease but after placing them in warm solution the tissue gets relaxed & shows motility within 5-10mins)

Record the rhythmic activity of ileum by using frontal writing lever & kymograph. Suspend the tissue organ bath of Tyrode solution (100ml) 37°C with adequate oxygen supply (mixture of 95% O_2 & 5% CO_2) Tie one end of the thread of the tissue to fixed point inside the organ bath and other end to the lever for recording contractions on the kymograph. Stabilise the tissue in the solution to the conditions for about 30mins. Ensure the lever should be placed horizontally and record the normal contractions followed by effects of drugs on muscles.

After recording normal contractions inject the drugs one by one & observe for force of contraction and tone (normal, increased or decreased), frequency of contraction (per minute) before & after drug administration.

Inject 0.1ml of drugs in the succession order in the organ bath & the responses are recorded. After noting the effect of every drug, drain the muscle bath & refill with fresh warm Tyrode solution (100ml). Take the control (without drug) reading before & after each drug response & check the next drug response.

Maintain wash out period for 15-20 minutes for change of every drug & check the next drug response. Only when the tone & amplitude returned to original value approximately. The drug & dose name should be mentioned in the record in after taking response of each drug.

ORDER OF ADDING DRUGS

1. 0.1ml of Ach-1.0ml of Ach-0.1ml of NE-1.0ml of NE(these drugs are postganglionic neurotransmitters of parasympathetic and sympathetic system. Ach increases contraction and NE relaxes the tissue.)
2. 1.0ml of phenylephrine-1.0ml of isoproterenol (phenylephrine causes contraction by inhibiting adenylate-cyclase-alpha adrenergic agonists: isoproterenol causes relaxation by showing beta agonist action.)

Add drugs one after one(1&2) to check for their activity.After taking response of each drug drain the solution & refill the bath with warm Tyrode solution & proceed with next drug.

3. 1.0ml of phentolamine (alpha adrenergic blocker) wait for 2 mins. Then proceed for adding 1.0ml of phenylephrine (to check for alpha receptor blockade,if they are blocked no response is seen)
4. 1.0ml of propranolol (beta adrenergic blocker) wait for 2mins. Then proceed for adding1.0ml of isoproterenol (to check for beta receptor blockade, if they are blocked no response is seen)
5. 1.0ml of atropine wait for 3 mins. Add 1.0ml of Ach(to check for parasympatholytic activity of atropine)

Conclusion

The effect of drugs on intestinal motility can be easily interpreted by the responses taken on kymograph.

Precautions

1. For experiment animal should be fasted 24hours.
2. Care to be taken during dissection.
3. Prepare fresh physiological salt solution.
4. Drug should be added in order.

Experiment No. 5

Aim: To Study the effect of agonist and antagonists on guinea pig ileum.

Introduction

Most of the drugs act by being either agonist or antagonist at receptors that respond to chemical messengers such as neurotransmitters.

Agonist: An agonist is a drug or chemical or ligand that binds to a receptor and activates the receptor to produce a biological response. An Agonist binds to the receptor and produces an effect within the cell.

Types of agonist

- a. Endogenous agonist
- b. Super agonist
- c. Full agonist
- d. Partial agonist
- e. Inverse agonist

Antagonist

An antagonist is a type of receptor ligand or drug or any other substance that blocks a biological response or produce opposite action by binding to blocking a receptor rather than activating it like an agonist. They are sometimes called blockers; examples beta blockers, calcium channel blockers and alpha channel blockers.

Types of antagonist

- a. Reversible
- b. Irreversible
- c. Pseudo-irreversible

Principle

Guinea pig ileum is a smooth muscle receive dual nerve supply from autonomic nervous system (sympathetic and parasympathetic). Parasympathetic tone is dominant in ileum which Ach cause contraction by stimulating M3-R

Types of Receptor Present in Guinea Pig Ileum

- ✓ Cholinergic receptor
- ✓ Serptnergic or tryptaminergic receptors(5-HT1 -5HT7)
- ✓ Histaminergic receptor
- ✓ Adrenergic receptor

Stimulation of presynaptic alpha-adrenoreceptor has been shown to reduce the release of non adrenaline from post ganglionic sympathetic nerve endings. Presynaptic alpha-adrenoceptors located on the endings of postganglionic sympathetic fibres are dissimilar from classical postsynaptic alpha-adrenoceptors. Clonidine tramazoline have been claim to stimulate preferentially presynaptic alpha-adrenoceptors whereas phenylephrine and methoxamine act preferentially on postsynaptic ones. Some alpha-adrenoceptors blocking agents such as phentolamine, yohimbin and piperoxan antagonize more readily the effects of clonidine on presynaptic alpha-adrenoceptors. In contrast prazocine is a preferentially postsynaptic alpha-adrenoceptor blocking agents and antagonizes the effects of alpha sympathomimetic agents such as phenylephrine and methoxamine on post synaptic e-adrenoceptors.

Requirements

Animal: Guinea pigs of either sex weighing 300-350 gm

Drugs and chemicals: tyrode solution (composition: NaCl 8g, KCl 0.2g, cAcL 0.2g, MgCl₂ 0.1 g, NaH₂PO₄ 0.05 G, GLUCOSE 0.5g, NaHCO₃ 1g for 1L of distilled water)

Procedure

1. Guinea pig of either sex weighing of 300-350 g were killed by a blow on the head and bled from the carotid arteries. Three or four segements of 1.5 -2cm were removed from tereminal part of ileum 6-12 cm above the ileocaecal junction.

2. The strips were placed in a 20 ml organ bath in Tyrode solution this medium was maintained at 37⁰ C and bubbled with a constant stream of 5% CO₂ in O₂.
3. Coaxial stimulation of the guinea pig ileum was performed with a neuro stimulator by means of two platinum electrodes, one placed in the lumen of ileum and the other in bath. The ileal preparation equilibrated for atleast 1 hour before the experiment started.
4. Supramaximal current(duration 1 ms, frequency 5Hz, supramaximal voltage from 4-10 V) was applied during a 5 s period every 30 s(without drug or 30s after addition of drug to the bath).
5. The contraction were recorded on a smoked drum by means of an isotonic lever. An initial load of 2 g was applied to preparation.
6. Drugs were added to bath after contraction elicited by electrical stimulation had become constant.

Precautions

1. Prepare Tyrodes solution carefully.
2. Carefully remove ileum of guinea pigs.
3. Provide constant supply of CO₂.
4. Drug should be added very carefully with exact concentration.

Experiment No. 6

Aim: Estimation of serum biochemical parameters by using semi auto-analyzer.

Introduction

Principle: An automated analyser is a medical laboratory instrument designed to measure different chemicals and other characteristics in a number of biological samples quickly, with minimal human assistance. These measured properties of blood and other fluids may be useful in the diagnosis of disease. These are mainly of two types

1. Semi-autoanalyzer: in this some procedures such as dilution of blood sample is done by the technician and can measure only a few parameters.
2. Automated: It needs only anticoagulated blood sample and can measure multiple parameters.

Analysis of some parameters



1. Calcium:

Calcium reacts with o-cresolphthalein complexone in the presence of 8-hydroxyquinoline-5-sulfonic acid to form a purple complex. The intensity of the final reaction color is proportional to the amount of calcium in the specimen.

Elevated total serum calcium levels are associated with idiopathic hypercalcemia, vitamin D intoxication, hyperparathyroidism, sarcoidosis, pneumocystic carinii pneumonia and blue diaper syndrome. Low calcium levels are associated with hypoparathyroidism, pseudohypoparathyroidism, chronic renal failure, rickets, infantile tetany, and steroid therapy.

Reagents used:

(a) Reagent 1 (R1) working solution: Ethanolamine buffer: 1 mol/l, pH 10.6 C until the °Use contents of the blank reagent, supplied "ready to use." Store at 2–8 expiration date on the package.

(b) Reagent 2 (R2) working solution: o-cresolphthalein complexone: 0.3 mmol/l; 8-hydroxyquinoline: 13.8 mmol/l; hydrochloric acid: 122 mmol/l Use contents of the o-cresolphthalein complexone/acetate buffer reagent, supplied "ready to C until the expiration date on the package. °use." Store at 2–8.

The method is linear up to 20.0 mg/dL. When reanalyzing any specimen with a concentration greater than 20 mg/dL, prepare a twofold (1+1) dilution of the specimen with physiological saline. The results must then be multiplied by 2 to account for this dilution.

Result: Serum calcium values are reportable in the range .2-20.0 mg/dL without dilution. If the value is >20.0 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

2. Cholesterol:

All cholesterol esters present in serum or plasma are hydrolyzed quantitatively into free cholesterol and fatty acids by microbial cholesterol esterase. In the presence of oxygen, free cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one. The H₂O₂ reacts in the presence of peroxidase (POD) with phenol and 4-aminophenazone to form an o-quinone-imine dye. The intensity of the color is proportional to the cholesterol concentration and is measured photometrically.

An elevated cholesterol level is associated with diabetes, nephrosis, hypothyroidism, biliary obstruction, and those rare cases of idiopathic hypercholesterolemia and hyperlipemia; low levels are associated with hyperthyroidism, hepatitis, and sometimes severe anemia or infection (8).

Reagents used:

Reagent 1 (R1) working solution: PIPES buffer (Piperazine-1,4-bis(2-ethane sulfonic acid)): 75 mmol/l, pH 6.8; Mg²⁺: 10 mmol/l; sodium cholate: 0.2 mmol/l; 4-aminophenazone D 0.15

mmol/l; phenol D 4.2 mmol/l; fatty alcohol polyglycol ether: 1%; cholesterol esterase (*Pseudomonas spec.*) D 0.5 U/ml; cholesterol oxidase (*E. coli*) D 0.15 U/ml; peroxidase (horseradish) D 0.25 U/ml; stabilizers; preservative. C until the expiration date on the package.°Use supplied "ready to use." Store at 2–8.

The method is linear up to 800.0 g/dL. When reanalyzing any specimen with a concentration greater than 800 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution. The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 3.0 mg/dL.

Result: Serum cholesterol values are reportable in the range 3-800 mg/dL without dilution. If the cholesterol value is >800 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

3. Glucose:

Hexokinase catalyzes the phosphorylation of glucose by adenosine triphosphate (ATP). G-6-PD is oxidized to 6-phosphogluconate in the presence of NAD by the enzyme glucose-6-phosphate dehydrogenase. No other carbohydrate is oxidized.

The glucose hexokinase method, based on the work of Schmidt, Peterson, and Young, has long been recognized as the most specific method for the determination of glucose. Glucose measurements are used in the diagnosis and treatment of pancreatic islet cell carcinoma and of carbohydrate metabolism disorders, including diabetes mellitus, neonatal hypoglycemia, and idiopathic hypoglycemia.

Reagents used:

(a) Reagent 1 (R1) working solution: Tris buffer*: 100 mmol/L, pH 7.8; Mg²⁺: 4 mmol/L; ATP @ 1.7 mmol/L; NADP @ 1.0 mmol/L; preservative;** Tris = Tris(hydroxymethyl)-aminomethane C until the expiration date on the package.°Use supplied "ready to use." Store at 2–8

(b) Reagent 2 (R2) working solution: R2 HEPES buffer*: 30 mmol/L, pH 7.0; Mg²⁺: 4 mmol/L; HK @ 8.3U/mL (yeast); G-6-PDH @ 15 U/mL (*E. coli*); preservative; * HEPES, 2-[4-(2-

hydroxyethyl)-1-piperaziny]]-ethane sulfonic acid. C until the expiration date on the package.°Use supplied "ready to use." Store at 2–8.

The method is linear up to 750 mg/dL. When reanalyzing any specimen with a concentration greater than 750 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

Result: Serum glucose values are reportable in the range 2.0–1000 mg/dL without dilution. If the value is >1000 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

Experiment No. 7

Aim: Effect of the saline purgative on the frog intestine.

Introduction

Any drugs used in the treatment of constipation or to promote the evacuation of feces are called laxatives. There are four types of laxatives

- a. Saline Purgatives
- b. Fecal softeners
- c. Contact purgatives
- d. Bulk purgatives

Saline purgative are the salts comprising of highly charged ion and do not cross the cell membrane freely. They remain inside the lumen and retain water through osmotic forces. They increase the volume of the contents of the bowel, stretch the colon and produce the normal stimulus for contraction of the muscle that leads to defecation. The aim of the present study is to examine the effect of saline purgative on frog intestine.

Requirement

Animal: Frog

Reagent: 0.9 to 0.45% of saline (hypotonic), 27% magnesium sulfate (hypertonic), frog Ringer solution (isotonic)

Instruments: frog's boards, pithing needles, dissecting instruments, needle with thread, tuberculin, syringe with needle.

Procedure

Pith the frog and place it on a dissecting board. Expose the abdominal cavity and carefully trace the small intestine. Make the small intestine into three compartments by tying threads of different colors in such a way that no fluid can move from one compartment to the other. Inject 0.2ml of each hypotonic solution into first compartment, 0.2ml of hypertonic solution to second

compartment and 0.2ml of isotonic solution into third compartment wait for 20min and observation are to be recorded.

Observation table

Drug	Compartment	Effect
Hypotonic solution (0.2ml of 0.9% of saline)	First compartment	Shrunk
Hypertonic solution(0.2ml of 27% magnesium sulphate	Second compartment	Swollen
Isotonic solution (0.2ml of frogs Ringer solution	Third compartment	No change

Conclusion

Hypotonic solution cause the fluid to move from lumen into circulation by process osmosis their by shrinks the tissue. Hypertonic solution moves the fluid from cell into lumen and swells the tissue. Isotonic solution did not show any fluid movement across the intestinal membrane.

Precautions

1. Immobilize frog carefully.
2. Use sterile surgical.
3. Carefully expose abdominal cavity.
4. Clean all apparatus and glassware before starting the experiment.
5. Prepare carefully hypotonic and hypertonic solution.

Experiment No. 8

Object: To Study Insulin Hypoglycemic effect in rabbit

Introduction

Hypoglycemia: Hypoglycemia, also called low blood glucose or low blood sugar, occurs when the level of glucose in blood drops below normal. For many people with diabetes, that means a level of 70 mg/dl or less.

Symptoms of Hypoglycemia: An irregular heart rhythm, fatigue, pale skin, shakiness, anxiety, sweating, hunger, irritability, tingling sensation around the mouth, crying out during sleep and visual disturbances etc.

Insulin is a peptide hormone produced by beta cells of the pancreatic islets. It regulates the metabolism of carbohydrates, fats and protein by promoting the absorption of carbohydrates, especially glucose from the blood into liver, fat and skeletal muscle cells.

Standard Preparation and Unit: It is a pure, dry and crystalline insulin. One unit contains 0.04082 mg. This unit is specified by Ministry of Health and Family Welfare, Govt. of India, which is equivalent to international unit.

Preparation of Standard and Best Solutions: Twenty units of insulin are accurately dissolved in normal saline solution. It is acidified using HCl to make pH 2.5 and 0.5% Phenol 1.8% glycerin are added as preservatives. Final volume of solution should contain 20 units / ml. store in a cool place and use within six months. Sample dilutions are freshly prepared by diluting with normal saline solution so they contain 1unit/ml(standard dilution 1) and 2units/ml (standard dilution 2).

Preparation of Test Sample Solution: The solution of test sample is prepared by same method as standard solution described above.

Methods of Bioassay: There are 2 official methods:

1). Rabbit method

2). Mouse method

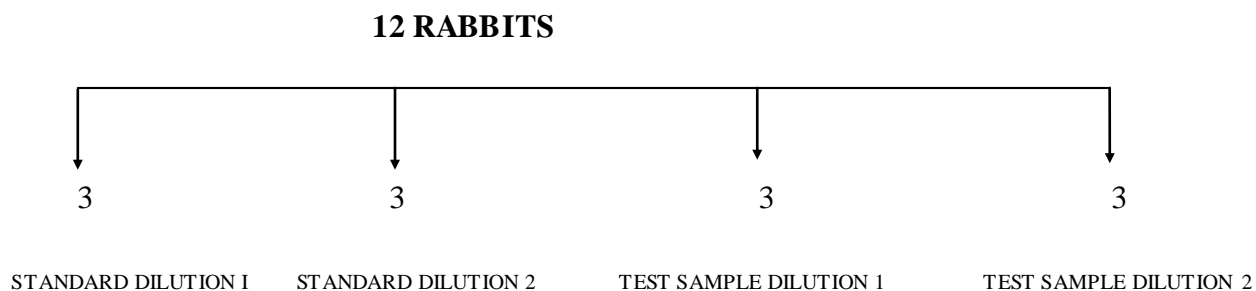
Rabbit method

Principle: The potency of test sample is estimated by comparing the hypoglycemic effect of the sample with that of standard preparation of insulin. Any other suitable method can also be applied.

Selection of Rabbit: they should be healthy weighing around 1800-3000gram, they should maintain a uniform diet and fasted for 18 hours before assay. Water is withdrawn during experiment.

Experimental Procedure: Animals are divided into 4 groups of 3 rabbits each. The rabbits are then put into animal holder. They should be handled with care to avoid excitement. In the first phase of the test sample of blood is withdrawn from marginal ear vein of each rabbit. Presence of reducing sugar is estimated per 100ml. of blood by a suitable method. This concentration is called '**Initial blood sugar level**'

The four groups of rabbits are then given subcutaneous injections of insulin as follows



From each rabbit, a sample of blood is withdrawn upto 5 hours at interval of 1 hour each. Blood sugar level is determined again, this is known as '**Final blood sugar level**'.

The second part of the test is cross over test. The same animals are used for second part of experiment. The second part of the experiment can be carried out after a week. Again they are fasted and initial blood sugar is determined. The grouping is reversed, those which received the test shall now receive standard and those who received higher dose standard earlier shall receive lower dose of test now and vice versa, this test is called "**Twin Cross Test**".

Mean percentage decrease in blood sugar level of the first and second part is calculated.

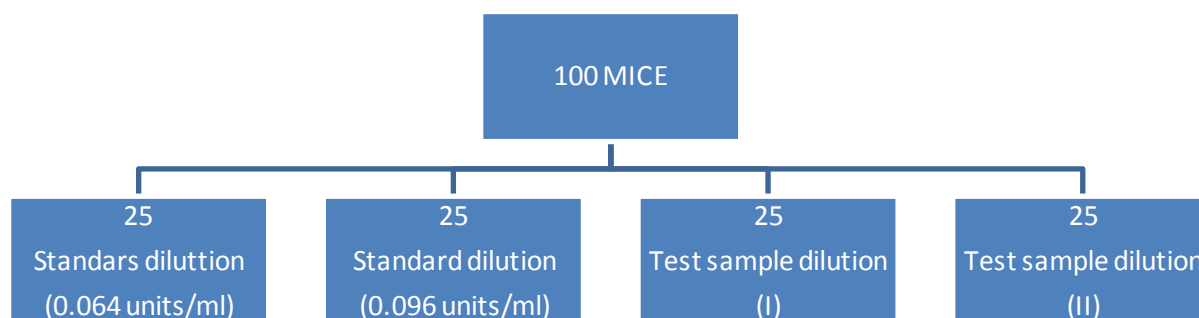
Other official methods

Mouse Method

Principle: Mice show characteristic convulsions after sub-cutaneous injection of insulin at elevated temperatures. The percentage convulsions produced by the test and standard preparation are compared.

Experimental Procedure: Minimum 100 mice weighing between 18-22 gm. Of the same strain are used. They are maintained on constant diet and fasted 18 hr. prior to the experiment.

Standard dilutions are prepared with sterile saline solution, so as to contain 0.064 units/ml. (Std. dilution 1) and 0.096 units/ml. (Std. dilution 2). Similarly, test sample solutions are also prepared. Mice are divided into 4 groups each containing 25 mice and insulin is injected s.c.as follows:



Mice are put in an air incubator at 33⁰ C and observed for one and a half hour. An air incubator with a glass front provided with six shelves is used. The temperature is thermostatically controlled. Two mice are kept in each of the boxes made up of perforated sheets of metal.

The mice which convulse or die are taken out of the incubator and observed. The reacting mice usually convulse severely but failure of the animal to upright itself when placed on its back,

should as well be considered as convulsion. Convulsive mice may be saved by an injection of 0.5 ml of 5% dextrose solution.

Percentage convulsions produced by the test sample are compared with those of the standard sample.

Those animals which survive may be used again for another experiment after an interval of one week.

Dose	S ₁ 0.25 ml	S ₂ 0.25 ml	T ₁ 0.25 ml	T ₂ 0.25 ml
Convulsion				
Percentage Convulsion				

Other non official methods

- 1. Rat Diaphragm Method:** In this method increase in glycogen content of the muscle or increase in glucose uptake by muscle in response to insulin is taken as the index of potency of insulin.
- 2. Rat Epididymal Fat-Pad Method:** HERE the ability of insulin to increase CO₂ production by the fat-pad is taken as the parameter for the measurement of potency of the insulin preparation.
- 3. Radioimmunoassay:** It is the estimation of the concentration of the substance in unit quantity of preparation using radiolabelled antigens. A number of drugs are estimated now a days by radioimmunoassay methods because these methods are highly specific and highly sensitive.

The radioimmunoassay of insulin is based on the ability of human insulin (unlabelled) to displace beef's insulin (which may be labeled) from the binding sites (i.e. antibodies). The method involves following steps:

- I. Bovine insulin is injected into the sheep. After a week the serum containing antibodies produced against bovine insulin is collected from the blood of sheep.

- II.** The serum containing antibodies is exposed to radiolabelled insulin and the bound vs free ratio is determined.
- III.** The mixture of antigen-antibodies (labelled) is then added in different test tubes labeled as standard and test. About 6 concentration of the standards are taken. They are then added to different tubes and the bound vs free ratio is again determined using gamma-counter (Scintillation counter).
- IV.** Standard curves are determined and the concentration of test insulin is determined using the standard curve.

Experiment No. 9

Aim: To Study Test for Pyrogens (Rabbit Method)

Introduction

In Greek word pyrogen means, pyro: fire, gen: beginning (Producing fire/ fever producer).

A Pyrogen is a substance i.e products of the growth of micro organisms or may be parts of dead cells or metabolic products.

Test for pyrogens

The Pyrogens test is designed to limit the risk of febrile reaction following parenteral administration administration of drugs. It includes both in vivo and invitro tests.

1. In vivo test (Rabbit test)
2. InVitro test (LAL test)

Pyrogen are originated from all type of bacteria and are all type bacteria and are closely related to bacterial antigen. They may get access to any parenteral solution. Certain drugs and injection are prone to pyrogenic contamination then other. A number of pharmaceutical solution in physiological saline dextrose calcium gluconate. Hartmanns and Darrow's solution and injection of sodium Sulphate and sodium Lactate are susceptible. Certain salts and drug are specially liable to pyrogenicity because of their preparation by fermentation process e.g sodium citrate solution, dextran and antibiotics certain preparation like liver extract and protein hydrolase can contaminant.

In a normal healthy person, pyrogenic reaction are not severe enough to be fatal, although the symptom can be extremely distressing various symptoms like hyperthermia, chill and body aches etc. Patients who require large volume of i/v fluid are already seriously ill and the introduction of pyrogenic solution instead of adding the patient recovery may cause adverse effect therefore pyrogen testing is important.

At present according to the rule every solution must be passed and than label as “ pyrogen free “ before use. The other qualities to be checked in the solution are sterility, particles, concentration.

The sample which are pyrogenic are either discarded or are made pyrogen free (if possible).

Various method use:

1. Adsorption on charcoal
2. Heating at 126°C for 5 hours
3. Carefully distillation

Pyrogen cause hyperthermia is not known 2 possibilities are as follow:

1. Pyrogen generate PGE IN brain which increase the temperature.
2. Temperature is controlled by thermoregulatory sample of hypothalamus. The ratio between sodium and calcium ions are appear to determine the discharge rate of neurons in hypothalamus . Pyrogen probably remove the inhibitory influence of calcium ions on the permeability of these neurons on the sodium and produce hyperthermia.
3. Glass apparatus syringes, hypodermic needle etc. are made pyrogen free in a muffle furnace at 250°C for half an hour. The product to tested should be warmed at 37°C . If the volume of the product should be less, than it should be diluted with pyrogen free saline or as per the monograph of the pharmacopoeia.

Precaution

1. Animal in no way should be excited.
2. Animal once used for pyrogen testing should not to be used again atleast for 3 days . If the pyrogen test have been positive it should be used after 3 weeks only.
3. Applicator should be interested to the depth of not less than 6cm and note more than 9cm.
4. Injection should be given by a single prick.
5. All apparatus should by pyrogen free.

Discussion

Pyrogens are water soluble, heat stable, non volatile by product of bacterial metabolism which have a characteristic property of producing rise in body temperature when administered parentally. They are macromolecules having mol. Wt. 150000 to 4000000.

Chemically they are lipid associated with complex polysaccharides or polypeptides molecules containing phosphorus and nitrogen. On hydrolysis, they may lead to the formation of glucosamine and glucose.

Table 1: Observation Table

Sr. No.	No. of Rabbits	Sample passes	Sample fails
1	3	1.15° C	2.65° C
2	6	2.80° C	4.30° C
3	9	4.45° C	5.95° C
4	12	6.60° C	6.60° C

Name of sample:

Batch no.

Name of manufacturer:

Trolley no.

Rabbit No.	Sex	Wt.	Dose initial temp. Rise in temp.				Max	Total
			2	3	mean		rise	rise

sign

Date:

Quality control officer

Head of Department

Conclusion

After completion of last step, find out the differences between initial and final temperature of each rabbit. The total maximum rise in temperature during three hours is summed up. If the sum of temperature difference is less than 1.15°C , the sample is considered to be pyrogen free. The sample fails if the response exceeds 2.65°C . If it is in between 1.15°C - 2.65°C the test is repeated again in 3 rabbits.

Limulus Amoebocyte Lysate Test(LAL Test)

Rabbit fever response test has been recommended so far by I.P, U.S.P, B.P.,and U.S.S.R.P. Recently another test has been introduced. This is LAL test it has been accepted by Q.C department of many countries including india, U.K and U.S. It has been given as alternative test to rabbit test.

LAL test based on the principle that pyrogen being an endotoxin when added to a suitable LAL preparation, causes gelation or increase in viscosity of the lysate which is directly proportional to the amount of endotoxin. In this test, 3 pyrogen free glass tubes are taken. To 1 test tube, the sample 0.1ml and to 2nd test tube, pyrogen free water or solution is added. To each test tube 0.1ml. of Entamoeba toxate is added and mixed. The test tube are incubated in water bath at 37°C . If there is an increase in viscosity within 30 minutes, the sample is considered as pyrogen positive.

LAL test has the advantage that

1. It require less time
2. Require less volume of the solution under test
3. Rules out biological variations

Disadvantage

1. It has limitation for biological products where product itself react with lysate, for example plasma, serum etc.
2. It require rigid control on lysate to be used.
3. It is expensive.

Experiment No. 10

Aim: Determination of acute oral toxicity (LD_{50}) of a drug from given data.

Acute toxicity studies demonstrate the adverse effects occurring within a short time, usually up to 2 months following administration of a single dose of a substance or multiple doses given in 24 hours.

LD_{50} : LD_{50} stands for Lethal Dose. LD_{50} is the amount of a substance given all at once, which causes the death of 50 % (one half) of a group of test animals. The LD_{50} is one way to measure the short term poisoning potential (acute toxicity) of a substance. Most often acute toxicity test is done with rats and mice.

Principle: The determination of ED_{50} (the dose effective in producing certain expected response in 50% of animal group) values helps in ascertaining the potency of a drug in terms of a reference standard. The calculation of ED_{50} value is done when a drug is showing graded response. But when the response is quantal or all-or-none, the ED_{50} value becomes LD_{50} (the dose lethal to 50% of animal group). Both these value i.e. ED_{50} and LD_{50} are important for knowing the safety of a drug. The ratio between LD_{50} and ED_{50} (LD_{50}/ED_{50}) represents therapeutic index. Greater the therapeutic index, safer is the drug. The therapeutic index of most of the drugs which have low margin of safety is generally close to unity. Ideally one would like to determine a dose that is effective in most of animals (ED_{99}) and least toxic to most of the animals of a group (LD_{10}). These value can, however, be calculated from the graph.

There are various methods used to calculate LD_{50} Values viz. the graphical method, arithmetical method and the statistical approach. For research purpose, the most widely used method is that of Litchfield and Wilcoxon (1949). For the routine practical class work, Reed and Muench, Miller and Tainter or the Karber's method can be used for calculating LD_{50} by either method, find out the least tolerated (smallest) dose (100% mortality) and most tolerated (highest) dose (0% mortality) by hit and trial method. Once these two doses are determined, select at least 5 doses in between the least tolerated and most tolerated doses, and observe the mortality due to these doses. Generally, mice are used for this purpose and each dose group should consist of 10 animals.

One can determine LD₅₀ value by different routes of administration. The LD₅₀ value of a new drug is determined by oral as well as by one of the parenteral routes (IP, IV or IM) of administration.

Requirement: Animal- Mice (20-25g)

Drug-Procaine hydrochloride

Procedure

1. Use overnight fasted mice. Each dose group should consist of 10 mice.
2. Inject the drug by intraperitoneal route and observe the animal for 2 hours for death due to acute toxicity.
3. The table below gives the result of a LD₅₀ Study Done With Procaine in The Mice using Miller and Tainter (1944). Note that a correction factor is applied to 0 and 100 per cent mortality group. The per cent mortality values are converted to probit values by reading the corresponding probit units from the probit table.
4. Plot the probit values against log doses and read LD₅₀ value as the dose that correspond to probit 5.
5. The LD₅₀ value of procaine hydrochloride was found to be 105.9mg/kg, IP.

Procaine hydrochloride toxicity data in mice

Group	Dose mg/kg, ip	Log dose	Dead/total	% Dead	Corrected Probit	Probit
1.	68	1.83	0/10	0	2.5*	3.04
2.	75	1.87	1/10	10	10	3.72
3.	91	1.96	2/10	20	20	4.16
4.	110	2.04	6/10	60	60	5.25
5.	146	2.16	9/10	90	90	6.28
6.	161	2.21	10/10	100	97.5*	6.96

* correction for 0% dead = $100(0.25/n)$ and 100 dead = $100 \times (n-0.25/n)$

EXPERIMENT NO. 11

Aim: Determination of Acute skin Irritation/Corrosion of a Test Substance.

Introduction

Dermal irritation is the production of reversible damage of the skin following the application of a test chemical for up to 4 hours.

Dermal corrosion is the production of irreversible damage of the skin like visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to 4 hrs. Corrosive reactions are classified by ulcers, bleeding and bloody scabs and by the end of observation at 14 days by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate lesions.

Principle:

1. The test chemical to be tested is applied in a single dose to the skin of an experimental animal; untreated skin areas of the test animal serve as the control. The degree of irritation/corrosion is read and scored at specified intervals and is further described in order to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effect observed.
2. 2. Animals showing continuing signs of severe distress and/or pain at any stage of the test should be humanely killed, and the test chemical assessed accordingly.

Requirements

Animal: Rabbit

Apparatus: Rabbit holder

Selection of animal species

The healthy young adult rabbits are used.

Preparation of the animals

1. Approximately 24 hours before the test, fur should be removed by closely clipping the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin, and only animals with healthy, intact skin should be used.
2. Some strains of rabbit have dense patches of hair that are more prominent at certain times of the year. Such areas of dense hair growth should not be used as test sites.

Housing and feeding condition

Animals should be individually housed. The temperature of the experimental animal room should be 20°C 3°C) for rabbits. Although the relative humidity should be at least 30 % and preferably not exceed 70 %, Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water.

Procedure:

Application of the test chemical

1. The test chemical should be applied to a small area (approximately 6 cm²) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In cases in which direct application is not possible (e.g., liquids or some pastes) the test chemical should first be applied to the gauze patch, which is then applied to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. If the test chemical is applied to the patch, it should attach to the skin in such a manner that there is good contact and uniform distribution of the test chemical on the skin.
2. Liquid test chemical are generally used undiluted .when testing solids (which may be pulverized, if considered necessary), the test chemical should be moistened with the smallest amount of water (or where necessary, of another suitable vehicle) sufficient to ensure good skin contact. When vehicles other than water are used, the potential influence on the vehicle on irritation of the skin by the test chemical should be minimal, if any.

3. At the end of the exposure period, which is normally 4 hours, residual test chemical should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Dose

A dose of 0.5 ml of liquid or 0.5 g of solid or paste is applied to the test site.

Initial test (using one animal)

1. The test is performed initially using one animal and applying the following approach. Three test patches are applied sequentially to the animal. The first patch is removed after three minutes. If no serious skin reaction is observed, a second patch is applied at a different site and removed after one hour. If the observations at this stage indicate that exposure can humanely be allowed to extend to four hours, a third patch is applied and removed after four hours, and the response is graded.
2. If a corrosive effect is observed after any of the three sequential exposures, the test is immediately terminated. If a corrosive effect is not observed after the last patch is removed, the animal is observed for 14 days, unless corrosion develops at an earlier time point.
3. In those cases in which the test chemical is not expected to produce corrosion but may be irritating, a single patch should be applied to one animal for four hours.

Confirmatory test (with additional animals)

If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals, each with one patch, for an exposure period four hours.

If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner or by exposing two additional animals simultaneously. In the exceptional case, in which the initial test is not conducted, two or three animals may be treated with a single

patch, which is removed after four hours. When two animals are used, if both exhibit the same response, no further testing needed. Otherwise, the third animal is also tested.

Observation period

The duration of the observation period should be sufficient to evaluate fully the reversibility of the effects observed. However, the experiment should be terminated at any time that the animal shows continuing signs of severe pain or distress. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches. If reversibility is seen before 14 days, the experiment should be terminated at that time.

Clinical observations and grading of skin reactions

1. All animals should be examined for signs of erythema and oedema, and the responses scored at 60 minutes, and then at 24, 48 and hours after patch removal. For the initial test in one animal, the test site is also examined immediately after the patch has been removed. Dermal reactions are graded and recorded according to the grades in the table. If there is damage to skin which cannot be identified as irritation or corrosion at 72 hours, observations may be needed until day 14 to determine the reversibility of the effects. In addition to the observation of irritation, all local toxic effects, such as defatting of the skin, and any systemic adverse effects (e.g., effect on clinical signs of toxicity and body weight), should be fully described and recorded. Histopathological examination should be considered to clarify equivocal responses.

2. The grading of skin responses is necessarily subjective. To promote harmonization in grading of skin response and to assist testing laboratories and those involved in making and interpreting the observations, the personnel performing the observations need to be adequately trained in the scoring system used.

Evaluation of results

The dermal irritation scores should be evaluated in combination with the nature and severity of lesions, and their reversibility or lack of reversibility. The individual scores do not represent an absolute standard for the irritant properties of material, as other effects of the test material are also evaluated. Instead individual scores should be viewed as reference which needs to be evaluated in combination with all other observations from the study.

Reversibility of dermal lesion should be considered in evaluating, irritant response. When responses such as alopecia (limited area), hyperkeratosis, hyperplasia and scaling, continue to the end of the 14 day observation period, the test chemical should be considered as an irritant.

Grading and skin reaction

Table 1: Erythema and Eschar formation

Sr. no	Skin reaction	Grade
1	No Erythema	0
2	Very Slight erythema	1
3	Well defined erythema	2
4	Moderate to severe erythema	3
5	Severe erythema(redness) to eschar formation preventing grading of erythema	4

Maximum possible: 4

Table 2: Oedema formation

Sr. No	Skin Reaction	Grade
1	No Oedema	0
2	Very slight Oedema	1
3	Slight Oedema(edges of area well defined by definite raising)	2
4	Moderate Oedema(raised approximately 1 mm)	3
5	Severe Oedema (raised more than 1 mm & extending beyond area of exposure)	4

Maximum Possible: 4

Experiment No. 12

Aim: Determination of Acute Eye Irritation/Corrosion of a Test Substance .

Introduction

Ocular corrosive: A substance that cause irreversible tissue damage to the eye.

Ocular irritant: A substance that produce a reversible change in the eye.

Ocular severe irritant: A substance that causes tissue damage in the eye that does not resolve within 21 days of application or causes serious physical decay of vision.

Principle:

Following pretreatment with a systematic analgesic and induction of appropriate topical anesthesia, the substance to be tested is applied in a single dose to one if the eyes of the experimental animal; the untreated eye serve as the control. The degree of eye irritation/corrosion is evaluated by scoring lesions of conjunctiva, cornea, and iris, at specific intervals. Other effects in the eye and adverse systematic effects are also described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effects.

Animals showing signs of severe distress and/ or pain at any stage of the test or lesions should be humanely killed, and substance assessed accordingly. Requirements:

Animal: Rabbit

Apparatus: Rabbit holder, binocular loupe, hand slit-lamp, biomicroscope etc.

Chemicals: Proparacaine or Tetracaine, Buprenorphne, Meloxicam etc.

Preparations of the test:

Selection of species:

The healthy young adult animals are used

Preparations of animals:

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hrs before testing starts. Animals showing eye irritation, ocular defects, or pre-existing corneal injury should be used.

Housing and feeding conditions:

Animals should be individually housed. The temperature of the experimental animal room should be 20°C ($\pm 3^\circ\text{C}$) for rabbits. The relative humidity should be at least 30% and preferably

not exceed 70%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Excessive light intensity should be avoided. For feeding conventional laboratory diets may be used with an unrestricted supply of drinking water.

Procedure:

Use of topical anesthetics and systemic analgesics:

The following procedures are recommended to minimize pain and distress in ocular safety testing procedures.

1. 60 minutes prior to test substance application (TSA), buprenorphine. 0.01 mg/kg is administered by subcutaneous injection to provide a therapeutic level of systematic analgesia. (Buprenorphine and other similar opioid analgesics administered systematically are not known or expected to alter ocular responses).
2. 5 minute prior to TSA, one or two drops of topical ocular anesthetic (e.g. 0.5 % proparacaine hydrochloride or 0.5 % tetracaine hydrochloride) are applied to each eye. In order to avoid possible interference with the study, a topical anesthetic that dose not contain preservatives is recommended.
3. The eye of each animal that is not treated with a test substance, but which is treated with topical anesthetics, serves as control. If the test substance is anticipated to cause significant pain and distress, it should not normally be tested in vivo. However, in case of doubt or where testing is necessary, consideration should be given to additional applications of the topical anesthetic at 5-minutes intervals prior to TSA.
4. 8 hours after TSA, buprenorphine 0.01 mg/kg S.C. and meloxicam 0.5mg/kg S.C. are administered to provide a continued therapeutic level of systemic analgesia. (While there are no data to suggest that meloxicam has anti-inflammatory effect on eye when administered s.c. once daily, meloxicam should not be administered until at least 8 hrs after TSA in order to avoid any possible interference with the study).
5. After initial 8 hrs post-TSA treatments, buprenorphine 0.01 mg/kg s.c. Should be administered every 12 hours, in conjunction with meloxicam 0.5mg/kg s.c. every 24 hrs, until the ocular lesions resolve and no clinical sign of pain and distress are present.
6. "Rescue" analgesia should be given immediately after TSA if pre-emptive analgesia and topical anesthesia are inadequate. If an animal shows signs of pain and distress during the study , a "rescue" dose of buprenorphine 0.03 mg/kg s.c. would be given

immediately and repeated as often as every 8 hours, if necessary, instead of 0.01 mg/kg s.c. every 12 hours. Meloxicam 0.5 mg/kg s.c. would be administered every 24 hours in conjunction with the “rescue” dose of buprenorphine, but not until at least post TSA.

Analgesic with anti inflammatory activity (e.g. meloxicam) should not be applied topically, and dose use systemically should not interfere with ocular effects

Application of the test substance

The test substance should be placed in the conjunctive sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to prevent loss of the material. The other eye, which remains untreated, serves as a control.

Dose

1. Testing of liquids

For testing liquids, a dose of 0.1 ml is not to be used. Pump sprays should not be used for installing the substance directly into the eye. The liquid spray should be expelled and collected in a container prior to installing 0.1 ml into the eye.

2. Testing of solids:

When testing solids pastes and particulates substances, the amount should have a volume of 0.1 ml or a weight of not more than 100 mg. The test material should be ground to a fine particle. The volume of solid material should be measured after gently compacting it, e.g. by tapping the measuring container. If the solid test substance has not been removed from the eye of the test animal by physiological mechanism at the first observation time point of 1 hour after treatment, the eye may be rinsed with saline or distilled water.

3. Testing of aerosols:

It is recommended that all pump sprays and aerosols be collected prior to instillation into the eye. The one exception is for substance in pressurized aerosols container which can't be collected due to vaporization. In such cases, the eye should be held open, and the test substance administered to the eye in simple burst of about one second, from a distance of 10 cm directly in front of eye. The distance may vary depending on the pressure of the spray and its contents. Care should be taken not to damage the eye from the pressure of the spray. In appropriate cases, there may be a need to evaluate the potential for “mechanical” damage to the eye from the force of the spray.

An estimate of the dose from an aerosol can be made by simulating the test as follow: the substance is sprayed on to weighing paper through an opening the size of rabbit eye placed directly before the paper. The weight increases of the paper is use to approximate the amount sprayed into the eye. For volatile substances, the dose may be estimated by weighing a receiving before and after removal of the test material.

Irrigation:

The eye of the test animals should not be washed for at least 24 hrs following installation of the test substance, except for solids and in case of immediate corrosive and irritating effects. At hours a washout may be used if considered appropriate.

A) Initial testing (using one animal)

It is strongly recommended that the in vivo be performed initially using one anima. Observation observation should allow for determination of severity and responsibility before proceeding to a confirmatory test in second animal.(If the result of this test indicate the substance to be corrosive or a severe irritant to the eyes using the procedure described , further testing for ocular irritancy should not be performed).

B) Confirmatory test (with additional animal) If a corrosive or severe irritant effect is not observed in the initial test , the irritant or negative response should be confirmed using upto two additional animals. If an irritant effect is observed in the initial test, it is recommended that that the confirmatory test be conducted in sequential manner in one animal at a time, rather than exposing the two additional animals simultaneously. If the second animals reveal corrosive or severe irritant effects the test is not continued. If the result from the second animal is sufficient to allow for a hazard classification determination, than no further testing should be conducted.

Observation period:

The duration of the observation period should be sufficient to evaluate fully the magnitude and reversibility of the effects observed. However, the experiment should be terminated at any time that the animal shows sign of severe pain or distress. To determine reversibility of effects, the animal should be observed normally for 21 days post administration of the test substance. If reversibility is seen before 21 days, the experiment should be terminated at that time.

Clinical observation and grading of eye reaction:

1. The eye should be comprehensively evaluated for the presence or absence of ocular lesions one hour post-TSA, follow by at least daily evaluations. Animals should be evaluated several times daily for three days to ensure that termination decisions are made in a timely manner.
2. Test animals should be routinely evaluated for the entire duration of the study for clinical signs of pain and \ or distress like repeated pawing or rubbing of the eye , excessive blinking and excessive tearing at least twice daily, with a minimum of 6 hrs between observations or more often if necessary. (This is necessary to adequately assess animals for evidence of pain and distress in order to make informed decisions on the need to increase the dosage of analgesics).
3. Fluorescein staining should be routinely used and a slit lamp biomicroscope used for assessing the depth of injury when corneal ulceration is present, as an aid in the detection and measurement of ocular damage.
4. Digital photographs of observed lesions may be collected for reference and to provide a permanent record of the extent of ocular damage. Animal should be kept on test no longer than necessary once definitive information has been obtained. Animal showing severe pain or distress should be humanely killed without delay, and the substance assessed accordingly.
5. Animals with the following eye lesions post-installation should be humanely killed. These lesions are corneal perforation or significant corneal ulceration including staphyloma, blood in anterior chamber of the eye, grade 4 corneal opacity , absence of a light reflex (iridial response grade 2} which persists for 72 hrs, ulceration of the conjunctive membrane , necrosis of the conjunctiva or nictitating membrane or sloughing. This is because such generally are not reversible.
6. Furthermore, it is recommended that the following ocular lesions be used to terminate studies before the end of the 21- day observation period. These lesions are considered predictive of severe irritant or corrosive injuries and injuries that are not expected to fully reverse by the end of 21-day observation period. These are severe depth of injury (e.g., corneal ulceration extending beyond the superficial layers of the stroma), limbus destruction >50 % (as evidence by blanching of the conjunctival tissue), and severe eye infection (purulent discharge). A combination of: vascularization of the cornea surface {i.e., pannus}, area of fluorescein staining not diminishing over time based on daily assessment and \ or lack of re-epithelialization 5 days after test substance application could also be considered as potentially useful criteria to influence the clinical decision on early study termination.

7. The grades of ocular reaction (conjunctivae, cornea and iris) should be obtained and recorded at 1, 24, 48, and 72 hrs following test substance application. Animals that do not develop ocular lesions may be terminated not earlier than 3 days post instillation. Animals with ocular lesions that are not severe should be observed until the lesions clear, or for 21 days, at which time the study is terminated.

8. Observation should be performed and recorded at a minimum 1 hour , 24 hour ,48 hours, 72 hours, 7 days, 14 days, 21 days in order to determine the status of lesions, and their reversibility or irreversibility.

9. The grades of ocular lesions should be recorded at each examination. Any other lesions in the eye (e.g. pannus, staining, anterior chamber changes) or adverse systemic effect should also be reported.

Examination of reaction can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observation at 24 hrs, the eyes may be further examined with the aid of fluorescein.

Evaluation of results:

The ocular irritation scores should be evaluated in conjunction with the nature and severity of lesions, and their reversibility or irreversibility. The individual scores do not represent an absolute standard for the irritant properties of a material, as other affects of the test material are also evaluated. Instead, individual scores should be viewed as reference values and are only meaningful when supported by a full description and evaluation of all observation.

Interpretation of the results:

Extrapolation of the results of eye irritation studies in laboratory animals to humans is valid only to a limited degree. In many cases the albino rabbit is more sensitive than humans to ocular irritants or corrosives. Care should be taken in the interpretation of data to exclude irritation resulting from secondary infection.

Grading of ocular lesions:

Sr.No	Cornea	Grade
1	Opacity: Degree of density (readings should taken from most dense area)* No ulceration or opacity.	0

2	Scattered or diffuse areas of opacity: (other than slight dulling of normal lustre): Details of iris clearly visible.	1
3	Easily discernible translucent areas: Details of iris slightly obscured.	2
4	Nacrous areas: No details of iris visible , size of pupil barely discernible	3
5	Opaque cornea: Iris not discernible through the opacity.	4

* The area of corneal opacity should be noted

Maximum possible : 4

Sr.No	Iris	Grade
1	Normal	0
2	Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperemia; or injection; iris reactive to light [a sluggish reaction is considered to be an effect.	1
3	Hemorrhage, gross destruction, or no reaction to light	2

Maximum possible : 2

Sr.No	Conjunctiva	Grade
1	Redness (refers to palpebral and bulbar conjunctivae; excluding cornea and iris) Normal	0
2	Some blood vessels hyperaemic (injected)	1
3	Diffuse crimson color , individual vessels not easily	2
4	Diffuse beefy red	3

Maximum possible: 3

Sr. No.	Chemosis	Grade
1	Swelling (refers to lids and /or nictitating membranes)Normal	0
2	Some swelling above normal	1
3	Obvious swelling , with partial eversion of lids	2
4	Swelling with lids about half closed	3
5	Swelling with lids more than half closed	4

Maximum possible: 4

EXPERIMENT NO: 13

Object: Calculation of Pharmacokinetic Parameters from a Given Data.

Objective: TO Study the pharmacokinetics parameters of a drug in rabbit

Introduction:

PHARACOKINETICS is defined as kinetics of drug absorption, distribution, metabolism and excretion [AMED] and their relationship with pharmacologic, therapeutic and toxicological response in man and animals.

PHARMACOKINETICS has become one of the most popular aspects in pharmacology. The importance of pharmacokinetics was realized by pharmaceutical industry and, drug control authorities and the pharmacologist when report of bioinequivalence caused by manufacturing factors has serious clinical effects. Pharmacokinetics is now considered as a well established discipline. Understanding of its principles and applications are not important only as an aid towards safer and more effective therapy but also in drug development and clinical studies of new drugs, therapeutics monitoring and clinical pharmacology and in toxicology.

PEAK PLASMA CONCENTRATION [C_{max}]: The point of maximum concentration of drug in plasma is called as the peak and the concentration of drug at peak is known as peak plasma concentration. It is also called peak height concentration and maximum drug concentration.

C_{max} is expressed in $\mu\text{g/ml}$. The peak level depends upon the administered dose and rate of absorption and elimination. The peak represents the point of time when absorption rate equals elimination rate of drug. Peak concentration is often related to the intensity of pharmacologic response and should ideally be above minimum effective concentration [MEC] but less than maximum safe concentration [MSC]

TIME OF PEAK CONCENTRATION [t_{max}]: The time for drug to reach concentration in plasma [after extra vascular administration] is called as the time of peak concentration. It is expressed in hours and is useful in estimating the rate of absorption. Onset time and onset of action

are dependent upon t_{max} . The parameter is of particular importance in assessing the efficacy of drug use to treat acute condition like pain and insomnia which can be treated by a single dose

AREA UNDER THE CURVE [AUC]: It present the total integrated area under the plasma level time profile and expresses the total amount of drug that come into the systemic circulation after it administration. AUC is expressed in $\mu\text{g/ml} \times \text{hours}$. It is the most dosage form as it represents the extent of absorption. AUC is also important for drugs are administered repetitively for the treatment of chronic conditions like asthma or epilepsy.

REQUIREMENTS: Rabbit, Rabbit holder, needles, syringes etc

Table 1: observation table

1	0 min	
2	30 min	
3	1 hour	
4	2 hour	
5	4 hour	
6	6 hour	
7	12 hour	
8	24 hour	

PROCEDURE:

1. Select healthy rabbit.
2. Animal are placed in rabbit holder. About 1 ml of blood is withdrawn from marginal ear vein by venepuncture.
3. Drug is administered intravenously into the marginal ear vein of the rabbit.
4. Blood sample are withdrawn at an interval of 30 minute 1, 2,4,8,12,18 and 24 hours from marginal ear by venepuncture.
5. The samples are analysed for concentration of drug in the blood.
6. The concentration of drug is entered in the observation table and it is plotted against time on a semi log paper .The concentration of drug should be on semi –log side {y-axis}.

Calculation:

1. Find out the concentration at zero time (c_0) by simple extra polation of the graph.
2. From the graph find out the half life of drug. $t_{1/2}$.
3. Calculate elimination constant (k_e) by the following equation.

$$K_e = 0.693 / t_{1/2}$$

4. Calculate volume of distribution (v_d) as follows.

$$V_d = \text{Dose} / c_0$$

5. Other parameters that can be determined from the graph are as follows.

a. AUC= Area under the curve

b. T_{max} = time to reach maximum effect

c. C_{max} = concentration of the drug at T_{max}

Importance of pharmacokinetics:

- a. The studies completed in laboratory animals may give useful indication for drug research and development. For eg less powerful molecules in vitro can turn out more effective in vivo because of their favourable kinetics (greater absorption, better distribution, etc.).
- b. Pharmacokinetics supports the studies of preclinical toxicology in animals (toxicokinetics) because the drug level in plasma or tissues are often more predictive than the dose to extrapolate to the toxicity data to man.

Toxicokinetics is also important to:

- i) Verify that the animals have measurable levels of drug in plasma and that these level are proportional to the administration dose.
- ii) Estimate the area under the curve and the maximum concentration of the drug in plasma because these parameter can be used to represent the exposure of the body to the drug.

- iii) Evidence difference in pharmacokinetics between the virus group of treatment, the days of treatment and other factors.
- iv) Estimate the variability between animals and identify cases with abnormal levels of the drug.
- c. Knowledge of the kinetics and of the effect (pharmacodynamics) of drug in Animals man is necessary for a correct use of drugs in therapy (choice of the best route of administration, choice of the best dose regimen, dose individualization).

Experiment No.14

Aim: To study Biostatics Methods in Experimental Pharmacology (Student t Test, ANOVA)

Statistics

It is the discipline that concerns the collection, organization, displaying, analysis, interpretation and presentation of data.

It is an important branch of mathematics which deals with collecting, recording, and presenting data collected from experiments, records and surveys.

Biostatistics

- The statistics applied to biological data is termed “Biostatistics”.
- It is the branch of statistics concerned with mathematical facts and data related to biological events.
- Biostatistics covers applications and contributions not only from health, medicines and nutrition but also from fields such as genetics, biology, epidemiology, and many others.
- Any science needs precision for its development.
- Precision is all the more important when it comes to health sciences. For precision; facts, observations or measurements have to be expressed in figures.
- Medicine is essentially an empirical science. It depends on observations and not on theories or theorems.
- As a part of clinical practice or research we deal with +many observations, which when systematically arranged, are called “Data”.
- The process of converting data into information is called “statistics”
- “Statistics” means a measured or counted fact or piece of the information stated as a figure such as height of one person, birth weight of a baby etc.
- The success of any scientific study obviously depends on the selection of a valid variable or parameter and statistical design of the study.

Methods of statistical analysis

The methods of statistical analysis will depend on the purpose of the study, the design of the experiment, and the nature of the resulting data.

1. Parametric statistical analysis

2. Non- parametric tests

1. Parametric statistical analysis- student's t test should not be used when more than two treatment groups are to be compared. If there are several groups, it lacks power, and multiple testing increases the chance of a false-positive result. Where there are two or more groups, and particularly with randomized block or more-complex designs, the ANOVA should be used.

2. Non- parametric tests- several of these methods, such as the Mann-Whitney test, replace the individual observations by their ranks, resulting in the loss of some information; hence, these methods often lack back power in situations where parametric tests are appropriate , but they may be more powerful in situations where the parametric test is not appropriate.

There are several non-parametric tests -; Wilcoxon rank sum test and Mann-Whitney test

Branches of biostatistics

Descriptive biostatistics

Methods of producing quantitative summaries of information in biological science Tabulation and graphical presentations

Inferential biostatistics

Methods of making generalizations about a larger group based on information about a sample of that group in biological sciences.

History

The earliest writings on probability and statistics date back to Arab mathematicians and cryptographers, during the Islamic Golden Age between the 8th and 13th centuries. Al-Khalil (717–786) wrote the Book of Cryptographic Messages, which contains the

first use of permutations and combinations, to list all possible Arabic words with and without vowels.

The earliest European writing on statistics dates back to 1663, with the publication of *Natural and Political Observations upon the Bills of Mortality* by John Graunt. Today, statistics is widely employed in government, business, and natural and social sciences.

The modern field of statistics emerged in the late 19th and early 20th century in three stages. The first wave, at the turn of the century, was led by the work of Francis Galton and Karl Pearson, who transformed statistics into a rigorous mathematical discipline used for analysis, not just in science, but in industry and politics as well. Galton's contributions included introducing the concepts of standard deviation, correlation, regression analysis and the application of these methods to the study of the variety of human characteristics—height, weight, eyelash length among others.

Sir Francis Galton is considered as the Father of Statistics.

Basis of biostatistics

Sources of medical uncertainties

- Intrinsic due to biological, environmental and sampling factors.
- Incomplete knowledge
- Errors in measurement

Intrinsic variation source of medical uncertainties

- Biological due to age, gender, heredity, party, height, weight etc.
- Also due to variation in anatomical, physiological and biochemical parameters
- Biostatistics is the term used when tools of statistics are applied to the data that is derived from biological sciences such as medicines
- Any science demands precision, for its development and so does medical science
- For precision, facts, observations and measurements have to be expressed in figures.

- Everything in medicine be it research ,diagnosis or treatment depends on counting or measurement
- High or low blood pressure has no meaning, unless it is expressed in figures.
- Thus medical statistics or biostatistics can be called Quantitative medicine.
- In nature, blood pressure, pulse rate, action of drug or any other measurement or counting varies not only from person to person but also from group to group.
- Variation more than natural limits may be pathological, abnormal due to play of certain external factors. Hence biostatistics may also be called a science of variation.
- The data after collection, are of no use,unless they are properly sorted, presented, compared, analyzed and interpreted.
- For such a study of figures, one has to apply certain mathematical techniques called statistical methods.

Data

1. The raw material of statistics is data.
2. Data are the values that the variable can assume, data can be as figures. Figures as a result from counting or taking measurements.
3. For example- when a hospital administrator counts number of patients(counting)
When a nurse weighs a patient (measurement)

Types of data

Qualitative data- consist of attributes, labels, non-numerical entries etc.

Quantitative data- consist of numerical, measures or counts etc.

Sources of data

1. Internal sources
2. External sources
3. Surveys
4. Experiments

Student's t Test

Introduction

The t-test is any statistical hypothesis test in which the test statistic follows a student's t-distribution under the null hypothesis.

A t-test is most commonly applied when the test statistic would follow a normal distribution if the value of a scaling term in the test statistic were known. When the scaling term is unknown and is replaced by an estimate based on the data, the test statistic (under certain conditions) follows a student's t distribution. The t-test can be used, for example, to determine if two sets of data are significantly different from each other.

History

The t-statistic was introduced in 1908 by William Sealy Gosset, a chemist working for the Guinness brewery in Dublin, Ireland. "student" was his pen name.

Gosset had been hired owing to Claude Guinness's policy of recruiting the best graduates from Oxford and Cambridge to apply biochemistry and statistics to Guinness's industrial processes. The t-test work was submitted to and accepted in the journal *Biometrika* and published in 1908.

Use of t-test

In medical research, t-test is among the three or four most commonly used statistical tests. The purpose of a t-test is to compare the means of a continuous variable in two research samples in order to determine whether or not the difference between the two observed means exceeds the difference that would be expected by chance from random samples.

Some important terms used in t-test –

1. Degree of freedom
2. The t-distribution

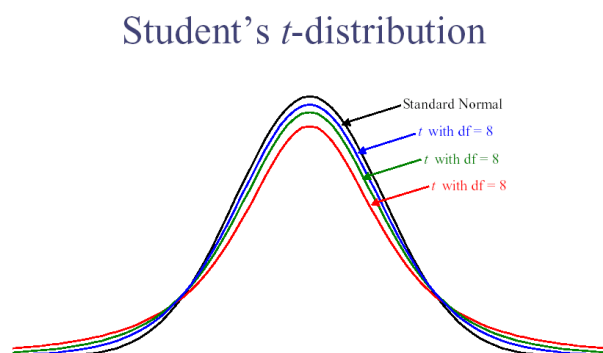
The term degree of freedom refers to the number of observations that are free to vary. The degrees of freedom for any tests are considered to be the total sample size minus 1 degree of freedom for each mean that is calculated.

The t-distribution was described by William Gosset, who used the pseudonym “student” when he wrote the description. The t distribution is necessary because when the sample size are small, the observed estimates of mean and variance are subject to considerable error. The larger the sample size is the smaller the errors are and the more the t size distribution looks like the normal distribution.

Assumptions

Most t-test statistics have the form $t = Z/s$, where Z and s are functions of the data. Typically, Z is designed to be sensitive to the alternative hypothesis (i.e., its magnitude tends to be larger when the alternative hypothesis is true), whereas s is the scaling parameter that allows the distribution of t to be determined.

The t-test (also called student’s t-test) compares two averages (mean) and tells you if they are different from each other. The t test also tells you how significant the differences are; In other words it lets you know if those differences could have happened by chance.



The t-distribution, used for the t-test. Image: Carnegie Mellon.

A very simple example: Let’s say you have a cold and you try a naturopathic remedy. Your cold lasts a couple of days. The next time you have a cold, you buy an over-the-counter pharmaceutical and the cold lasts a week. You survey your friends and they all tell you that their colds were of a shorter duration (an average of 3 days) when they took the homeopathic remedy. What you *really* want to know is, are these results repeatable? A t test can tell you by comparing

the means of the two groups and letting you know the probability of those results happening by chance.

Another example: Student's T-tests can be used in real life to compare means. For example, a drug company may want to test a new cancer drug to find out if it improves life expectancy. In an experiment, there's always a control group (a group who are given a placebo, or "sugar pill"). The control group may show an average life expectancy of +5 years, while the group taking the new drug might have a life expectancy of +6 years. It would use a Student's t-test seem that the drug might work. But it could be due to a fluke. To test this, researchers would to find out if the results are repeatable for an entire population.

The T Score

The t score is a ratio between the **difference between two groups and the difference within the groups**. The larger the t score, the more difference there is between groups. The smaller the t score, the more similarity there is between groups. A t score of 3 means that the groups are three times as different *from* each other as they are within each other. When you run a t test, the bigger the t-value, the more likely it is that the results are repeatable.

A large t-score tells you that the groups are different.

A small t-score tells you that the groups are similar.

T-Values and P-values

How big is "big enough"? Every t-value has a p-value to go with it. A p-value is the probability that the results from your sample data occurred by chance. P-values are from 0% to 100%. They are usually written as a decimal. For example, a p value of 5% is 0.05. **Low p-values are good;** They indicate your data did not occur by chance. For example, a p-value of .01 means there is only a 1% probability that the results from an experiment happened by chance. In most cases, a p-value of 0.05 (5%) is accepted to mean the data is valid.

Calculating the Statistic / Test Types

There are **three main types of t-test**:

- An Independent Samples t-test compares the means for two groups.
- A Paired sample t-test compares means from the same group at different times (say, one year apart).
- A One sample t-test tests the mean of a single group against a known mean.

What is a Paired T Test (Paired Samples T Test / Dependent Samples T Test)?

A paired t test (also called a correlated pairs t-test, a paired samples t test or dependent samples t test) is where you run a t test on dependent samples. Dependent samples are essentially connected — they are tests on the same person or thing. For example:

- Knee MRI costs at two different hospitals,
- Two tests on the same person before and after training,
- Two blood pressure measurements on the same person using different equipment.

When to Choose a Paired T Test / Paired Samples T Test / Dependent Samples T Test

Choose the paired t-test if you have two measurements on the same item, person or thing. You should also choose this test if you have two items that are being measured with a unique condition. For example, you might be measuring car safety performance in Vehicle Research and Testing and subject the cars to a series of crash tests. Although the manufacturers are different, you might be subjecting them to the same conditions.

With a “regular” two sample t test, you’re comparing the means for two different samples. For example, you might test two different groups of customer service associates on a business-related test or testing students from two universities on their English skills. If you take a random sample each group separately and they have different conditions, your samples are independent and you should run an independent samples t test (also called between-samples and unpaired-samples).

The null hypothesis for the independent samples t-test is $\mu_1 = \mu_2$. In other words, it assumes the means are equal. With the paired t test, the null hypothesis is that the pairwise difference between the two tests is equal ($H_0: \mu_d = 0$). The difference between the two tests is very subtle; which one you choose is based on your data collection method.

Paired Samples T Test By hand

Sample question: Calculate a paired t test by hand for the following data:

Subject #	Score 1	Score 2
1	3	20
2	3	13
3	3	13
4	12	20
5	15	29
6	16	32
7	17	23
8	19	20
9	23	25
10	24	15
11	32	30

Step 1: Subtract each Y score from each X score.

Subject #	Score 1	Score 2	X-Y
1	3	20	-17
2	3	13	-10
3	3	13	-10
4	12	20	-8
5	15	29	-14
6	16	32	-16
7	17	23	-6
8	19	20	-1
9	23	25	-2
10	24	15	9
11	32	30	2

Step 2: Add up all of the values from Step 1.

Set this number aside for a moment.

Subject #	Score 1	Score 2	X-Y
1	3	20	-17
2	3	13	-10
3	3	13	-10
4	12	20	-8
5	15	29	-14
6	16	32	-16
7	17	23	-6
8	19	20	-1
9	23	25	-2
10	24	15	9
11	32	30	2
		SUM:	-73

Step 3: Square the differences from Step 1.

Subject #	Score 1	Score 2	X-Y	(X-Y) ²
1	3	20	-17	289
2	3	13	-10	100
3	3	13	-10	100
4	12	20	-8	64
5	15	29	-14	196
6	16	32	-16	256
7	17	23	-6	36
8	19	20	-1	1
9	23	25	-2	4
10	24	15	9	81
11	32	30	2	4
		SUM:	-73	

Step 4: Add up all of the squared differences from Step 3.

Subject #	Score 1	Score 2	X-Y	(X-Y)^2
1	3	20	-17	289
2	3	13	-10	100
3	3	13	-10	100
4	12	20	-8	64
5	15	29	-14	196
6	16	32	-16	256
7	17	23	-6	36
8	19	20	-1	1
9	23	25	-2	4
10	24	15	9	81
11	32	30	2	4
		SUM:	-73	1131

Step 5: Use the following formula to calculate the t-score:

$$t = \frac{(\sum D)/N}{\sqrt{\frac{\sum D^2 - \frac{(\sum D)^2}{N}}{(N-1)(N)}}$$

$\sum D$: Sum of the differences (Sum of X-Y from Step 2)

$\sum D^2$: Sum of the squared differences (from Step 4)

$(\sum D)^2$: Sum of the differences (from Step 2), squared.

$$t = \frac{(\sum D)/N}{\sqrt{\frac{\sum D^2 - \frac{(\sum D)^2}{N}}{(N-1)(N)}}$$

$$t = \frac{-73/11}{\sqrt{\frac{1131 - \frac{(-73)^2}{11}}{(11-1)(11)}}$$

$$t = \frac{-73/11}{\sqrt{\frac{1131 - \frac{5329}{11}}{110}}}$$

$$t = -2.74$$

Step 6: Subtract 1 from the sample size to get the degrees of freedom. We have 11 items, so $11-1 = 10$.

Step 7: Find the p-value in the t-table, using the degrees of freedom in Step 6. If you don't have a specified alpha level, use 0.05 (5%). For this sample problem, with $df=10$, the t-value is 2.228.

Step 8: Compare your t-table value from Step 7 (2.228) to your calculated t-value (-2.74). The calculated t-value is greater than the table value at an alpha level of .05. The p-value is less than the alpha level: $p < .05$. We can reject the null hypothesis that there is no difference between means.

Note: You can ignore the minus sign when comparing the two t-values, as \pm indicates the direction; the p-value remains the same for both directions

ANOVA

Analysis of variance (ANOVA) is a collection of statistical models and their associated estimation procedures (such as the "variation" among and between groups) used to analyze the differences among group means in a sample. ANOVA was developed

by statistician and evolutionary biologist Ronald Fisher. The ANOVA is based on the law of total variance, where the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether two or more population means are equal, and therefore generalizes the t -test beyond two groups.

ANOVA is useful for comparing (testing) three or more group means for statistical significance.

There are two main types; *one-way and two-way*

Two-way tests can be with or without replication

One-way ANOVA between groups ; used when you want to test two groups to see if there's a difference between them.

Assumptions of ANOVA

Like so many of our inference procedures, ANOVA has some underlying assumptions which should be in place in order to make the results of calculations completely trustworthy. They include:

- (i) Subjects are chosen via a simple random sample.
- (ii) Within each group/population, the response variable is normally distributed.
- (iii) While the population means may be different from one group to the next, the population standard deviation is the same for all groups. Fortunately, ANOVA is somewhat robust (i.e., results remain fairly trustworthy despite mild violations of these assumptions). Assumptions (ii) and (iii) are close enough to being true if, after gathering SRS samples from each group, you
 - (i) look at normal quantile plots for each group and, in each case, see that the data points fall close to a line.
 - (ii) compute the standard deviations for each group sample, and see that the ratio of the largest to the smallest group sample s.d. is no more than two.

Types of tests

There are two main types; one-way and two-way. Two-way tests can be with or without replication.

One-way ANOVA between groups-used when you want to test two groups to see if there's a difference between them.

Two way ANOVA without replication- used when you have one group and you're double-testing that same group. For example, you are testing one set of individuals before and after they take a medication to see if it works or not.

Two way ANOVA with replication- two groups, and the members of those groups are doing more than one thing, for example, two groups of patients from different hospitals trying two different therapies.

One way ANOVA

A one way ANOVA is used to compare two means from independent (unrelated) groups using the F-distribution. The null hypothesis for the test is that the two means are equal. Therefore, a significant result means that the two means are unequal.

When to use a one way ANOVA

Situation 1; you have individuals randomly split into smaller groups and completing different tasks. For example, you might be studying the effects of tea on weight loss and form three groups; green tea, black tea, and no tea.

Situation 2; Similar to situation 1, but in this case the individuals are split into groups based on an attribute they possess. For example, you might be studying leg strength of people according to weight. You could split participants into weight categories (obese, overweight and normal) and measure their leg strength on a weight machine.

Limitations of the One Way ANOVA

A one way ANOVA will tell you that at least two groups were different from each other. But **it won't tell you which groups were different**. If your test returns a significant f-statistic, you

may need to run an ad hoc test (like the Least Significant Difference test) to tell you exactly which groups had a difference in means.

Two Way ANOVA

A Two Way ANOVA is an extension of the One Way ANOVA. With a One Way, you have one independent variable affecting a dependent variable. With a Two Way ANOVA, there are two independents. Use a two way ANOVA when you have one measurement variable (i.e. a quantitative variable) and two nominal variables. In other words, if your experiment has a quantitative outcome and you have two categorical explanatory variables, a two way ANOVA is appropriate.

For example, you might want to find out if there is an interaction between income and gender for anxiety level at job interviews. The anxiety level is the outcome, or the variable that can be measured. Gender and Income are the two categorical variables. These categorical variables are also the independent variables, which are called **factors** in a Two Way ANOVA.

The factors can be split into levels. In the above example, income level could be split into three levels; low, middle, and high income. Gender could be split into three levels; male, female, transgender. Treatment groups are all possible combinations of the factors. In this example there would be $3 \times 3 = 9$ treatment groups.

Main Effect and Interaction Effect

The results from a Two Way ANOVA will calculate a main effect and an interaction effect. The main effect is similar to a One Way ANOVA: each factor's effect is considered separately. With the interaction effect, all factors are considered at the same time. Interaction effects between factors are easier to test if there is more than one observation in each cell. For the above example, multiple stress scores could be entered into cells. If you do enter multiple observations into cells, the number in each cell must be equal.

Two null hypotheses are tested if you are placing one observation in each cell. For this example, those hypotheses would be:

H_{01} : All the income groups have equal mean stress.

H_{02} : All the gender groups have equal mean stress.

For multiple observations in cells, you would also be testing a third hypothesis;

H_{03} : The factors are independent or the interaction effect does not exist.

An F-statistic is computed for each hypothesis you are testing.

Assumptions for Two Way ANOVA

- The population must be close to a normal distribution.
- Samples must be independent.
- Population variances must be equal.
- Groups must have equal sample sizes.

What is MANOVA

MANOVA is just an ANOVA with several dependent variables. It's similar to many other tests and experiments in that its purpose is to find out if the response variable (i.e. your dependent variable) is changed by manipulating the independent variable. The test helps to answer many research questions, including:

Do changes to the independent variables have statistically significant effects on dependent variables?

What are the interactions among dependent variables?

What are the interactions among independent variables?

MANOVA Example

Suppose you wanted to find out if a difference in textbooks affected students' scores in math *and* science. Improvements in math *and* science means that there are **two** dependent variables, so a MANOVA is appropriate.

An ANOVA will give you a single (univariate) f-value while a MANOVA will give you a multivariate F value. MANOVA tests the multiple dependent variables by creating new, artificial, dependent variables that maximize group differences. These new dependent variables are linear combinations of the measured dependent variables.

Interpreting the MANOVA results

If the multivariate F value indicates the test is statistically significant, this means that something is significant. In the above example, you would not know if math scores have improved, science scores have improved (or both). Once you have a significant result, you would then have to look at each individual component (the univariate F tests) to see which dependent variable(s) contributed to the statistically significant result.

Advantages and Disadvantages of MANOVA vs. ANOVA

Advantages

MANOVA enables you to test multiple dependent variables.

MANOVA can protect against Type-1 errors.

Disadvantages

MANOVA is many times more complicated than ANOVA, making it a challenge to see which independent variables are affecting dependent variables.

One degree of freedom is lost with the addition of each new variable.

What is Factorial ANOVA?

A factorial ANOVA is an Analysis of Variance test with more than one independent variable, or “**factor**“. It can also refer to more than one **Level of Independent Variable**. For example, an experiment with a treatment group and a control group has one factor (the treatment) but two levels (the treatment and the control). The terms “two-way” and “three-way” refer to the number of factors or the number of levels in your test. Four-way ANOVA and above are rarely used because the results of the test are complex and difficult to interpret.

A two-way ANOVA has two factors (independent variables) and one dependent variable. For example, time spent studying and prior knowledge are factors that affect how well you do on a test.

A three-way ANOVA has three factors (independent variables) and one dependent variable. For example, time spent studying, prior knowledge, and hours of sleep are factors that affect how well you do on a test

Factorial ANOVA is an efficient way of conducting a test. Instead of performing a series of experiments where you test one independent variable against one dependent variable, you can test all independent variables at the same time.

Assumptions of Factorial ANOVA

- Normality: the dependent variable is normally distributed.
- Independence: Observations and groups are independent from each other.
- Equality of Variance: the population variances are equal across factors/levels.

Variability

In a one-way ANOVA, variability is due to the differences between groups and the differences within groups. In factorial ANOVA, each level and factor are paired up with each other (“crossed”). This helps you to see what interactions are going on between the levels and factors. If there is an interaction then the differences in one factor depend on the differences in another.

Let's say you were running a two-way ANOVA to test male/female performance on a final exam. The subjects had either had 4, 6, or 8 hours of sleep.

IV1: SEX (Male/Female)

IV2: SLEEP (4/6/8)

DV: Final Exam Score

A two-way factorial ANOVA would help you answer the following questions:

Is sex a main effect? In other words, do men and women differ significantly on their exam performance?

Is sleep a main effect? In other words, do people have had 4, 6 or 8 hours of sleep differ significantly in their performance?

Is there a significant interaction between factors? In other word, how do hours of sleep and sex interact with regards to exam performance?

Can any differences in sex and exam performances be found in the different levels of sleep?

How to run an ANOVA

These tests are very time-consuming by hand. In nearly every case you'll want to use software. For example, several options are available in Excel:

Two way ANOVA in Excel with replication and without replication.

One way ANOVA in Excel 2013.

Running the test in Excel

ANOVA tests in statistics packages are run on parametric data. If you have rank or ordered data, you'll want to run a non-parametric ANOVA (usually found under a different heading in the software, like "nonparametric tests")

Steps

It is unlikely you'll want to do this test by hand, but if you must, these are the steps you'll want to take:

Find the mean for each of the groups.

Find the **overall mean** (the mean of the groups combined).

Find the **Within Group Variation**; the total deviation of each member's score from the Group Mean.

Find the Between Group Variation: the deviation of each group mean from the overall mean.

Find the F-static: the ratio between group variations to within group variation.

Experiment No. 15

Aim: To Study Biostatics methods in experimental Pharmacology (Chi square test, Wilcoxon Signed Rank test)

Chi Square Test

The term "chi-squared test," also written as χ^2 test, refers to certain types of statistical hypothesis tests that are valid to perform when the test statistic is chi-squared distributed under the null hypothesis. Often, however, the term is used to refer to *Pearson's* chi-squared test and variants thereof. Pearson's chi-squared test is used to determine whether there is a statistically significant difference (i.e., a magnitude of difference that is unlikely to be due to chance alone) between the expected frequencies and the observed frequencies in one or more categories of a so-called contingency table.

In the standard applications of this test, the observations are classified into mutually exclusive classes. If the so-called null hypothesis is true, the test statistic computed from the observations follows a χ^2 distribution. The purpose of the test is to evaluate how likely the observed frequencies would be assuming the null hypothesis is true.

Test statistics that follow a χ^2 distribution occur when the observations are independent and normally distributed, which assumptions are often justified under the central limit theorem. There are also χ^2 tests for testing the null hypothesis of independence of a pair of random variables based on observations of the pairs.

The term "chi-squared test" is often used to refer to tests for which the distribution of the test statistic approaches the χ^2 distribution *asymptotically*, i.e., the sampling distribution (if the null hypothesis is true) of the test statistic approximates a chi-squared distribution more and more closely as sample sizes increase.

Chi square statistic

The chi-square test is the most common of the goodness of fit tests and is the one you'll come across in AP statistics or elementary statistics. The chi square can be used for discrete distributions like the binomial distribution and the Poisson distribution, while the The

Kolmogorov-Smirnov and Anderson-Darling goodness of fit tests can only be used for continuous distributions.

Two potential disadvantages of chi square are:

1. The chi square test can only be used for data put into classes (bins). If you have non-binned data you'll need to make a frequency table or histogram before performing the test.
2. Another disadvantage of the chi-square test is that it requires a sufficient sample size in order for the chi-square approximation to be valid.

There is another type of chi-square test, called the chi-square test for independence. The two are sometimes confused but they are quite different.

- The chi-square test for independence compares **two** sets of data to see if there is a relationship.
- The chi-square Goodness of fit is to fit **one** categorical variable to a distribution.

History

In the 19th century, statistical analytical methods were mainly applied in biological data analysis and it was customary for researchers to assume that observations followed a normal distribution, such as Sir George Airy and Professor Merriman, whose works were criticized by Karl Pearson in his 1900 paper.

At the end of 19th century, Pearson noticed the existence of significant skewness within some biological observations. In order to model the observations regardless of being normal or skewed, Pearson, in a series of articles published from 1893 to 1916, devised the Pearson distribution, a family of continuous probability distributions, which includes the normal distribution and many skewed distributions, and proposed a method of statistical analysis consisting of using the Pearson distribution to model the observation and performing a test of goodness of fit to determine how well the model really fits to the observations.

Goodness-of-Fit Test

The goodness of fit test is used to test if sample data fits a distribution from a certain population (i.e. a population with a normal distribution or one with a weibull distribution). In other words, it

tells you if your sample data represents the data you would expect to find in the actual population. Goodness of fit tests commonly used in statistics are:

- The chi-square.
- Kolmogorov-Smirnov.
- Anderson-Darling.
- Shipiro-Wilk.

The formula for the chi-square statistic used in the chi square test is:

$$\chi^2_c = \sum \frac{(O_i - E_i)^2}{E_i}$$

The subscript “c” are the degrees of freedom. “O” is your observed value and E is your expected value. It’s very rare that you’ll want to actually *use* this formula to find a critical chi-square value by hand. The summation symbol means that you’ll have to perform a calculation for every single data item in your data set. As you can probably imagine, the calculations can get very, very, lengthy and tedious. Instead, you’ll probably want to use technology:

- Chi Square Test in SPSS.
- Chi Square P-Value in Excel.

A chi-square statistic is one way to show a relationship between two categorical variables. In statistics, there are two types of variables: numerical (countable) variables and non-numerical (categorical) variables. The chi-squared statistic is a single number that tells you how much difference exists between your observed counts and the counts you would expect if there were no relationship at all in the population.

There are a **few variations** on the chi-square statistic. Which one you use depends upon how you collected the data and which hypothesis is being tested. However, all of the variations use the same idea, which is that you are comparing your expected values with the values you actually collect. One of the most common forms can be used for contingency tables:

$$\chi^2 = \sum_{i=1}^k \left[\frac{(O_i - E_i)^2}{E_i} \right]$$

Where O is the observed value, E is the expected value and “i” is the “ith” position in the contingency table.

A low value for chi-square means there is a high correlation between your two sets of data. In theory, if your observed and expected values were equal (“no difference”) then chi-square would be zero - an event that is unlikely to happen in real life. Deciding whether a chi-square test statistic is large enough to indicate a statistically significant difference isn’t as easy it seems. It would be nice if we could say a chi-square test statistic >10 means a difference, but unfortunately that isn’t the case.

You could take your calculated chi-square value and compare it to a critical value from a chi-square table. If the chi-square value is more than the critical value, then there is a significant difference.

You could also use a p-value. First state the null hypothesis and the alternate hypothesis. Then generate a chi-square curve for your results along with a p-value (See: Calculate a chi-square p-value Excel). Small p-values (under 5%) usually indicate that a difference is significant (or “small enough”).

Tip: The Chi-square statistic can only be used on numbers. They can’t be used for percentages, proportions, means or similar statistical value. For example, if you have 10 percent of 200 people, you would need to convert that to a number (20) before you can run a test statistic

Chi Square P-Values

A chi square test will give you a p-value. The p-value will tell you if your test results are significant or not. In order to perform a chi square test and get the p-value, you need two pieces of information:

Degrees of freedom. That’s just the number of categories minus 1.

The alpha level(α). This is chosen by you, or the researcher. The usual alpha level is 0.05 (5%), but you could also have other levels like 0.01 or 0.10.

In elementary statistics or AP statistics, both the degrees of freedom(df) and the alpha level are usually given to you in a question. You don't normally have to figure out what they are. You *may* have to figure out the df yourself, but it's pretty simple: count the categories and subtract 1.

Degrees of freedom are placed as a subscript after the chi-square (χ^2) symbol. For example, the following chi square shows 6 df:

$$\chi^2_6.$$

And this chi square shows 4 df:

$$\chi^2_4.$$

The chi-square distribution (also called chi-squared distribution) is a special case of the gamma distribution; A chi-square distribution with n degrees of freedom is equal to a gamma distribution with $a = n/2$ and $b = 0.5$ (or $\beta = 2$)

Assumptions of the chi-square test

The assumptions of the chi-square test are the same whether we are using the goodness-of-fit test or the test-of-independence.

The standard assumptions are:

- Random sample.
- Independent observations for the sample(one observation per subject).
- No expected counts less than five.

Notice that the last two assumptions are concerned with the expected counts, not the raw observed counts.

The chi squared distribution has many uses in statistics including,

- Confidence interval estimation for a population standard deviation of a normal distribution from a sample standard deviation.

- Independence of two criteria of classification of qualitative variables.
- Relationship between categorical variables (contingency tables).
- Sample variance study when the underlying distribution is normal.
- Tests of deviations of differences between expected and observed frequencies (one-way tables).
- The chi-square test (a goodness of fit test).

Test a Chi Square Hypothesis: Steps

Sample question: Test the chi-square hypothesis with the following characteristics:

1. 11 Degrees of Freedom
2. Chi square test statistic of 5.094

Note: Degrees of freedom equals the number of categories minus 1.

Step 1: Take the chi-square statistic. Find the p-value in the chi-square table. If you are unfamiliar with chi-square tables, the chi square table link also includes a short video on how to read the table. The closest value for $df=11$ and 5.094 is between .900 and .950.

Note: The chi square table doesn't offer exact values for every single possibility. If you use a calculator, you can get an exact value. The exact p value is 0.9265.

Step 2: Use **the p-value** you found in Step 1. Decide whether to support or reject the null hypothesis. In general, small p-values (1% to 5%) would cause you to reject the null hypothesis. This very large p-value (92.65%) means that the null hypothesis should *not* be rejected.

What does a chi-square test do

Chi square is used to test hypotheses about the distribution of observations in different categories. The null hypothesis (H_0) is that the observed frequencies are the same as the expected frequencies. If the observed and expected frequencies are the same, then, $\chi^2=0$. If the frequencies you observe are different, the value of, χ^2 goes up.

Wilcoxon Signed Rank Test

The **Wilcoxon signed-rank test** is a non-parametric statistical hypothesis test used to compare two related samples, matched samples, or repeated measurements on a single sample to assess whether their population mean ranks differ (i.e. it is a paired difference test). It can be used as an alternative to the paired Student's t-test (also known as "t-test for matched pairs" or "t-test for dependent samples") when the distribution of the differences between the two samples cannot be assumed to be normally distributed. A Wilcoxon signed-rank test is a nonparametric test that can be used to determine whether two dependent samples were selected from populations having the same distribution.

HISTORY

The test is named for Frank Wilcoxon (1892–1965) who, in a single paper, proposed both it and the rank-sum test for two independent samples (Wilcoxon, 1945). The test was popularized by Sidney Siegel (1956) in his influential textbook on non-parametric statistics. Siegel used the symbol T for a value related to, but not the same as W . In consequence, the test is sometimes referred to as the **Wilcoxon T test**, and the test statistic is reported as a value of T .

Assumptions

1. Data are paired and come from the same population.
2. Each pair is chosen randomly and independent.
3. The data are measured on at least an interval scale when, as is usual, within-pair *differences* are calculated to perform the test (though it does suffice that within-pair comparisons are on an ordinal scale).

Test procedure

Carrying out the Wilcoxon signed rank sum test

Case 1: Paired data

1. State the null hypothesis - in this case it is that the median difference, M , is equal to zero.

2. Calculate each paired difference, $d_i = x_i - y_i$, where x_i, y_i are the pairs of observations.
3. Rank the d_i s, ignoring the signs (i.e. assign rank 1 to the smallest $|d_i|$, rank 2 to the next etc.)
4. Label each rank with its sign, according to the sign of d_i .
5. Calculate W^+ , the sum of the ranks of the positive d_i s, and W^- , the sum of the ranks of the negative d_i s. (As a check the total, $W^+ + W^-$, should be equal to $n(n+1)/2$, where n is the number of pairs of observations in the sample).

Case 2: Single set of observations

1. State the null hypothesis - the median value is equal to some value M .
2. Calculate the difference between each observation and the hypothesised median, $d_i = x_i - M$.
3. Apply Steps 3-5 as above.

Under the null hypothesis, we would expect the distribution of the differences to be approximately symmetric around zero and the the distribution of positives and negatives to be distributed at random among the ranks. Under this assumption, it is possible to work out the exact probability of every possible outcome for W . To carry out the test, we therefore proceed as follows:

6. Choose $W = \min(W^-, W^+)$.
7. Use tables of critical values for the Wilcoxon signed rank sum test to find the probability of observing a value of W or more extreme. Most tables give both one-sided and two-sided p-values. If not, double the one-sided p-value to obtain the two-sided p-value. This is an exact test.

Example

The table shows the hours of relief provided by two analgesic drugs in 12 patients suffering from arthritis. Is there any evidence that one drug provides longer relief than the other?

Case	Drug-A	Drug-B	Case	Drug-A	Drug-B
1	2.0	3.5	7	14.9	16.7
2	3.6	5.7	8	6.6	6.0
3	2.6	2.9	9	2.3	3.8
4	2.6	2.4	10	2.0	4.0
5	7.3	9.9	11	6.8	9.1
6	3.4	3.3	12	8.5	20.9

Solution

1. In this case our null hypothesis is that the median difference is zero.
2. Our actual differences (Drug B – Drug A) are:
+1.5, +2.1, +0.3, -0.2, +2.6, -0.1, +1.8, -0.6, +1.5, +2.0, +2.3, +12.4
Our actual median difference is 1.65 hours.
3. Ranking the differences and affixing a sign to each rank(steps 3&4 above)

Diff.	0.1	0.2	0...	0.6	1.5	1.5	1.8	2.0	2.1	2.3	2.6	12.4
Rank	1	2	3	4	5	6	7	8	9	10	11	12
Sign	-	-	+	-	+	+	+	+	+	+	+	+

Calculating W_+ and W_- gives:

$$W_- = 1+2+4 = 7$$

$$W_+ = 3+5.5+5.5+7+8+9+10+11+12 = 71$$

Therefore, we have $n = 12$

$$\frac{12*13}{2} = 78W = \max(W-, W+) = 71$$

We can use a normal approximation in this case. We have one group of 2 tied ranks, so we must reduce the variance by 8-2.

$$\frac{8-2}{48} = 0.125 \text{ we get,}$$

$$71 - \frac{12*13}{4}$$

$$Z = \frac{71 - 12*13/4}{\sqrt{12*13*25/24 - 0.125}} = \frac{71-39}{\sqrt{162.5-0.125}} = 2.511$$

This gives a two-sided p-value of p=0.012. There is strong evidence that Drug B provides more relief than Drug A.

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