Chhatrapati Shahu Ji Maharaj University, Kanpur

SCHOOL OF PHARAMCEUTICAL SCIENCES

Subject: Pharmacological and Toxicological Screening Techniques
Topic: Cardiovascular Pharmacology

DEPARTMENT OF PHARMACY

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THE TOPICS COVERED ARE:

"Preclinical Screening of New Substances for the Pharmacological Activity Using In-Vivo, In-Vitro, and Other Possible Animal Alternative Models of

CARDIOVASCULAR PHARMACOLOGY (II)"

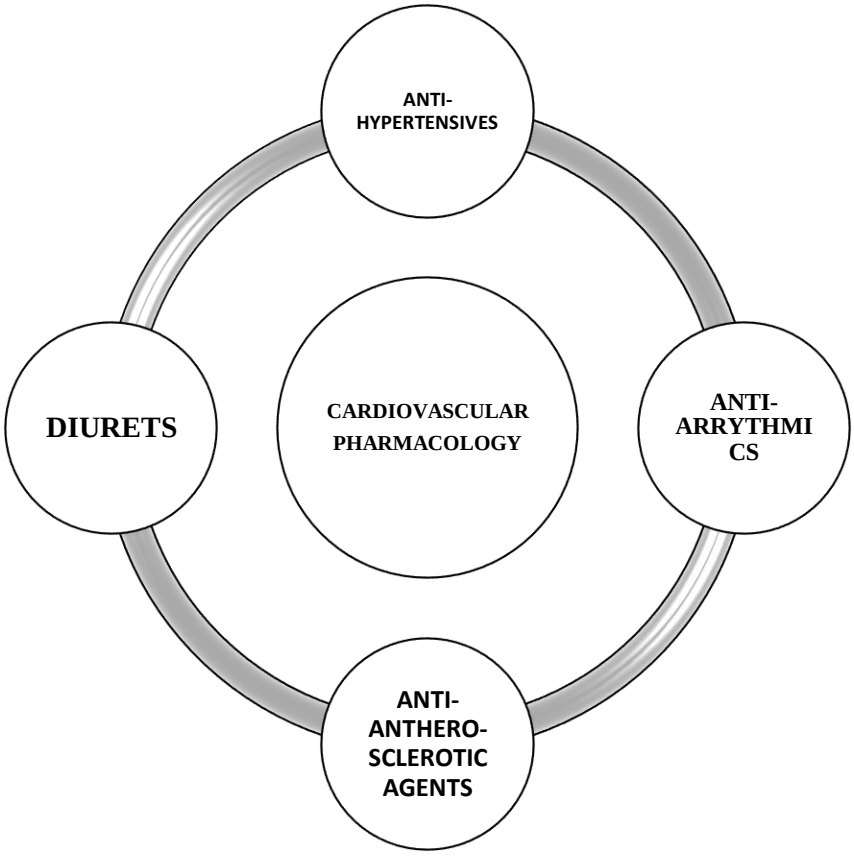


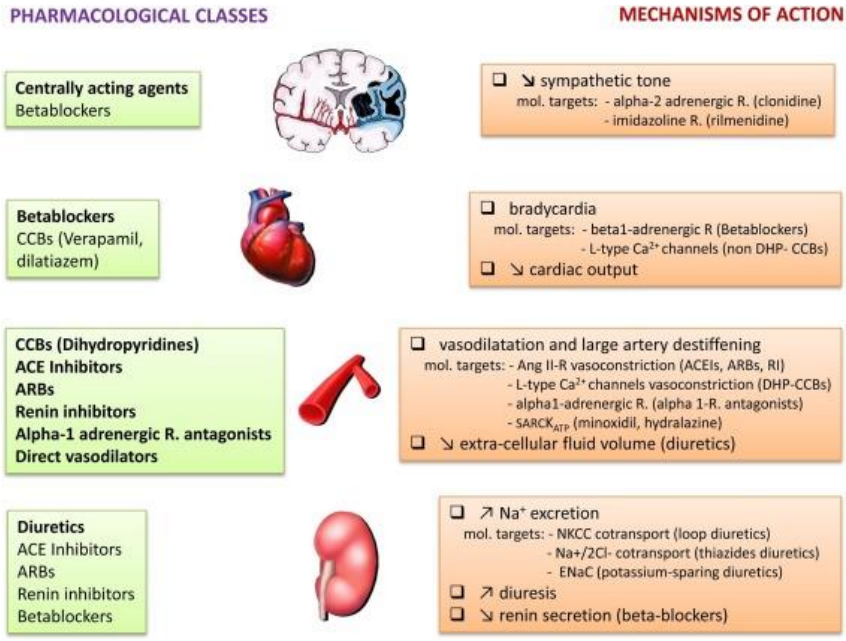
Fig 1: A flow chart represents about the topics which will be covered in this following assignment.

CARDIOVASCULAR PHARMACOLOGY

- Cardiovascular Drugs refers to Prescription drugs and medicines for diseases relating to the structure and function of the heart and blood vessels. Studies in these areas include: heart failure, coronary artery disease, high cholesterol, blood clots, circulation disorder.
- Cardiovascular pharmacology focuses on the fundamental mechanisms of cardiovascular cells and how drugs influence the heart and vascular system and others
- Cardiovascular drugs affect heart function in three main ways:
  - ❖ by affecting the force of contraction of the heart muscle (inotropic effects);
  - ❖ by affecting the frequency of the heartbeat, or heart rate (chronotropic effects); or
  - ❖ by affecting the regularity of heartbeat (rhythmic effects)

ANTI HYPERTENSIVES

- Antihypertensives are a class of drugs that are used to treat hypertension (high blood pressure). Antihypertensive therapy seeks to prevent the complications of high blood pressure, such as stroke and myocardial infarction. Evidence suggests that reduction of the blood pressure by 5 mmHg can decrease the risk of stroke by 34%, of ischemic heart disease by 21%, and reduce the likelihood of dementia, heart failure, and mortality from cardiovascular disease.There are many classes of antihypertensives, which lower blood pressure by different means. Among the most important and most widely used medications are thiazide diuretics, calcium channel blockers, ACE inhibitors, angiotensin II receptor antagonists (ARBs), and beta blockers.



- Hypertension can be classified as either essential or secondary. Essential hypertension indicates that no specific medical cause can be found to explain a patient's condition. Secondary hypertension indicates that the high blood pressure is a result of (i.e. secondary to) another condition, such as kidney disease or certain tumors (especially of the adrenal gland)

IN VITRO MODELS

1. Endothelin receptor antagonism in porcime isolated hearts
2. Monocrotaline induced pulmonary hypertension
3. ACE inhibition in isolated guinea pig Ileum

4. Beta Sympatholytic activity in Guinea pig Atria

IN VIVO MODELS

1. Rat models of hypertension two kidney one clip
2. Chronic renal hypertension
3. Tail cuff method
4. Salt sensitive DAHL rats
5. Angiotensin –II Antagonism

IN VITRO MODELS

1. ENDOTHELINS RECEPTOR ANTAGONISM IN PORCINE ISOLATED HEARTS

- Endothelins (ET) have been implicated in the pathophysiology of cardiovascular disorders. In this model isolated porcine coronary artery is used since the smooth musculature of artery is considered to contain the ETA receptors. ET results in potent long lasting contractions of isolated blood vessel strips. An increase of blood pressure in vitro and in vivo studies has been elicited byendothelin peptides. Six domestic, 12 weeks old, crossbred female pigs (30 to 40 kg) are studied. The pigs are anesthetized with a combination of ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/ min).

- Through a left thoracotomy, the heart is exposed. From porcine hearts left anterior descending coronary arteries are isolated. They are cleaned to remove the fat and connective tissue. The endothelium-denuded arteries are cut into spiral strips about 10 mm long and 1 mm wide. The intimal surface of the spiral rings is then rubbed gently with filter paper to remove the vascular endothelium. Each strip is suspended in an organ bath containing Krebs Henseleit solution bubbled with 95% O2/ 5% CO2 at 37°C. Once the isolated preparation is stabilized reference contraction is isometrically obtained with 50 mM KCl.

- Concentration response curves for ET-1 is obtained by cumulative addition of ET-1. Twenty min before the addition of ET-1 the endothelin receptor antagonist/test drug is added to the organ bath and concentration response curve is recorded. The pA2 values and slopes are obtained by analysis of Schild plots.

2. MONOCROTALINE-INDUCED PULMONARY HYPERTENSION

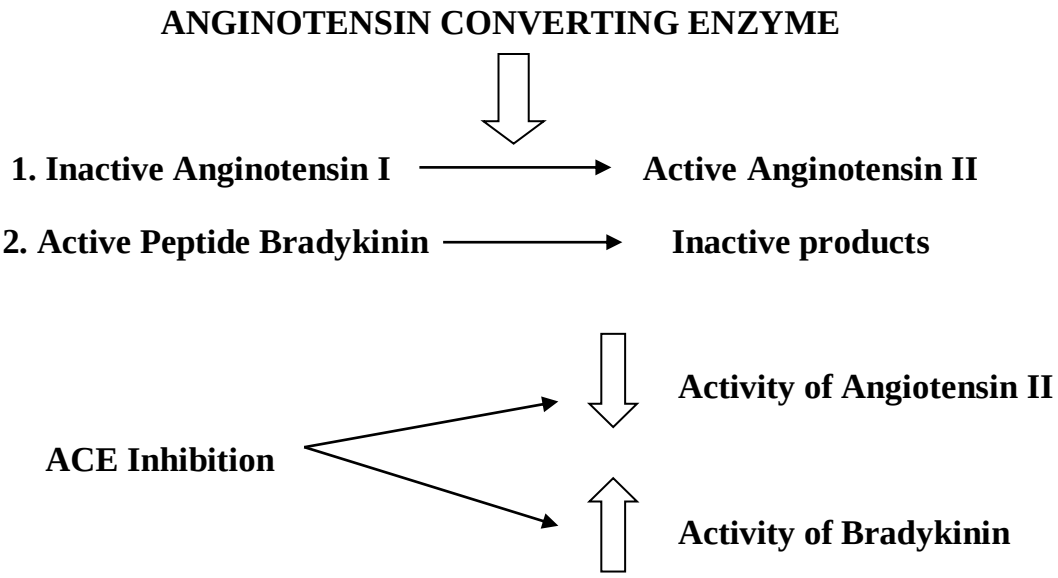
- Monocrotaline is a hepatotoxic and pneumotoxic agent used in rats to induce pulmonary hypertension. It is a pyrrolizidine alkaloid derived from Crotaloria spectabilis and its single injection leads to progressive pulmonary hypertension followed by right ventricular hypertrophy and cardiac failure.
- Ultrastructural changes such as degeneration and fragmentation of endothelial cells, perivascular edema, extravasation of red blood cells and muscularization of pulmonary arteries and arterioles are also observed.

- Monocrotaline administration in rats may result in severe right ventricular hypertrophy accompanied by ascitis and pleural effusion.
- Sprague Dawley rats (200 to 225 g) are fed with the test drug for one week prior to single subcutaneous injection of 100 mg/kg monocrotaline. The animals are sacrificed with an overdose of anesthesia (hexobarbital sodium) 4, 7 or 14 days latter and their hearts and lungs are excised from thoracic cavity.
- The left ventricle and left lung are weighed. Their pulmonary artery segments, main pulmonary artery, right extra pulmonary and an intra pulmonary artery are isolated. Each vessel is suspended between stainless steel hooks in tissue baths containing Krebs Hensleit buffer aerated with 95% O2 and 5% CO2 at 37°C. At the end of the experiment vessel segments are blotted, weighed and their dimensions are measured.
- Cross sectional area of artery is determined from tissue weight and diameter. After 1 h arteries are made to contract to KCl (6x10-2 M). Maximum active force generated by an artery is plotted as a function of applied force and changes in isometric force are monitored using force displacement transducers and recorded on a polygraph. Contractile and relaxant agonist responses are assessed in pulmonary arteries.

Cumulative concentration–response curves to KCl, angiotensin IInorepinephrine are plotted.
Contractions are expressed as active tension development, force generated per cross-sectional area.

- Both contractile and relaxation responses are plotted as a function of negative logarithm of agonist concentration.5-7 t-test for grouped data is used to compare differences in mean responses.

3. <u>ACE INHIBITION IN ISOLATED GUINEA PIC ILEUM</u>



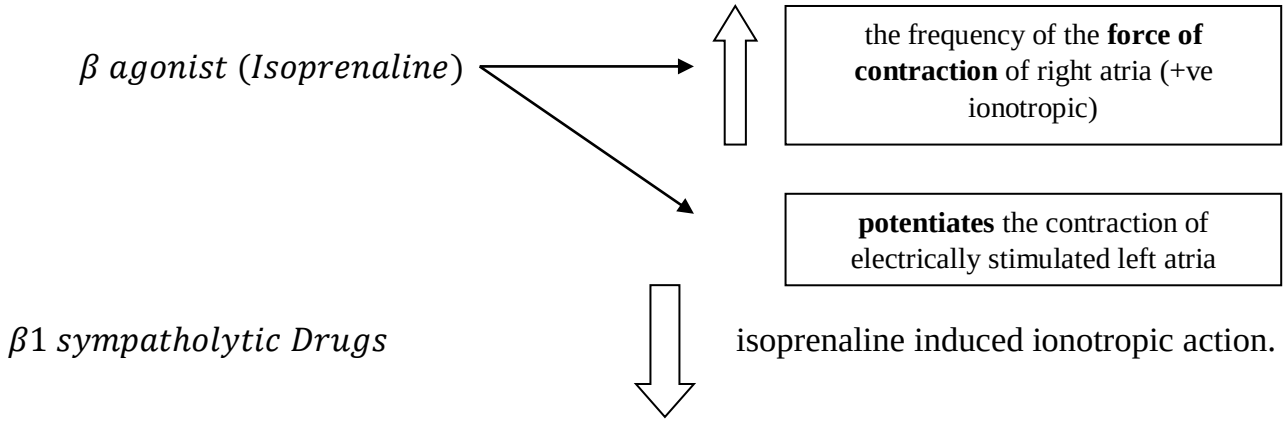
The Guinea Pig Ileum responds powerfully for both Angiotensin II and Bradykinin.

Procedure:-

- Guinea pig of either sex (300-500gm) is selected

- Animals are sacrificed by stunning and abdomen is opened.
- A chord is tied around the starting of intestine.
- The intestine is gradually removed from the bottom and the mesentry is cut away.
- When reached the colon, the intestine is cut halfway by passing Tyrode solution to clean the surface
- The distal pieces (more sensitive) are fixed in the tissue clamp and brought into organ bath of 37º (oxygenated)
- Angiotensin I is added (10mg/mL) after 30 minutes of equilibrium in bath solution and contraction is recorded.
- 5 min after the addition 10mg/mL after 30 minutes of equilibrium in bath solution and contraction is recorded.
- 5 min after the addition of ACE Inhibitor (test drug) the diminished contraction is recorded.
- The opposite response can be observed while using Bradykinin.

4.BETA-1 SYMPATHOLYTIC ACTIVITY GUINEA PIG ATRIA



β_1 receptor blocking activity can be evaluated by this technique.

Procedure:-

- Pirbright- Guinea pig of either sex (250-300gm) is used.
- The animals is sacrificed by stunning and exsanguinations
- Heart is removed and right or left atrium is mounted in 50 mL organ bath containing Krebs-Henseleit buffer with aeration (95% O2, 5% CO2) AT 37°C Contraction are recorded using lever transducer.

RIGHT ATRIUM

- Isoprenaline is administered in the organ bath after 30 minutes to induce ionotropy.
- Cumulative dose is maintained starting from 0.5 µgm/mL and consecutive doses at 3 kin intervals.
- The bath is flushed for 3-5 times after the stable max plateau is achieved.

- The test drug is added into the organ bath
- 5 min later again isoprenaline is added in above concentrations and observe the response.

LEFT ATRIUM

- LA is stimulated by square wave stimulator (2 impulses at 15V, duration- 1 min) After equilibrium repeat the same as above using Isoprenaline at concentrations of 0.05-0.1, 5µgm/mL.
- The bath is flushed for 3-5 times after the stable max. plateau is achieved.
- The test drug is added into the organ bath, 3 min later again isoprenaline is added in above same doses.
- If it has β1 receptor blocking activity, the ISP induced activity is inhibited.
- IC50 values are determined from the individual dose responses.

OBSERVATIONS

β_1 receptor blocking activity

Higher conc. of ISP is necessary to attain same response (potentation and ionotropy).	The increase in the frequency and ionotropy is reduced at same ISP Conc. added before.

 # <u>IN VIVO MODELS</u>

1.Rat Models of Hypertension Two-Kidney One Clip (Goldblatt hypertension, 2K1C)

- Ischemia of the kidneys causes elevation of blood pressure by activation of rennin angiotensin system. In rats clamping the renal artery for 4 h can activates peripheral RAAS and sympathetic nervous system and induce renal hypertension. After re-opening of the vessel, accumulated renin is released into circulation leading to acute hypertension.
- Renin is secreted by kidneys when sympathetic activity is increased. Renin converts angiotensinogen to angiotensin-I.
- Angiotensin-I is converted to angiotensin-II by angiotensin converting enzyme (ACE). Angiotensin-II is a potent vasoconstrictor and increases BP. Angiotensin-II also causes release of aldosterone leading to salt and water retention resulting in increased blood volume and hypertension.
- This model is used to evaluate anti-hypertensive activities of drugs.8,9 Sprague Dawley rats (300 g) are anesthetized with hexobarbital sodium (100 mg/kg,

intraperitoneally). In the left lumbar area a flank incision is made parallel to the long axis of the rat. The kidneys are identified.

- A PVC coated clip is placed into the left hilum of the kidney and fixed to the back muscles. The renal artery is occluded for 3.5-4 h. Ganglionic blockade is performed with pentolinium and after obtaining stable reduced blood pressure values, the 'renal arterial clip' is removed. Subsequently the animals are anaesthetized with pentobarbitone sodium (30-40 mg/kg, intraperitoneally).
- The trachea is cannulated to facilitate spontaneous respiration. Through a pressure transducer connected to carotid artery, blood pressure is measured. Jugular vein is cannulated for administration of test compound. As a consequence of elevated plasma renin level, blood pressure rises.
- Test compound is administered by intravenous route. Blood pressure is monitored continuously. Increase in blood pressure after re-opening of renal artery and reduction of blood pressure after administration of test compound is determined.
- Percent reduction of blood pressure values under drug treatment is calculated as compared to pre-treatment values. The renal artery is constricted on only one side with the other artery (or kidney) left untouched.

  This results in increased plasma rennin activity. However, there is no salt and water retention because of the other normal kidney being intact. Thus, the resultant hypertension at this stage is renin-angiotensin dependent.

2.**Chronic Renal Hypertension in Rats (1-kidney-1-clip method**)

- Sprague-Dawley Rats (200-300gm) are anesthetized Phenobarbitone sodium (100mg/kg) Intra peritonially.
- A flank parallel incision is made in the left lumbar area.
- Renal artery is dissected, cleaned and U shaped silver clip is shipped around near the aorta.
- The internal gap between the clip is adjusted to 0.25-0.38nm
- The right kidney is removed after tying off the renal pedicel
- After 4-5 weeks the BP is measured and the animals are divided into groups of different doses.
- Test drug is administered for 3 days.
- Pressure before and after drug drug administration (3min) are recorded.
- Percent reduction in pressure is calculated and compare to the standard.

3.TAIL CUFF METHOD(BLOOD PRESSUREINCONSCIOUS RATS)

- Method without any surgical procedure. Similar to sphygomanometry in humans (systolic pressure)
- Widely used to evaluate the anti hypertensive drugs in experimentally induced animals.
- Charles River male rats (300-350g) are anaesthized using IP injection of 0.8mL of 4% of charcoal hydrate.
- Both the kidneys are exposed, hypertension is induced by placing a silver clip on both renal arteries. (0.2mm dm & 4mm)
- After 5 to 6 weeks operated animals attain renal hypertension with systolic BP of 170 to 200 mm Hg.
- A tubular inflatable cuff is placed around the base of tail and a Pizo-electric pulse detector is positioned distal to the cuff.
- The cuff is inflated to approximately 300mg/Hg
- As the pressure in the cuff is released slowly, the ystolic pressure is detected and recorded in the poly graph.
- Test substance is administered intra peritonially for alternative days in three times.
- Decrease in the systolic pressure is determined by the following steps:-
    - Day 1 :- Predose and 2 hour postdrug.
    - Day 2:- Predose & 2 hours postdrug
    - Day 3 :- Predose, 2 hours post drug and 4 hour post drug

4.SALT SENSITIVE DAHL RATS:-

- These rats develop severe fatal hypertension when fed high-salt diets whereas salt resistance rats.
- Sprague- Dawley Rats (250-300gm) are used in this study.
- Drinking water is replaced with 85 NaCl solution high salt diet is prepare by mixing salt with regular diet.
- The animals are fed with the above diet and saline
- The test group rats are administered the drug orally for 1 month.
- Blood pressure changes are recorded.
- After the experiment the animals (both central and test) are sacrificed.
- The hearts are isolated, the mas, weight of LV and RV is compared and measured.
- Upon salt feeding animals BP rises steeply (up to 36%)
- The ability of the drug to reverse these changes is studied.
- Cardiac failure occurs at 4 to 5 months of age in these kind off rats.

5.ANGIOTENSIN-II ANTAGONISM:-

- Male Sprague-Dawley rats (200-225gm) are selected.
- They are anaesthetized with 60mg/kg of phenobarbitone sodium IV.
- One carotid artery is cannulated and connected with a Statham transducer and the BP is recorded in a polygraph.
- Both the jujular veins are cannulated to administer the these drug.

- Peritoneum (10mg/kg) is injected to block the ganglionic activity.
- Atleast 5 animals are used for the evaluation of test drug.
- In 10 min interval doses of 0.5, 1.0 and 2µg/kg of angiotensin II are injected to establish the dose response curves.
- After 10 min a continuous infusion is started of the Angiotensin II blocker in a dose of 10µg/kg/0.1 mL.
- Again doses of 0.5, 1.0, 2µg/kg of angiotensin II are injected.
- Intensity and duration of fall in BP is recorded.
- The result are compared with the known standard drug.

ANTIARRHYTHMIC

- An arrhythmia is any deviation from or disturbance of the normal heart rhythm. The basic rhythm of the heart is a tightly regulated phenomenon designed to insure maximal efficiency and optimal performance. It is a dynamic phenomenon that changes according to the metabolic needs of the body. The cardiac rhythm involves several different microscopic and macroscopic structures within the normal heart.
- An arrhythmia may occur when any portion of this sequence is interrupted or disturbed. Among arrhythmic disturbances is the failure of the pacemaker or electrical system to trigger appropriately and conduct impulses properly.

SCREENING METHOD OF ANTI ARRHYTHMIC

IN-VITRO METHOD

1. ACETYCHOLINE OR POTASSIUM INDUCED ARRHYTMIA
2. LANGENDROFF TECHNIC

IN-VIVO METHOD

1. **CHEMICALLY INDUCED ARRHYTHMIA**

A. DIGOXIN INDUCED ARRHYTHMIA IN GUINEA PIGS

B. ACONITINE ANTAGONISM IN RATS

C. ADRENALINE INDUCED ARRHYTHMIA

2. **ELECTRICALLY INDUCED ARRHTHMIA**

A. VENTRICULAR FIBRILLATION ELECTRICALTHRESHOLD

B. SUDDEN CORONARY DEATH MODELS IN DOGS

3. **EXERCISE IDUCED VENTRICULAR FIBRILATION**

INVIVO METHOD -

CHEMICALLY INDUCED ARRHTYHMIA:-

A large number of agents alone or in combination are capable of inducing arrhythmias. Administration of anesthetics likes chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus, such as intravenous adrenaline, ouabain alkaloids cause arrhythmia. The sensitivity of these arrhythmogenic substances differs among various species

1.**DIGOXIN INDUCED ARRHYTHMIAS IN GUINEA PIG**

Digoxin overdose causes ventricular extrasystoles, fibrillation and death

⇩

Male marioth guinea pigs (350-500g) anesthetize with pentobarbitone 35mg/kg intraperitonially

⇩

Trachea, jugular vein and carotid artery catheterized and animals is maintained on artificial respiration. (45 breath/min)

⇩

Through jugular vein digoxin is infused @85mcg/kg in 0.266 mL/min untill cardiac arrest

⇩

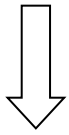
ECG recorded with steel needle during experiment

⇩

Test drug is administered either 1 hour before if orally or 1 min before if IV, prior infusion of digoxine

⇩

The onset of ventricular extra systoles, fibrillation, cardiac arrest



Compared results in test and control group

2.ACONITINE ANTAGONISM IN RATS

- Aconitine, a plant alkaloid from aconitine root acts persistently on sodium channels and activates it resulting in ventricular arrhythmias.
- Drugs considered to have anti-arrhythmic properties can be tested in aconitine intoxicated rats.
- Males Ivanovas rats (300-400 g) are anesthetized intraperitoneally with urethane (1.25 g/kg).
- Aconitine (5 µg/kg) is dissolved in 0.1 N HNO3 and continuously infused into the rat's saphenous vein at a rate of 0.1 ml/min. Lead II ECG is recorded every 30 s.
- Test compound is injected orally or intravenously 5 min before the aconitine infusion.
- A higher dose of aconitine in the test group compared to untreated group gives an index of antiarrhythmic activity.
- The antiarrhythmic effect of test compound is measured by the amount of aconitine/100 g animal (infusion duration) and includes ventricular extrasystoles, tachycardia, fibrillation and death.

3.ADRENALINE INDUCED ARRHYTHMIA

- Adrenaline at high dose may precipitate arrhythmia. Dogs (10-11 kg) are anesthetized with pentobarbitone sodium (30-40 mg/kg) intraperitoneally.
- The femoral vein is cannulated. Adrenaline is infused at a rate of 2-2.5 µg/kg through femoral vein. Lead II ECG and atrial ECG are recorded.
- Test drug is administered 3 min after adrenaline infusion.
- A test compound is considered to have antiarrhythmic effect if the extrasystoles disappear immediately after drug administration.

ELECTRICALLY INDUCED ARRHYTHMIA

- Serial electrical stimulation results in flutter and fibrillation and it is possible to reproduce some of the main types of arrhythmias of clinical importance. The flutter threshold or the ventricular multiple response thresholds may be determined in anesthetized dogs before or after administration of test drug.

1. VENTRICULAR FIBRILLATION ELECTRICAL ARRHYTHMIA

- The maximum frequency at which atria would follow a stimulus used to compare anti fibrilati compound. Several method:-
- Single pulse method
- Train of pulses stimulation
- Continue 50Hz stimulation
- Sequential pulse method

Dogs (8-12kg) anesthetized with pentobarbitone intraperitonially and maintained on artificial respiration

⇩

Chest opened and heart is suspended on pericardial cradle

⇩

SA nod crushed and Ag-AgCl stimulating electrode embedded with Teflon disc sutured to anterior surface of LV

⇩

Abodal constant current for 400ms is applied through electrode and which was programmed by digital stimulator

⇩

ECG recorded through lead II of body monitoring

⇩

To determine Ventricular Fibrillation (VFT) 0.2 to 1.8 sex train of 50Hz pulses delivered

⇩

The current intensity of pulse train required to induced sustained ventricular fibrillation is defined as VFT

⇩

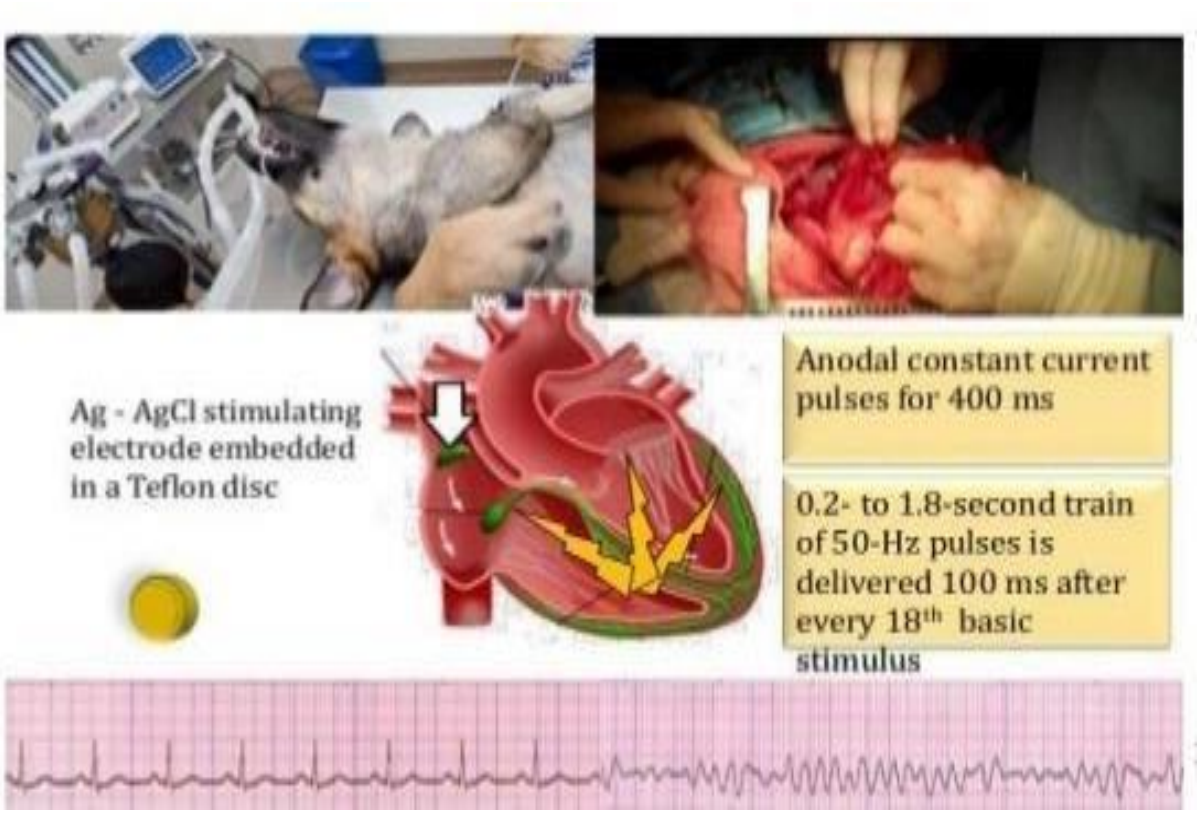
When VF occurs, heart immediately defibrillate and allow to recover as controlled condition for 15-20 min

⇩

Test drug administered through femoral vein

⇩

VFT is determined before and after administered of test drug and compared by using student **T-TEST**



2.SUDDEN CORONARY DEATH NODELS IN DOGS

Sudden coronary death is one of the most leading causes in developed countries

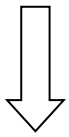
This model of dogs used to test protection offered against sudden coronary death

⇩

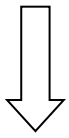
Male mongrel dogs (14-22days) anesthetized with pentobarbitone IV

⇩

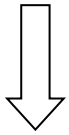
Tracheal is cannulated and maintained artificial breathing: Jugular veins cannulated for test drug administration



Chest opened and heart exposed, **left anterior descending (LAD)** isolated and 20G needle placed on LAD



Ligature tide across artery and needle followed by removal of needle resulting critical stenosis of vessel



LAD is occuluded for 2 hours using rubber snare and allowed to re-prfused for 2 hours in presence of stenosis

An epicardial bipolar electrode is sutured on left ventricular wall; one @the distribution of LAD distal to the occlusion and second in distribution of **Left Circumflex Coronary (LCX)** artery

Silver coated electrodes is passed through the wall and into lumen of LCX sutured to the adjunct surface of heart

Surgical incision closed and animals is allowed to recover

After recovery they are treated with test drug

Anodal 15µA current from 9 V Ni-Ca battery is passed through 25Ω resistor and applied to electrode in luman of LCX

Animals are sacrificed after 24 hours on constant anodal current

Hearts are removed and thrombus mass in LCX is removed and weighed

Heart is sectioned and stained with Tetrazolium Triphynyl Chloride (TTC) to study the area of infarct

Time of onset of ventricular ectopy and lethal arrhythmia is studied using recording of cardiac cassette recorder.

EXERCISE INDUCED VENTRICULAR FIBRILATION

Mongral dogs (15-19kg) anesthetized with pentobarbitone IV (10mg/kg)

↓

Chest cavity opened, heart exposed and supported with pericardial cradle

↓

Around LCX 20 MHz pulsed Doppler flow transducer and hydraulic acclder are placed

↓

Pair of insulated silver wire sutured to epicardial surface of both left and rigt ventricle. ECG and HR ministered.

↓

Pre Calibrated solid state pressure transducer is inserted into left ventricle and finally, a two stage occlusion of LAD is performed

↓

Leads from cardiovascular instrumentation are tunneled under skin to exit on the back of animals's neck

↓

3-4 weeks after the production of myocardial ischemia; animal are walked on treadmill and trained to lie quietly on lab table during recovery period

↓

Protocol starts with 3 min warm up followed by run @6.4km/hrs (0% grade)

↓

Grade is increased every 3 min run 0% 4% 8% 12% 16%

↓

During last min of exercise LCX is occluded, treadmill stapped and occlusion maintained for additional 1 min

↓

Exercise and ischemia test is repeated after pretreated with test drug and compared with control reading.

MECHANICALLY INDUCED ARRHYTHMIA

- Arrhythmias can be induced directly by ischemia or by reperfusion. By ischemia induced infarction or by coronary ligation several phases of arrhythmia can be studied. The two stage coronary ligation technique focuses on late arrhythmia. The influence on reperfusion arrhythmia can be tested in various species.

1. **REPERFUSION ARRHYTHMIA IN RATS**
2. **REPERFUSION ARRHYTHMIA IN DOGS**

1. REPERFUSION ARRHYTHMIA IN RATS

- Ligature of left main coronary atery results in ventricular arrhythmia and MI
- ECG is recorded during ligation and subsequent reperfusion
- The amount of infracted tissue is measured by means of P-nitro blue tetrazolium chloride staining in myocardium section
- Spargue-Dawley rats (350-400g) are anesthetized with pentobarbitone (60mg/kg) intra peritonially and maintained on artificial respiration
- Jugular vain is cannulated for administration of test drug
- BP is recorded from the carotid artery using pressure transducer connected to polygraph
- Chest id opened and heart is exposed. The left coronary artery is located and ligated for 15-90 minutes and subsequently reperfusion for 30 min.
- Test drug is administered 5 min before the ligation
- BP and ECG lead II recorded continuously during experiment
- The number of ventricular premature beats, ventricular tachycardia and fibrillation are couted in occlusion and reperfusion period.
- At the end of reperfusion period, the animal is sacrificed and TTC staining is done to quantity the infarct size
- Heart is dissected and cut into transverse section and stained with TTC in order to visualize the infarct (Blue/Violate stained healthy; Unstained necrotic tissue)
- Slices are photographed and infarct area is measured by planimetry from projection of all slice
- Changes in hemodynamics parameters and infarct size in drug treated animal are compared to control values.

2. REPERFUSION ARRHYTHMIA IN DOGS:-

- Coronary artery ligation in dogs may result in increased heart rate, heart contractility, left ventricular end diastolic pressure, blood pressure and ventricular arrhythmias especially in the reperfusion duration.

- Dogs (20-25 kg) are anesthetized with thio-butobarbital sodium (30 mg/kg) intraperitoneally and maintained on intravenous chloralose (20 mg/kg) and 250 mg/kg urethane intravenously followed by subcutaneous administration of 2 mg/kg morphine.
- Animal is subsequently maintained on artificial respiration. A peripheral vein (saphenous vein) is cannulated for the administration of test compound.
- ECG is recorded continuously in lead II. Femoral artery is cannulated to measure blood pressure and connected to a pressure transducer.
- Left ventricular end diastolic pressure and heart rate are determined from the left ventricular pressure curves.
- Myocardial contractility is measured as a rise of left ventricular pressure. The experimental procedure followed is similar to that in rats.
- Coronary artery is ligated for 90 minutes. Twenty minutes prior to ligation the test compound is administered. Animal is reperfused for 30 min.
- All the above mentioned parameters are recorded during the whole experiment. Changes in parameters (mortality, hemodynamic and arrhythmia) in drug treated animals are compared to vehicle controls.

ANTI ANGINAL

- Angina pectoris is the chest pain due to anoxia of heart muscles generally due to obstruction or spasm in coronary artery. The drugs used in angina pectoris prevent terminate attacks of angina pectoris are called anti-anginal drugs.
- Obesity, insulin resistance, and type 2 diabetes mellitus are increasing and are powerful risk factors for IHD.

This imbalance may be caused by :

- An increase in myocardial oxygen demand (which is determined by heart rate, ventricular contractility, and ventricular wall tension)
- A decrease in myocardial oxygen supply (primarily determined by coronary blood flow, but occasionally modified by the oxygen-carrying capacity of the blood).
- Sometimes by both

SCREENING MODEL RELATED TO ANTI ANGINAL:-

IN VITRO MODELS

1.LANGENDORFF HEART PREPARATION:-

- Langendorff is a highly reproducible preparation, which can be studied quickly in large number at relatively low cost. It allows measurement of broad spectrum of biochemical, physiological and morphological indices. Measurements are made in absence of the confounding effects of other organs.

- Both global and regional ischemia can be studied using this model. It allows experiments to be continued in face of events (MI, arrhythmias), which would normally jeopardize the survival of an in vivo experiment.
- However, it is a deteriorating preparation though capable of study for several hours. The basic principle involved is that heart is perfused in a retrograde direction from the aorta either at constant pressure or constant flow with oxygenated saline solutions. Retrograde perfusion closes the aortic valves, just as in the in situ heart during diastole.
- The perfusate is displaced through the coronary arteries flowing off the coronary sinus and the opened right atrium. Parameters usually measured are contractile force, coronary flow and cardiac rhythm
- Guinea pigs of either sex weighing 300 to 500 g are used for the study. They are sacrificed by stunning. Diaphragm is assessed by transabdominal incision and cut carefully to expose the thoracic cavity. Thorax is opened by bilateral incision along the lower margins of the last to first ribs. Thoracic cage is reflected over the animal's head exposing the heart. The heart is cradled between fingers and lifted before incising the aorta, vena cava and pulmonary veins. Immediately after excision, heart is dipped in cold perfusion solution (4°C to limit ischemic injury during period between excision and restoration of vascular perfusion).
- The aorta is located and cut below the point of division. A cannula is inserted into the aorta and tied and the heart is perfused with oxygenated Ringer's solution. The heart is transferred to a double wall Plexiglas perfusion apparatusmaintained at 37°C.1 Oxygenated Ringer's solution is perfused at a constant pressure of 40 mm Hg at a temperature of 37°C from a reservoir. A small steel hook with a string is attached to the apex of the heart.
- Contractile force is measured isometrically by a force transducer and recorded on a polygraph. Heart rate is measured through a chronometer coupled to the polygraph.

  Drugs are injected in to the perfusion medium.2 The antianginal effect of the test drug is indicated by an increase in coronary blood flow. The incidence and duration of ventricular fibrillation, coronary flow, inotropic state and K+ levels after treatment with drug are compared with control.

  This method is very useful for testing coronary vasodilator drugs. It has wide applications in the fields of pharmacology and physiology. It is useful to study positive inotropic effects, negative inotropic effects, coronary vasodilator effect, calcium antagonism, effect on potassium outflow induced by glycosides and determination of hypoxic damage. Metabolic studies, arrhythmogenic, antiarrhythmic and antifibrillatory effects can also be assessed using Langendorff method. Recently this model has been also used to study endothelium derived relaxing factor (EDRF) release from the coronary vascular bed and electrophysiological evaluation of cardiovascular agent.

2.ISOLATED RABBIT AORTA PREPARATION:-

- Aortic rings are used to evaluate the smooth muscle relaxant/contractile activity in this method. Adding potassium chloride or nor-epinephrine to the organ bath containing

slightly modified Krebs' bicarbonate buffer induces contraction of aorta rings. Using an overdose of pentobarbital sodium, rabbits of either sex weighing 3 to 4 kg are sacrificed. Thorax is opened by bilateral incision. The descending thoracic aorta is rapidly removed and placed in Krebs' bicarbonate buffer maintained at 37°C.

- Tissue is cleaned, fat and connective tissue is carefully removed. Eight rings of 4-5 mm width are obtained and each is mounted in 20 ml tissue bath containing Krebs' solution. A stabilization period of 2 hr is allowed wherein the Krebs' solution is frequently changed followed by stabilization period of 1 h. A tension of 1 g is maintained during these times. A sustained contraction is generated by addition of KCl. Twenty minutes after addition of agonist, the test drug is added. The percent relaxation reading is taken every 30 min after addition of the test drug.
- There is a 30 min time interval between additions of different test drugs. Active tension is calculated for the tissue at time point just prior to the addition of the test compound and also at the point 30 min after the addition of each concentration of the test compound. ID50 and percentage relaxation caused by the test drug from the precontracted level is calculated.4 Test drug with calcium channel blocking activity have a relaxing effect and can be evaluated using this method.

3.CALCIUM ANTAGONISM IN PITCHED RAT

- This model can differentiate calcium entry blockers from other agents that do not directly block entry of calcium. Sprague-Dawley rats (250 to 350 g) are anesthetized intraperitoneally with methohexitone sodium (50 mg/kg). The trachea is cannulated. Thereafter the rats are pitched through one orbit and immediately maintained on artificial respiration.
- The pithing rod is used as a stimulating electrode and continuous electrical stimulation of the thoracic spinal cord with square wave pulses at supramaximal voltage (frequency 0.5 Hz and duration 0.5 min) produces a cardio-accelerator response. Only rats with a resulting tachycardia (100 beats/min) are included for the study.
- The jugular vein is cannulated for administration of drugs and blood pressure is recorded via carotid artery using a pressure transducer. In the femoral region, an indifferent electrode is inserted subcutaneously.
- When cardioaccelerator response is established for 3-5 min, calcium channel blockers and β-blockers are administered. These test compounds dose dependently block tachycardia. The level of tachycardia immediately prior to drug administration is taken as 100% and response to drug.

4.ISOLATED HEART LUNG PREPARATION:-

- The isolated heart–lung preparation of the dog has been used to study various physiological and pharmacological processes. Now this model has also been established in rats. Wistar rats (300 to 350 g) are anesthetized intraperitoneally with pentobarbitione sodium (50 mg/kg). The trachea is cannulated and the animal is maintained on artificial respiration. The chest cavity is opened and ice-cold saline is injected to arrest the heart. The aorta, superior and inferior vena cava are cannulated. The heart lung preparation is

perfused with Krebs-Ringer bicarbonate buffer (pH 7.4) containing rat RBC (hematocrit 25%).

- The perfusate is pumped from the aorta and is passed through the pneumatic resistance and collected in a reservoir maintained at 37°C. It is then returned to the inferior vena cava thus perfusing only the heart and the lung. Test drug is administered into the perfusate 5 min after start of experiment. Cardiac output is recorded with an electromagnetic blood flowmeter and mean arterial pressure from the pneumatic resistance. With the help of a bioelectrical amplifier heart rate is recorded.

  Hemodynamic data and recovery time of the test drug group and control group (without any treatment) is compared using ANOVA and Kruskal-Wallis test respectively.

#IN VIVO METHOD:-

1. **OCCLUSION OF CORONARY ARTERY:-**

- Compounds that reduce infarct size are studied using this model. Infarct size is studied after proximal occlusion of the left anterior descending coronary artery in open chest dogs. Nitroblue tetrazolium chloride stain in myocardial sections is used to visualize infarct size in coronary arteriograms made after injection of $BaSO_4$ gelatin mass into the left coronary ostium. Dogs of either sex (30 kg) are used in this model.
- The animals are anesthetized with pentobarbitone sodium (35 mg/kg, intraperitoneally) which is followed by its continuous infusion at 4 mg/kg/hr. Trachea is cannulated and the animal is maintained on artificial respiration. Peripheral vein (saphenous vein) is cannulated for administration of test compound. ECG is recorded continuously.
- Femoral vein is cannulated and connected to a pressure transducer for measuring peripheral systolic and diastolic pressure. Left ventricular end diastolic pressure, left ventricular pressure and heart rate are also measured using a Millar microtip catheter (PC 350) inserted via the left coronary artery. Heart is exposed through a left thoracotomy between 4th and 5th intercostal space.
- The pericardium is opened and the left anterior descending coronary artery is exposed and then ligated for 360 min. Test substance or vehicle is administered by intravenous bolus injection. Hemodynamic parameters are monitored and at the end of the experiment, animals are sacrificed with an overdose of pentobarbital sodium. Area at risk of infarction is measured using coronary arteriograms. The left ventricle is cut into transverse sections. From each slice angiograms are made with X-ray tube at 40 kV to assess the area at risk of infarction by defect opacity: reduction of $BaSO_4$ filled vessels in infarct tissue.
- The slices are then incubated in p-nitro-blue-tetrazolium (0.25 g/L) in order to visualize the infarct tissue (blue/violet stained healthy tissue, unstained necrotic tissue). The slices are photographed for determination of infarct area. Mortality, hemodynamic parameters and infarct size are determined. Changes in parameters in drug treated animals are compared to vehicle controls.

2.ISPPROTERENOL INDUCED MYOCARDIAL NECROSIS

- Synthetic catecholamines like isoproterenol when injected at high dose produce cardiac necrosis. Rona, et al have studied the infarct like lesions in the rat myocardium. Several drugs such as sympatholytics or calcium antagonists can totally or partially prevent these lesions. Wistar rats (150 to 200 g) are pretreated with test drug or standard drug orally or subcutaneously for at least a week.
- These rats are then injected with 85 mg/kg isoproterenol subcutaneously on two consecutive days. Mortality as well as symptoms are recorded in each group and compared to group injected with isoproterenol only. After 48 hr of first dose of isoproterenol the animals are sacrificed.
- The heart is removed, weighed and preserved for histological evaluation or processed for estimation of various biochemical parameters. Before sacrificing, the animal's hemodynamic parameters such as systolic/diastolic blood pressure and heart rate can be recorded by cannulating the carotid artery and connecting it to a pressure transducer. By inserting a cannula in the left ventricle, parameters such as LVEDP and dP/dt can be measured.
- The degree of histopathological changes can be graded as follows:

- **Grade 0**: No change
- **Grade 1**: Focal areas of necrosis
- **Grade 2**: Focal areas of necrosis and muscle fiber fragmentation
- **Grade 3**: Confluent areas of necrosis, edema and inflammation and muscle fiber fragmentation
- **Grade 4:** Massive areas of necrosis, edema and inflammation and mural thrombi

Changes of parameters (histological, biochemical and hemodynamic) of drug treated animals are compared to isoproterenol controls.

3.STENOSIS INDUCED CORONARY THROMBOSIS MODEL:-

- Thrombosis can be induced by stenosis in dogs. This model is characterized by alterations in coronary blood flow with transient platelet aggregation at the site of coronary constriction. Dogs (15 to 20 kg) are anesthetized with pentobarbitone sodium (30 to 40 mg/kg, intraperitoneally) and then maintained on artificial respiration through a tracheal tube using a positive pressure respirator.
- Through a left thoracotomy the heart is exposed at the 4th and 5th intercostal space and the pericardium is removed. An electromagnetic flowprobe is placed on the proximal part of the left coronary artery to measure coronary blood flow. Distal to the flowmeter, the vessel is clamped for 5 sec.
- A small plastic constrictor is placed around the artery at the site of damage. The constrictor is changed several times until the required narrowing of the coronary artery is achieved. In case the artery is occluded, the coronary artery is lifted to induce reflow.

Dogs with regular repeated cyclic flow variations of same intensity with in a pretreatment phase of 60 min are used for experimental purpose.

- Hemodynamic parameters are recorded. Test compound is administered intravenously and the cyclic flow variations are registered for 2 to 5 hr and compared to pre-treatment values In case simple clamping of the coronary artery does not produce cyclic flow variations, additionally adrenaline (0.2 µg/kg) is infused into the peripheral vein, thirty min before and thirty min following drug administration. Also platelet activating factor (PAF; 0.2 nmol/kg/ min) when infused for a similar duration as adrenaline into the cannulated lateral branch of the coronary artery may produce cyclic flow variations. Cyclic flow variations are registered and compared to the drug treated group.

ANTI- ATHREOSCHLEROTIC

- Athreoschlerotic is a hardening and narrowing of your arteries caused by cholesterol plaques lining the artery over time. It can put blood flow at risk as your arteries become blocked.
- Atherosclerosis is a disease in which fatty plaque build up in the walls of arteries.
- The fatty plaque deposition is due to the increased level of cholesterol and other triglycerides in the blood which is characterized as hyperlipidemia.
- So the hyperlipidemia is the main cause of atherosclerosis.
- So the treatment include against hyperlipidemia and atherosclerosis.

#IN VIVO MODELS

1. TRITON WISTAR RAT INDUCED HYPERLIPIDEMIA
2. HYPOLIPIDEMIC ACTIVITY IN RATS
3. CHOLESTEROL DIET INDUCED ATHEROSCLEROSIS IN RABBITS
4. HEREDITARY HYPERLIPIDEMIA IN RABBITS

#INVITRO METHOD:-

1.Inhibition of the isolated enzyme HMG CoAreductase in vitro

2.ACAT inhibitory activity

#IN VIVO MODELS

1. Hereditary Hyperlipidemia in Rabbits:-

PURPOSE AND RATIONAL:-

To produced hereditary hyperlipidemia in rabbit. To study the effect of potential anti arteriosclerotic drugs.

REQUIREMENT:-

- **CHEMICAL**:- Probucol
- **ANIMAL**:- Female mice, Homozygous Wistar, Hereditary Hyperlipidemic rabbits

PROCEDURE:-

- Homozygous wistar hereditary hyperlipidemic rabbits were raised in Kyoto by mating heterozygous and homozygous male Wistar hereditary hyperlipidemic rabbits.
- At 2 months of age, 8 rabbits were divided into two groups (group A and group B). Rabbits in group A (2 males, 2 females) were fed standard rabbit chow for 6 months.
- Rabbits in group B (two males, two females) were raised with rabbit chow enriched with 1 % (wt/wt) probucol for 6 months.
- The amount of daily diet for each animal was restricted to 100g during the study period. Water was supplied. Six months later (at the age of 8 months), the rabbits were sacrificed and their blood and aortas were taken for analysis.

EVALUATION:-

- Plasma levels of cholesterol were measured by the enzymatic method.
- Statistical significance was determined by the t test.

2.<u>Triton Wistar Rat Induced Hyperlipidemia</u>

PURPOSE AND RATIONAL:- The systemic administration of the surfactant triton to rats results in a biphasic elevation of plasma cholesterol and triglycerides.

REQUIREMENT:-

- **CHEMICAL:-** Surfactant, Triton, Momordiciadiociaroxb
- **ANIMAL:-** Wistar strain male albino rats

PROCEDURE:-

- Fourty two male wistar rats weighing 190g to 230g were randomly divided into 7 groups
- In each group contains 6 male rats and kept in their cages for 5 days prior dosing to allow for acclimization to laboratory condition.
- The animals were starved for 18hours and IP with 10% aqueous solution of triton at 400mg/kg body weight.
- The test drugs employed the solvent for control was administered simultaneously with triton injection
- Serum analyzed made on 24 hours and 48 hours after triton injection. The drug was administered in the vehicle in the same volume orally.

- After administration of triton in the 24 hours blood was collected by retro orbital puncture under ether anesthesia and subject to centrifugation to obtain serum
- Again 48hour blood was collected by retro orbital puncture under ether anesthesia and subject to centrifugation to obtain serum with 2 mL syringe.

EVALUATION:-

- Serum was analyzed for serum triglyceride, serum total cholesterol, serum HDL cholesterol, serum LDL cholesterol, serum VLDL cholesterol, serum glucose.
- The result is evaluated by **ANOVA TEST** and **DUNNET MULTIPLEcomparison test.**

3.<u>Hypolipidemic activity in rats:-</u>

PURPOSE AND RATIONAL:-

- Hyperlipoprotein with **increased concentration of cholesterol** and triglyceride carrying lipoproteins is considered to be the cause of arteriosclerosis with its dual sequelae of **thrombosis** and **infarction.**
- **HDL** promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver.
- Increased levels of HDL are desirable
- High levels of VLDL and LDL promote arteriosclerosis. LDL in its oxidized form
- Anti arteriosclerotic drugs should reduce VLDL and LDL and or elevate HDL

REQUIREMENT:-

- **CHEMICAL**:- Methanol extract of trianthemaportulacasstrum
- **ANIMALS:-** Wistar albino rats

PROCEDURE:-

- Male Wistar rats (groups of 10)
- Daily once in morning over a period of 8 days the test compounds or the standard in various doses ranging from 1 to 100 mg/kg via stomach tube in a volume of 5 ml/kg
- Body weight of each animal is registered at the beginning and at the end of the experiment
- Dose is applied
- Blood sample are taken by retro orbital puncture
- Immediately the animals sacrificed and the liver removed blotted free from blood and weighed.
- Samples of liver are frozen in liquid nitrogen and stored at 25°C for lipid analysis.
- Blood samples are centrifuged for 2 min at 16000 rpm.

4.<u>Cholesterol diet induced atherosclerosis in rabbits:-</u>

PURPOSE AND RATIONALE:-

- Rabbits are known to be susceptible to hypercholesterolemia and artheriosclerosis after excessive cholesterol feedings.

- Therefore, this approach has been chosen by to study the effect of potiential anti arteriosclerotic drugs.

REQUIREMENT:-

- **CHEMICAL**:- Dimethyl sulfoxide
- **ANIMALS**:- White New Zealand male rabbits

- **PROCEDURE:-**

Male rabbits from an inbred strain are used (GROUP 0F 10)

↓

Blood is withdrawn from the marginal ear vein

↓

Rabbits are diet supplemented with 0.3-2% cholesterol for 10-12 weeks

↓

One group is kept on normal diet and at the end of the experiment blood is taken for analysis

↓

Animal sacrificed and thoracic aorta removed and longitudinally cut and fixation with formaldehyde

↓

Tissue is stained with oil red

EVALUATION:-

- Cholesterol fed rabbits the aorta shows severe atherogenic lesions.
- Data are expressed as means ± standard deviation. Statistical evaluation is performed Scheffe's test.

INVITRO METHOD:-

1.Inhibition of the isolated enzyme HMG CoA-reductase in vitro

2.ACAT inhibitory activity

1.<u>INHIBITION OF THE ISOLATED ENZYME HMG-CoA-REDUCTASE INVITRO</u>

PURPOSE:-

For screening purposes, studies on the inhibition of HMG-CoA reductase obtained from rat liver microsomal fraction can be used.

- **REQUIREMENTS:-**
- **Chemical:** Dithiothreitol (reducing agent)
- **Animal:** Rats

- The inhibitory activity of the test compound on HMGCoA reductase is estimated with soluble enzyme preparations obtained from the microsomal fraction of rat liver.
- HMG-COA reductase is a rate controlling enzyme of mevalonate pathwaythat produces cholesterol.
- Inhibitors of HMG-COA reduce the cholesterol production in body by inhibiting HMGCOA

PROCEDURE

The enzyme reaction is carried out with 50 µl partially purified HMGCoA reductase in buffer containing 25 mM Tris, 10 mM EDTA, and 10 mM dithiothreitol at pH 7.5, 20 µl of 910µMHMG-CoA solution containing 100nCi of 14C-HMG-CoA and 20 µl of NADPH regenerating system , with the actual concentration of 50mM NADPH. The final incubation volume is 200ul.

↓

The main reaction is preceded by 20 min preincubation with the NADPH regenerating system at 37 °C, followed by 20 min incubation at 37 °C of the completed samples with the test compound or the standard and stopped by addition of 75 µl 2 N HClO4

↓

After 60 min at room temperature, the samples are cooled in an ice-bath and neutralized by addition of 75 µl 3 N potassium acetate Supplementing the volume with water to 500 µl, the precipitate is centrifuged and 250 µl of the clear supernatant are applied to a column (0.6 × 8.0 cm) of BIORAD AG1-X8 (100–200 mesh)

↓

Mevalonolactone is eluted with water discarding the first 750 µl and collecting the next 3 500µl. Five hundred µl of the eluate are used for measurement in duplicate, mixed in vials with 10 ml Quickscint (Zinsser) and measured in a liquid scintillation counter (Beckman)

↓

The assay is generally performed in triplicate. Lovastatin sodium is used as standard

EVALUATION:-

- The mean values with and without inhibitors are compared for the calculation of inhibition.
- IC50 values are calculated.

2.ACAT INHIBITORY ACTIVITY:-

- ACAT stands for Aceyl COA cholesterol acyltransferase.
- It is an intracellular protein located in the endoplasmic reticulum that forms cholesteryl esters from cholesterol.
- Excess cellular cholesterol is stored as cholesteryl esters.
- In the disease atherosclerosis, chronic accumulation of CE in macrophages causes these cells to appear foamy and is a hallmark of early stages in atherosclerosis.

➢ **PROCEDURE:-**

Male Sprague-Dawley rats weighing 200–225 g arefed with a diet containing 5.5% peanut oil, 0.5%cholic acid and 1.5% cholesterol

On the last day, food is removed and the isotopes are administered. [3H]cholesterol (13 mCi/rat) isgiven by oral gavage and[14C]cholesterol(1.5 mCi/rat)given is given by tail vein injection

Each animal receives 1ml of oral dose and 0.5ml of i.v. dose. The rats are allowed to consume their respective diets , and are sacrificed 48 h after the isotope administration.

EVALUATION:-

- % oral dose of cholesterol absorbed is calculated by:

Plasma isotope ratio= % of oral dose in 2ml plasma *100 % of i.v. dose in 2ml plasma

DIURETICS

- A type of drug that causes the kidneys to make more urine. Diuretics help the body get rid of extra fluid and salt. They are used to treat high blood pressure, edema (extra fluid in the tissues), and other conditions. There are many different types of diuretics. They are sometimes called water pills.
- Urine consists of metabolic waste materials, water and some electrolytes. Diuretics are agents that facilitate the removal of salt or especially sodium ion.

SCREENING METHODS OF DIURETICS AGENTS:-

IN VITRO:-

- Isolated tubule preparation

- Carbonic anhydrase inhibition
- Patch clamp technique

INVIVO

- Saluretic activity in rats
- Stop flow technique
- Lipschitz test

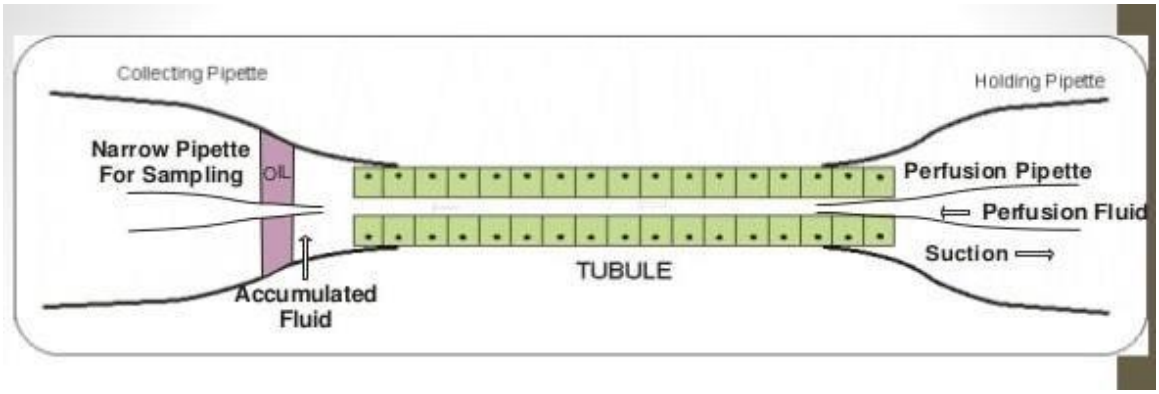
IN VITRO METHODS

1.ISOLATED TUBULE PREPARATION:-

PRINCIPLE : Measurement of change in concentration of solutes in perfusion fluid

PROCEDURE :

- This technique has been used in the kidney segments of several species like rat, mouse, hamster, rabbit etc.
- The thin (>1mm) tubule segments are dissected from kidney slices Segment is transferred into perfusion chamber.



- To perfuse a suitable tubule, one end of the tubule is holded by micropipette
- A perfusion pipette is inserted into tubule lumen
- The other end of the tubule is sucked into collecting pipette
- The oil inside the collecting pipette prevents the evaporation
- All the accumulated fluid is collected at periodic intervals by inserting a narrow calibrated pipette in the collecting pipette
- To approximate the in vivo situation, an isotonic rabbit serum is perfused while the tubule is immersed in a bath of rabbit serum

EVALUATION:-

- The absolute volume of reabsorption is determined from the change in the concentration of an impermeable marker like (3H) inulin, (125I) isothalamate in the collecting fluid.

- Leaks around the perfusion pipette is detected from the appearance of the marker in the external bath.

2.CARBONIC ANHYDRASE INHIBTION:-

PRINCIPLE:-

Carbonic anhydrase is Zn containing enzyme.

$H_2O + CO_2$

CARBONIC ANHYDRASE

↓

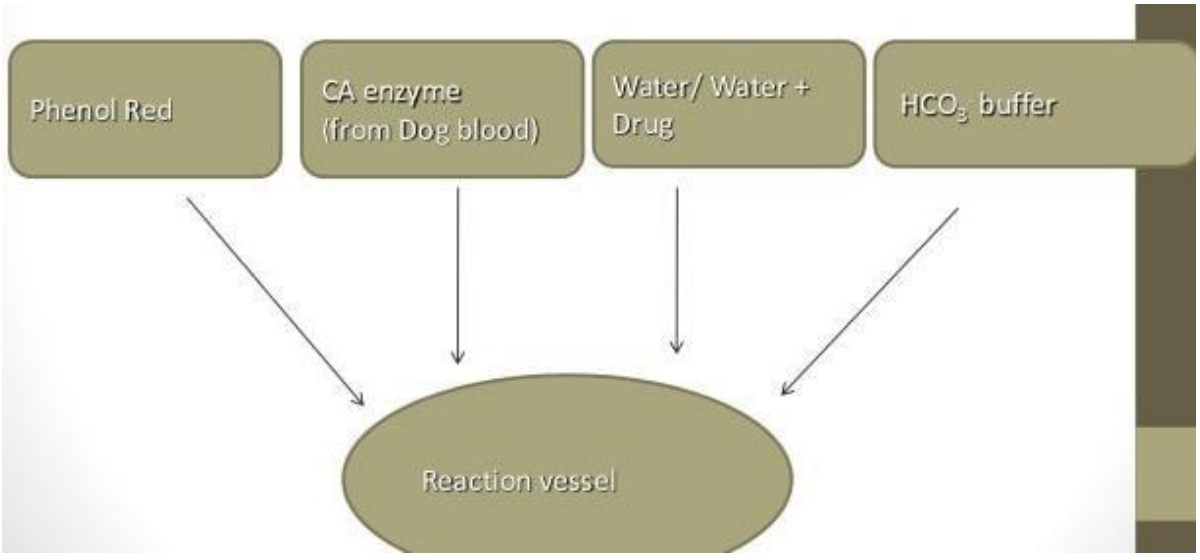
H_2CO_3

↓

$H^+ + HCO_3^-$

PROCEDURE:-

- Here the reaction vessel is used.
- CO_2 flow rate is adjusted to 30-45 ml/min.



EVALUATION:-

Following parameters are determined.

Tu = Time for color change in absence of enzyme

Te = Time for color change in presence of enzyme

Tu – Te = Enzyme rate

Ti = Enzyme rate in presence of inhibitor

$$\% \text{ Inhibition} = 1 - \frac{(T_u - T_e) - (T_i - T_e)}{T_u - T_e} \times 100$$

PATCH CAMP TECHNIQUE

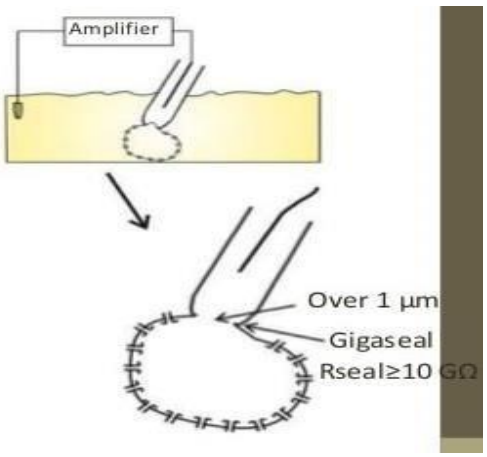
- This technique allows the study of single-ion channels as well as whole-cell ion channel currents
- It requires a patch electrode with a relatively large tip (>1 mm) that has a smooth surface

PROCEDURE :

- The patch-clamp electrode is pressed against a cell membrane and suction is applied to pull the cell membrane inside the electrode tip
- The suction causes the cell to form a tight, high-resistance seal with the rim of the electrode, usually greater than 10 giga Ohms, which is called a gigaseal
- Cell-attached, cell-excised, whole-cell mode of this technique allow investigation of ion channels

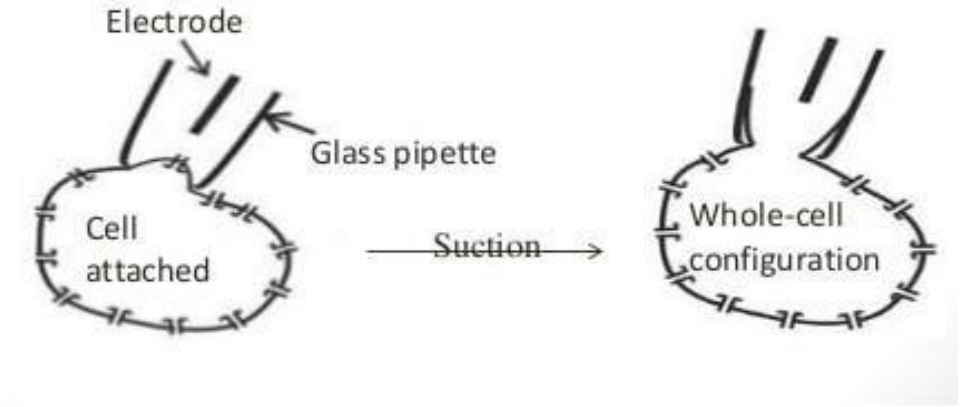
CELL ATTACHED MODE:-

- With this mode, the patch electrode remains sealed to the cell membrane, permitting the recording of currents through single-ion channels from the patch of membrane surrounded by the tip of the electrode.



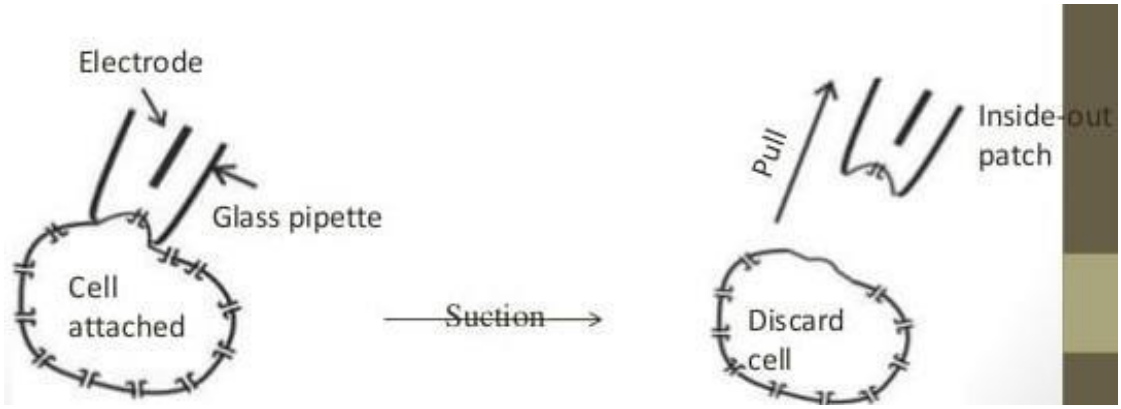
WHOLE CELL MODE:-

- From the initial cell-attached configuration, additional suction is applied to rupture the cell membrane, thus providing access to the intracellular space of the cell.
- The soluble contents of the cell will slowly be replaced by the contents of the electrode
- Whole-cell mode records currents through all channels from the entire cell membrane at once



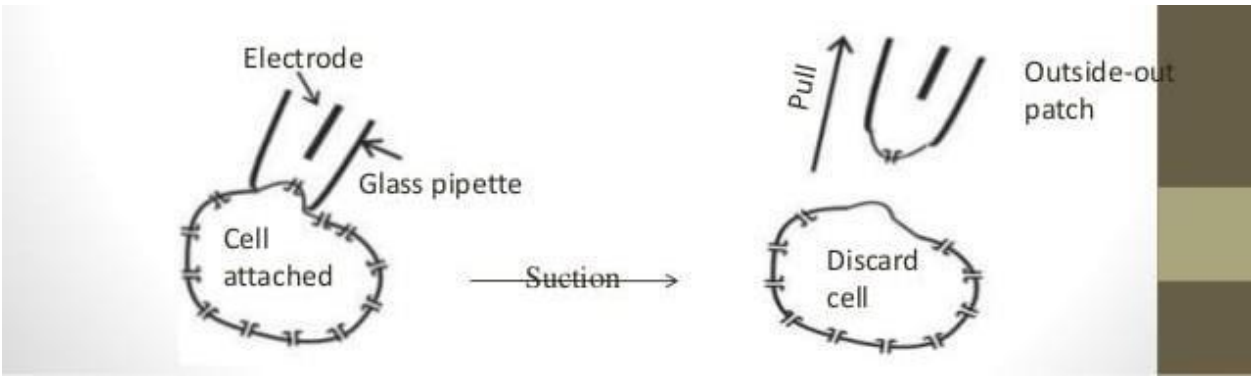
INSIDE OUT MODE:-

- From the cell-attached configuration, the electrode is quickly pulled out from the cell.
- This leaves the patch of membrane attached to the electrode exposing the intracellular surface of the membrane to the external environment, allowing pharmacological manipulations to the intracellular side of the ion channels.



OUTSIDE OUT MODE:-

- After achieving cell-attached configuration, the electrode is slowly withdrawn from the cell, allowing a membrane patch to be Excised
- Which will then reorganize on the edge of the electrode with the original interior of the cell membrane facing inside the electrode solution
- This mode allows researchers To examine ion channel properties, studying effects of membrane non permeable molecules on the intracellular part of the channel.



EVALUATION:-

- Concentration response curve of the drugs which inhibit ion channels can be obtained
- Single ion channel events studied by cell-attached & cell-excised technique
- Co transport system only studied by whole cell patch clamp technique as transport rate of single event is too small to detect

INVIVO METHOD:-

1.LIPSCHITZ TEST:-

PRINCIPLE : Based on water & Na+ excretion in test animal & compared to rats treated with std. drug

PROCEDURE: Male Wistar rats weighing 100-200 g are used & placed in Metabolic cages

Metabolic cages

- Wire mesh at bottom
- Funnel to collect urine
- Stainless-still sieves are placed into the funnel to retain feces and to allow the urine to pass
- Rats are fed with std diet & water
- 15 hr. before the experiment, food & water are withdrawn Animals are divided as treated with test and standard drug.

1. TEST ----------> 2 groups (6rats)
2. STANDARD----------> 2 groups (6 rats)

- Urine excretion recorded up to 5 hr& 24 hr.
- Na+ content of urine estimated by flame photometer & Urine vol. excreted calculated for each group.

EVALUATION:-

- Results expressed in LIPSCHITZ value for both urine excretion & for electrolyte
- LIPSCHITZ value =

Urine output in test animal ---------- Urine output in std. drug treated animal

- Lipschitz value ≥ 1 indicates positive effect
- Lipschitz value ≥ 2 potent diuretic activity
- For studying prolonged effect, 24 hr urine sample collected & analyzed
- For Saluretic drugs
  - Hydrochlorothiazide = 1.8
  - For loop diuretics ≥ 4

SALURETIC ACTIVITY IN RATS

PRINCIPLE :

- Excretion of electrolytes is important for the treatment of peripheral edema, CHF, hypertension so, need to develop diuretic with saluretic& K+ sparing effect
- Diuresis test in rats is designed to determine Na+ ,K+ , Cl- , water content & osmolarity of urine
- Ratio b/w electrolytes can be calculated indicating carbonic anhydrase inhibition or K+ sparing effect

PROCEDURE:

- Male Wistar Rats weighing 100-200 g are fed with std diet & water
- 15 hrs prior to experiment food is withdrawn but not water
- 3 animals placed in one metabolic cage
- Urine excretion measured every hr up to 5 hr& collected urine is analyzed for Na+ ,K+ & Cl-
- 2 groups, each of 3 rats used for drug test & std
- Furosemide, HCT, triamterene & amiloride are used as standards.

❖ ***For Saluretic activity :***

$Na^+ + Cl^-$ excretion calculated

❖ ***For Natriuretic activity :*** $\frac{Na^+}{K^+}$ is calculated

Natriuretc effects > 2

Potassium sparing effect > 10

EVALUATION:-

❖ ***For estimating CA inhibition :***

- Inhibition can be excluded at $\frac{Cl^-}{Na^+ + K^+}$ is calculated ratio between 1 to 0.8 with decreasing ratio slight to strong carbonic anhydrase inhibition can be assumed

STOP FLOW TECHNIQUE

PRINCIPLE :

- Useful in localization of transport process along the length of nephron
- During clamping of ureter, GFR is grossly reduced
- Contact time for tubular fluid in respective nephron segment increases & conc. of constituents of tubular fluid approximate the static head situation
- After releasing clamp, rapid passage of tubular fluid modify composition of fluid only slightly
- Urine is sampled sequentially

- **PROCEDURE :**

- This method can be Performed in different animals during anesthesia
- Ureter of animal is clamped allowing static column of urine to remain in contact with tubular segments for longer than usual time period
- Clamp released & urine is sampled sequentially
- Substances examined are administered along with inulin before the application of occlusion

EVALUATION :

- Concentration of insulin and substance under study is measured

- Fractional excretion of substance & insulin are plotted against cumulative urine volume.