EXPERIMENT NO.: 5 **DATE:**

AIM: A. INTRODUCTION OF COMMON LABORATORY TECHNIQUES USED FOR BLOOD WITHDRAWAL, SERUM AND PLASMA SEPARATION FROM EXPERIMENTAL ANIMALS

Blood sample from experimental animals are frequently required for study of effects of drugs on biochemical parameters and for the study of pharmacokinetics of drugs in the experimental animals.

The sampling procedures for blood collections are of two types:

A) Non-terminal Blood collection:

In this type, blood is collected from the conscious or unconscious experimental animals through a single or multiple withdrawals. Animal are not sacrificed after non-terminal blood collection.

a) Lateral Tail Vein or Ventral/Dorsal Artery:

- Can be used in both rats and mice by cannulating the blood vessel or by nicking it superficially perpendicular to the tail.
- Obtainable volume: Mouse - small to medium [50-100 ul]
  : Rat – medium [0.2-0.4 ml]
- Procedure is carried out in the conscious mice or rat. The tail is dipped in warm water (about 50-60^{o}C) or xylol is applied to the tail to increase circulation through tail vein. The needle (25-27 gauge, 0.5 to 1 length) is inserted, bevel up in the distal portion of tail vein. The blood is slowly aspirated avoiding the collapse of vein.
- Sample collection using a needle minimizes contamination of the sample, but is more difficult to perform in the mouse.
- Sample collection by nicking the vessel is easily performed in both species, but produces a sample of variable quality that may be contaminated with tissue and skin products.
- Sample quality decreases with prolonged bleeding times and tail stroking.
- Repeated collection possible.
- Relatively non-traumatic.
- Routinely done without anesthesia, although effective restraint is required.
- In most cases warming the tail with the aid of a heat lamp or warm compresses will increase obtainable blood volume.

- Arterial sampling produces larger volumes and is faster, but special care must be taken to ensure adequate hemostasis.
- Piercing the tail vein with a needle is also a way to collect a very small blood sample.

b) Mandibular Vein/Artery:

- Can be used in both rats and mice by piercing the mandibular vein or artery with a needle [20G] or stylet.
- Obtainable volume: medium to large [100-200 ul, mouse; 0.4-0.5 ml rat]
- Sample quality is good.
- The procedure is customarily done on an unanesthetized animal, but effective restraint is required.
- Arterial sampling produces large volumes very rapidly.
- Venous sampling produces medium volumes more slowly.
- Ensure that gentle pressure is applied for approximately 30 seconds post-collection to ensure hemostasis.

c) Saphenous/Lateral Tarsal:

- Can be used in both rats and mice by piercing the saphenous vein with a needle [23-25G: mouse, 21-23G: rat].
- Obtainable blood volumes: small to medium [mouse: 100 ul; rat: 0.4 ml]
- Repeat sampling is possible.
- Variable sample quality.
- The procedure is customarily done on an unanesthetized animal, but effective restraint is required.
- Can be more time-consuming than other methods due to time required for site preparation.
- After training, it requires more practice than tail or retro-orbital sampling to reliably withdraw more than a minimal amount of blood. Prolonged restraint and site preparation time can result in increased animal distress when handling an unanesthetized animal.
- Temporary favoring of the limb may be noted following the procedure.
- Care must be taken to ensure adequate hemostasis following the procedure.

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d) Retro-orbital:

*Note: Due to the increased risk of complications associated with this procedure, the CPCSEA recommends that other routes of blood collection be considered prior to use of this method. The mandibular technique permits an equivalent volume of blood to be collected in a rapid manner with less risk or complications.

- Individuals performing the procedure must be certified by Animal Ethical Committee (AEC).
- Can be used in mice by penetrating the retro-orbital sinus with a glass capillary tube [0.5 mm in diameters] or via the retro-orbital plexus in rats with a capillary tube.
- Must be performed by a skilled operator.
- Follow-up required 24-48 hours after blood collection. If complications such as squinting or bulging of the eye are noted, an animal health report must be completed.
- Obtainable volume: medium to large
- Collection is limited to once per eye.
- In the hands of an unskilled operator, retro-orbital sampling has a greater potential than other blood collection routes to result in the following complications:
  - Hematoma and excessive pressure on the eye resulting from retro-orbital hemorrhage
  - Corneal ulceration, keratitis, rupture of the eyeball or micro-ophthalmia caused by pressing on the eye to stem persistent bleeding or from a hematoma
  - Damage to the optic nerve and other intra-orbital structures leading to vision deficits or blindness
  - Fracture of the bones of the orbit and neural damage by the pipette; loss of vitreous humour due to penetration of the eyeball
- Skilled personnel can conduct retro-orbital bleeding in unanesthetized mice. Anesthesia is recommended for retro-orbital blood collection in mice and is required during the training of personnel.
- In rats, the presence of a venous plexus rather than a sinus can lead to greater orbital tissue damage than in the mouse. General anesthesia must be used unless scientific justification is provided and approved by the CPCSEA. In addition, a topical ophthalmic anesthetic, e.g. proparacaine or tetracaine, is recommended prior to the procedure. Retro-orbital bleeding performed in rats by a trained practitioner

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represents more than "minimal or transient pain or distress" and therefore should be considered a Category 2 procedure.

- Care must be taken to ensure adequate hemostasis following the procedure.

B) Terminal/Post-Mortem blood collection: In this type, large volume of blood is collected in single or multiple withdrawals from the anesthetized experimental animals. Animal is generally sacrificed during or after such blood collection.

Blood withdrawal by cardiac puncture or axillary cut down are considered terminal procedures and must be performed only after ensuring that the animal is under surgical anesthesia. The post-mortem collection from the aorta is performed immediately after euthanasia.

a) Cardiac Puncture

- Can be used in both rats and mice by penetrating the heart.
- Must be performed by a skilled operator.
- Obtainable volume: medium to large.
- Animal must be euthanized immediately after blood collection.

b) Axillary cut down

- Can be used in both rats and mice.
- Axillary vessels are cut with a scalpel blade or scissors and the pooled blood is collected via capillary tube.
- Obtainable volume: medium to large.
- Animal must be euthanized immediately after blood collection prior to recovery from anesthesia.

c) Pre-mortem collection from the aorta or vena cava

- Can be used in both rats and mice as a pre-mortem procedure on anesthetized animals.
- Blood is collected using a needle.
- Animal must be euthanized immediately after blood collection prior to recovery from anesthesia.
- Obtainable volume: medium to large.

d) Post-mortem collection from the aorta

- Can be used in both rats and mice as a post-mortem procedure in a euthanized animal.
- Must be done rapidly after euthanasia to ensure blood flow.

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- Aorta is cut and the blood pools in the pleural cavity.
- Blood is collected in a mini capillary tube. The tube must be held continuously in a horizontal position during the blood draw.
- Obtainable volume: medium to large

Summary of Blood Sampling Techniques

Route	Anesthesia Required		Speed		Sample Quality		Repeat Samples		Relative Obtainable Volume *(approximations)*		Potential for Complications	
	Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat
Tail Vein	No	No	Med	Med	Fair	Good	Yes	Yes	Small (50 ul)	Small *(.2 mls)*	Low	Low
Tail Artery	No	No	Fast	Fast	Good	Very Good	Yes	Very Good	Medium *(100 ul)*	Medium *(.4 mls)*	Low	Low
Retro-orbital	No	Yes	Fast	Med	Very Good	Good	Alternate eyes	Alternate eyes	Med.-Large *(200 ul)*	Med.-Large *(.5 mls)*	Moderate-High	Moderate-High
Saphenous	No	No	Med.	Med	Good	Good	Yes	Yes	Small-Med. *(100 ul)*	Small-Med. *(.4 mls)*	Low	Low
Mandibular Vein	No	No	Slow.-Med.	Slow-Med.	Fair-Good	Fair-Good	Yes	Yes	Small-Med. *(100 ul)*	Small-Med. *(.4 mls)*	Moderate	Moderate
Mandibular Artery	No	No	Very Fast	Very Fast	Very Good	Very Good	Yes	Yes	Large *(200 ul)*	Large *(.5 mls)*	Moderate	Moderate

Blood Collection Limits

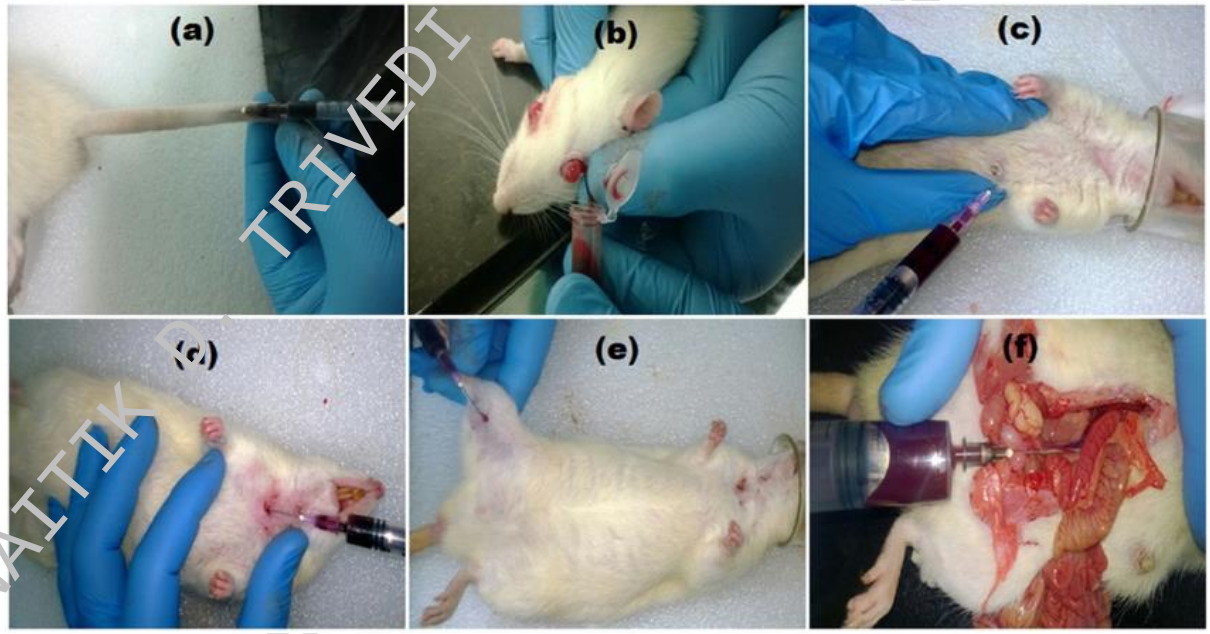
The AEC limits one time survival blood collection to 15% of an animal's blood volume in most circumstances. Serial blood sampling limit vary by species, strain, and frequency of blood collection as outlined in Tables 1 and 2. The AEC may require monitoring for anemia (using assays such as hematocrit and/or serum protein levels) when repeated collections or collection of larger volumes are required. Blood collected for diagnostics or other veterinary procedures must be considered when evaluating total volume available for experimental use. In all cases blood collection volumes should be limited to the minimum volume that will allow for successful experimentation or diagnostics.

Table 1:

Species	Blood Volume Mean (ml/kg)	Blood Volume Range (ml/kg)	Blood Volume (average)		
Mouse (25 g average Wt.)	58.6	55-80	7.5%	10%	15%
Rat (250 g)	64	58-70	1.2 ml	1.6 ml	2.4 ml
Rabbit (4 kg)	56	44-70	17 ml	22 ml	34 ml
Nonhuman primate (NHP; 8 kg)	56	55-75	34 ml	45 ml	67 ml

Table 2:

Single sampling		Multiple sampling	
% Circulatory blood volume removed	**Approximate recovery period**	**% Circulatory blood volume removed (cumulative volume)**	**Approximate recovery Period**
7.5%	1 week	7.5%	1 week
10%	2 weeks	10-15%	2 weeks
10-15%	4 weeks	20%	3 weeks



Rat blood sampling sites: (a) Lateral tail vein, (b) Retro-orbital sinus, (c) Cardiac puncture, (d) Jugular vein, (e) Saphenous (lateral tarsal) vein, and (f) Inferior vena cava.

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B. ANAESTHETICS AND EUTHANASIA USED FOR ANIMAL STUDIES.

THEORY:

A fundamental responsibility of individuals that use animals in research, teaching or testing is to anticipate and eliminate or minimize any potential that procedures may cause animal pain, distress, or discomfort.

Although animals that are in pain may not behave like humans, (e.g., pain in animals may be accompanied by immobility and silence, in contrast to the groans and cries of human patients), it is assumed that procedures that cause pain in humans cause pain in animals.

The presence of pain in animals can be recognized by alterations in animal behavior (e.g., reduced activity, reduced grooming, hunched- up posture, altered gait, changes in temperament, vocalizations, reduced food and water intake, reduced urinary and fecal output), and in physiological variables, (e.g., reduced depth of respiration, increased heart rate, and reduced hydration status).

Animal pain, distress, and discomfort can produce a range of undesirable physiological changes, which may radically alter measured responses to experimental stimuli, as well as the rate of recovery from surgical procedures, hence, its avoidance and alleviation are in the best interest of both the animal and researcher. Reducing post-procedural/post-operative pain, distress, and discomfort is accomplished by good nursing care, (e.g., keeping the animal warm, clean, dry and well padded), and by the administration of analgesic drugs.

In addition to the avoidance and alleviation of pain and discomfort, adequate post-procedural postoperative animal care also includes efforts to prevent and/or treat post-anesthetic complications, (e.g., aspiration, hypostatic pneumonia, cardiovascular and respiratory depression, dehydration, and infection). The prevention or minimization of animal pain, distress, or discomfort by the proper use of tranquilizers, anesthetics, and analgesics is scientifically and ethically essential to the humane care, use, and treatment of research animals.

1. **ANAESTHESIA:**

   The word anaesthesia has been derived from Greek word that means “without perception of insensibility”. Anesthesia is the act of providing sensation-free relief from pain or pain-producing procedures. Anesthesia must be performed by a person with knowledge of and familiarity with the drugs to be used in the animal species under consideration.

   There are numerous anesthetics available for use in rodents. Some of the more popular agents include:

- Chloralose
- Urethane
- Barbiturates
- Paraldehyde
- Magnesium Sulphate
- Ketamine
- Tribromoethanol

CHLORALOSE:

- It is a compound of chloral and glucose prepared by heating equal parts of anhydrous glucose and charcoal, when both α- chloralose (active form) and β-chloralose (in active form) are formed.
- It is prepared as one percent solution by boiling in 0.9% NaCl or in distilled water, and administered intravenously or intraperitoneally at a temperature of 30-40^{0}C before the chloralose comes out of solution.
- Disadvantages: It is suitable only for acute experiments, usually in dogs and cats, inducing surgical anaesthesia for 3-4 hours or longer.
- Advantages: It has the advantage of greater constancy of the depth of anaesthesia. The respiration and circulation are not depressed, and the blood pressure is well maintained usually on the higher side. Reflexes are not depressed but may be slightly exaggerated including responses to bilateral carotid occlusion.

URETHANE (Ethyl Carbamate):

- It is readily soluble in water giving a neutral solution. Usually 25% solution in water is used.
- Disadvantages: It is suitable only for acute experiments since it has delayed toxic effect on liver, and may also cause agranulocytosis and pulmonary adenomata.
- Mice develop an exceptionally high incidence of lung tumours regardless of the route of administration.

BARBITURATES:

- Barbiturates interfere with nerve impulse transmission both in the central nervous system and in the ganglia producing depression of cardiovascular and spinal cord reflexes.
- In rabbits pedal reflex (leg retraction) is lost first, then pupillary and finally palpebral reflex.

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- Pentobarbital Pentobarbital is a barbiturate and, historically, the most commonly used anesthetic in rodents.
- Advantages: At recommended doses, it causes minimal cardiovascular depression. It is also relatively long acting and can provide approximately 45 minutes of surgical anesthesia. Disadvantages:- Pentobarbital is a potent inducer of the hepatic microsomal enzyme system. Causes pronounced respiratory depression as well as hypothermia, particularly when repeated doses are given.
- Phenobarbitone sodium Phenobarbitone sodium and barbitone sodium are used for prolonged experiments.
- Tiopentone sodium Thiopentone sodium (pentothal) is used for surgical operations of short duration. It produces rapid induction with minimum excitation.

PARALDEHYDE:

- Advantages: It has a wide margin of safety because it depresses only the cerebrum and not the medullary centres. Intravenous injection is likely to produce cardiac dilatation and pulmonary congestion and oedema.
- Disadvantages: Under its influence the basal blood pressure as well as the response to vasopressor and depressor drugs are low. Bilateral carotid occlusion produces poor pressor response or even a depressor response.

MAGNESIUM SULPHATE:

- A 20% magnesium sulphate solution 5ml/kg intravenously produces anaesthesia for about an hour. Calcium gluconate intravenously will counteract its depressant effect immediately. Its principal use is in producing euthanasia.

TRIBROMOETHANOL

- Advantages: In most rodents, tribromoethanol produces good surgical anesthesia, with good skeletal muscle relaxation and only a moderate degree of respiratory depression. It is relatively inexpensive and not a controlled agent.
- Disadvantages:- It is a potential for causing peritonitis. When exposed to either light or temperatures >40^0C, tribromoethanol degrades into two byproducts: hydrobromic acid and dibromoacetaldehyde. Both of these compounds are highly irritating when administered IP and result in peritonitis and visceral adhesions which may be fatal.

KETAMINE HYDROCHLORIDE:

- Ketamine hydrochloride, a dissociative anesthetic, disrupts pain transmission and suppresses spinal cord activity with some action at opioid receptors. Visceral pain is not abolished with dissociative anesthetics and there is poor muscle relaxation and analgesia.
- Disadvantages: Ketamine is a poor anesthetic when used alone, but is more often combined with other agents. When combined with other drugs, it is usually administered IP. Ketamine is acidic, can be irritating, and cause muscle necrosis when administrated IM. Ketamine-induced nerve damage can cause selfmutilation in rodents. Ketamine is a controlled substance. Store in a locked cabinet and maintain a log of its use.

2. **EUTHANASIA:**

The term euthanasia is derived from the Greek terms eu meaning good and thanatos meaning death. The act of inducing humane death in an animal by a method that induces rapid loss of consciousness and death with a minimum of pain, discomfort or distress. Methods of euthanasia fall into two broad categories:

A. Chemical methods:

i. **Inhalant agents:**

Eg.: ether, halothane, methoxyflurane, isoflurane, enflurane, chloroform, nitrogen, nitrous oxide, carbon di oxide, carbon monoxide, argon, hydrogen cyanide.

ii. **Injectable agents**:

Eg.: barbiturates, chloral hydrate, ethanol, ketamine, magnesium sulphate, potassium chloride, neuromuscular blocking agents.

B. **Physical methods**:

Eg.: Penetrating Captive Bolt, Euthanasia by a Blow to the Head, Gunshot, Cervical Dislocation, Decapitation, Electrocution, Microwave Irradiation, Thoracic (Cardiopulmonary, Cardiac) Compression, Kill Traps, Maceration, Adjunctive Methods, Exsanguination, Stunning, Pithing

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Introduction about some Common Methods of Euthanasia

Inhalation of anesthesia gas – acceptable with conditions for rodents and other small animals (< 7 kg). Typically used as part of a two-step process with a secondary physical method of euthanasia such as decapitation or cervical dislocation.

Inhalation of CO2 - acceptable with conditions, including the special considerations listed below.

Immersion agents – e.g. MS 222/Tricaine. Acceptable for aquatic species, usually in connection with a secondary physical method.

Cervical Dislocation – acceptable for small birds, mice and immature rats. Requires training and should be performed under anesthesia unless specifically approved by the CPCSEA.

Decapitation – acceptable for rodents and small rabbits. Requires training; anesthesia recommended unless approved by the CPCSEA. Guillotines must be sharpened and adjusted frequently to ensure proper performance.

Injectable barbiturate agents – e.g. sodium pentobarbital, Euthasol®, Eutha 6®, Fatal Plus® - acceptable for most species.

Exsanguination/Cardiac Perfusion – acceptable with conditions; animals must be anesthetized.

Special Considerations for the use of carbon dioxide gas as a euthanasia agent:

The following additional guidelines must be followed when using CO2:

- CO2 must be delivered from compressed gas canister only. Gas should be delivered using a gradual fill method- a displacement rate from 10% to 30% of the chamber volume per minute is recommended. Use of a flowmeter is strongly recommended.
- Use of dry ice to deliver CO2 gas is unacceptable.
- High concentrations of CO2 have been determined to cause pain and distress. The practice of immersion, where conscious animals are placed directly into a container filled with 100% CO2, is unacceptable.
- Use of the rodents' home cage is recommended as it minimizes stress in the animals.
- Chambers used for CO2 euthanasia must not be overcrowded. Overcrowding in this situation is defined as less than one half the normal housing space normally required for the animals.

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- If animals are removed from their home cages prior to euthanasia, the container used to transport animals between the housing area and procedure room/euthanasia chamber must not be overcrowded.
- Male mice from different cages should not be mixed in transport cages or the euthanasia chamber to prevent distress and/or fighting.
- Detailed instructions for the use of CO2 euthanasia are posted in all vivarium procedure rooms and can be reviewed on the CPCSEA website.

TEACHER'S SIGNATURE

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