Thrombinase, as a new thrombolytic drug, its efficacy, effects on the coagulation parameters, haematological parameters, respiration and cardiovascular system (ECG, BP) were evaluated in different animal models. Along with these, its pyrogenicity and hypersensitivity were investigated. The drug Thrombinase was also evaluated for its pharmacokinetic parameters such as plasma clearance, organ distribution and excretion.

**Animals**

The pharmacokinetic studies of Thrombinase were evaluated in healthy male and female adult inbred Wistar strain albino rats (body weight 200 - 250 gm) (colony maintained) and New Zealand white rabbits (body weight 2 - 2.5 Kg). The efficacy study was evaluated in male and female New Zealand white rabbits and Mongrel dogs (body weight 10 -15 Kg). The rabbits used in this study were purchased from Department of Laboratory Animal Medicine (Tamil Nadu Veterinary University, Chennai). The dogs were procured from Corporation of Chennai.

Rat and rabbits were housed in air-conditioned room and accustomed to a light/dark cycle of 12h/12h. The animals were well maintained at standard laboratory conditions with food and water *ad libitum*. Dogs were allowed to acclimatize for a week and fed with dog feed. All the animal experiments were approved by Committee for Purpose of Control and
Supervision of Experiments on Animals (CPCSEA), Govt. of India and Institutional Animal Ethics Committee (IAEC).

EFFICACY STUDY OF THROMBINASE

THROMBINASE

Thrombinase, a fibrinolytic enzyme, required for these experiments was obtained from Malladi Drugs and Pharmaceuticals Limited (MDPL), Chennai, India, in the form of lyophilized powder (in mg of protein).

IN VITRO STUDY OF THROMBINASE

10 mg of lyophilized powder containing 1.25mg of Thrombinase was dissolved in one ml of distilled water and was used for in vitro studies.

IN VITRO EFFICACY OF THROMBINASE IN RABBIT CLOT

From this stock solution 50µl (62µg), 75 µl (94µg) and 100µl (125µg) of enzyme was taken and clot lysing ability was tested in rabbit plasma by blood clot assay method.

Blood clot assay

Principle

The blood samples with haemoglobin concentration of 16- 17-gm/dl were used. In fresh blood clot (derived from the blood samples) with similar size and shape, known concentration of Thrombinase was added and the thrombolytic activity of the enzyme corresponds to the number of red blood
cells (RBC) released, which were lysed with distilled water and the optical density was measured at 540 nm using spectrophotometer. (Toombs, 2001)

**Materials**

Fresh rabbit blood clot, rabbit plasma, spectrophotometer, water bath (37°C), 1ml micro centrifuge tubes (eppendorfs), petridish, cork borer and Phosphate Buffer Saline (PBS).

**Procedure**

1. Fresh blood of rabbit was allowed to clot in a petridish. Using cork borer small sized uniform clots were made. Clots were washed with PBS for many times till the blood stains were removed.

2. To determine the thrombolytic efficacy of Thrombinase, a clot was put into three micro centrifuge tubes and enzyme at three different concentrations 50µl (62µg), 75 µl (94µg) and 100µl (125µg) was dispensed in micro centrifuge tubes by using micropipettes. The total volume of the reaction solution was made up to 500µl by adding rabbit plasma. Four duplicates were used for each concentration of Thrombinase.

3. One micro centrifuge tube containing clot and PBS served as control.

4. The micro centrifuge tubes containing clot and testing enzyme were incubated in water bath at 37°C for 20 to 45 minutes. In between the incubation time, micro centrifuge tubes were taken out and shaken slightly. This helps the enzyme to act all over the surface of the clot.
5. After incubation, the maximum supernatant with RBC which was released from clot, of each reaction solution was taken in a micro centrifuge tube and was centrifuged at 10,000 rpm for 10 minutes at 4°C.

6. The precipitate, which contains the red blood cell released from the blood clot, was washed with PBS and centrifuged once again.

7. After washing the precipitate thoroughly, 1ml of cold water was poured into the micro centrifuge tubes to disintegrate the RBCs. The haemoglobin released from the RBCs gives colour to the solution.

8. The solution was centrifuged again and its optical density was measured with spectrophotometer.

9. From the OD the activity of enzyme was calculated using the following formula,

\[
\text{Activity} = \text{OD} \times \text{Time factor} \times \text{volume factor} \times \text{assay factor}
\]

in which,

- **OD** - Optical density of the haemoglobin released from the RBCs after clot lysis
- **Time factor** - The Time taken for the assay
- **Volume factor** - The volume of reaction solution
- **Assay factor** - The factor that is standardized with known thrombolytic drug, Streptokinase for this assay
IN VITRO EFFICACY OF THROMBINASE IN HUMAN CLOT

Since species variation can affect the drug action, it is essential to understand whether the drug can act on human blood clots. To understand this, different concentrations of enzyme were added in human blood clot in vitro and the clot lysing capability was confirmed by detecting Fibrinogen Degradation Products (FDP) with reference to streptokinase (Bookstein and Saldinger, 1985).

Materials

Human blood clot of 50 and 100µl volume, cross linked Fibrinogen Degradation Products (XL FDP) kit, water bath (37°C), stopwatch, 1ml micro centrifuge tubes (eppendorfs), Phosphate Buffer Saline (PBS), Thrombinase and streptokinase (Streptase, Hoechst Roussel Laboratories Ltd., India)

XL FDP kit

This kit provides qualitative test materials for detecting cross-linked fibrin degraded product in human plasma

1. XL FDP reagent: A uniform suspension of polystyrene latex particles coated with mouse monoclonal Anti D – dimer antibody against FDP. The reagent is standardized to detect XL FDP 200 ng/ml
2. Positive control - reactive with XL FDP latex reagent
3. Negative control – non reactive with XL FDP latex reagent
4. Glass slide with six reaction circle, disposable sample dispensing dropper, mixing sticks
Principle

XL FDP slide test for detection of cross-linked fibrin degradation products is based on principle of agglutination. The test specimen (plasma or FDP containing solution) is mixed with XL FDP latex reagent and observed for agglutination. The sensitivity of the reagent is 200ng / ml, below which samples are negative and above which the samples give positive agglutination reaction.

Procedure

1. Human blood, volume of 50 and 100μl of were placed in micro centrifuge tubes and were allowed to clot to make the uniform sized clots. Clots were washed with PBS for many times till the blood stains were removed.

2. The thrombolytic efficacy of Thrombinase and streptokinase were determined based on the FDP released from the clot. The clots were placed in micro centrifuge tubes and the two drugs each at two different concentrations 50μl (62μg) and 100μl (125μg) were dispensed by using micropipettes. The total volume of the reaction solution was made up to 1ml by adding PBS. Four duplicates were used for each concentration of Thrombinase and Streptokinase.

3. One micro centrifuge tube containing clot and PBS served as control.

4. The micro centrifuge tubes containing clot and testing enzyme were incubated in water bath at 37°C for 20 to 45 minutes. In between the
incubation time, the tubes were taken out and were shaken slightly. This helps the enzyme to act all over the surface of the clot.

5. After incubation, one drop of supernatant solution from each tube was dispensed accurately on one circle of glass slide using pipette with disposable dropper provided with the kit.

6. Immediately one drop of XL FDP latex reagent was placed adjacent to the drop of supernatant solutions and mixed uniformly.

7. After mixing, the slide was tilted back and forth for three minutes. Agglutination was observed macroscopically.

IN VIVO EFFICACY OF THROMBINASE

It is essential to confirm the effect of a drug on different animal models. The efficacy of Thrombinase was tested in rabbit and dog, in venous as well as in arterial thrombosis models.

1. A clot was induced in rabbits, the drug was administered as slow infusion in one set and as a bolus injection in another set of animals.

2. As Thrombinase is a new thrombolytic enzyme, its effect on coagulation was tested both in pathological state (clot induced animals) and in normal healthy animals.

3. The efficacy of Thrombinase was compared with that of existing standard thrombolytic drug, Streptokinase, in dog thrombosis model.
IN VIVO EFFICACY OF THROMBINASE IN RABBIT JUGULAR VEIN THROMBOSIS MODEL (SLOW INFUSION) (n = 6)

Materials

1. Anaesthetic agent – Ketamine (Themis Medicare Ltd., India)
2. Thromboplastin - It was used to induce clot within vein. (Biopool international, USA)
3. Slow infusion peristaltic pump (Miclins, India).
4. Dop II Doppler flow meter (Kody Corporation, India)

Method of determining blood flow

Doppler flow meter was used to monitor the pattern of blood flow, both in artery and vein. The doppler flow probe was placed on the artery or vein at an angle of 45 degree against the direction of blood flow.

Method of conversion doppler sound in to graphical pattern

The blood flow was monitored using a doppler flow meter. The doppler sound was recorded using an audio recorder (BPL, India). The sound waves were played back and fed into a Personal computer as *.wav files. The noise during recording was filtered from the sound waves using the software GOLD WAVE, which is a sound editor, player, recorder, and converter software. (Gold Wave is a product of Gold Wave Incorporate, a Canadian corporation founded in 2001 by Chris Craig) Later the *.wav files were converted into a data array using MATLAB software program.
(Version6.0, The Math Works, Inc.) is an integrated technical computing environment that combines numerical computation, advanced graphics and visualization and a high level programming language. The sound wave was sampled at a rate of 44 KHz for a time interval of one minute. The sampled data was normalized to a magnitude scale of $-1$ to $+1$ and plotted as a graph. In order to appreciate the difference between the arterial and venous blood flow pattern, an exclusive blood flow pattern of dog was plotted in detail (44 KHz, 2.5 milliseconds) (Figure- IV).

**Clot induction procedure**

Rabbits were anaesthetized by intramuscular (IM) injection of Ketamine (50mg / kg of body weight). After anesthesia, the external jugular veins on both sides were exposed through a medial incision in neck. Each vein was cleared and separated from the neighboring structures. The blood flow pattern of the right jugular vein was recorded with doppler flow meter. Then, in the right jugular vein two bulldog clamps were placed 1 cm apart. To produce an occlusive thrombus, Thromboplastin (50μl) was injected through a micro syringe into this separated segment of the vein. The oozing blood was ceased by applying cotton. In all instances, from the time of occlusion, the clot was allowed to age for 30 minutes before the vessel clamps were removed (A modification of the thrombosis model is described by Collen *et al.*, 1983). Doppler flow pattern after the thrombus formation was recorded.
Figure – IV Comparison of Doppler pattern of normal blood flow in Carotid Artery and Jugular vein – Dog
Thrombinase infusion

A butterfly needle was introduced in the contralateral earlobe vein, through which Thrombinase was infused into the systemic circulation, at a flow rate of 6.7ml/hour (0.37mg/ml) (dose randomly selected) by using slow infusion pump.

Blood flow of occluded vessel was continuously monitored by doppler flow meter. After restoration of blood flow in the vein was confirmed by doppler recording, the perfusion of Thrombinase was immediately stopped. The volume of Thrombinase used to lyse the clot was determined.

Coagulation and haematological profile

2 ml of blood sample was withdrawn in the contralateral marginal ear lobe veins before the clot induction and 30 minutes after the clot lysis in a Sodium citrate (3.2%) containing syringe. 1 ml of blood sample was centrifuged and plasma was separated. This plasma sample was used for the measurement of the following coagulation parameters namely, Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), Fibrinogen concentration. With another one ml of blood sample haematological parameters namely, Total White blood cell count (TC), Total Red blood cell count. Haemoglobin concentration (Hb), Platelet count and Haematocrit (PCV) were studied. Coagulation time was determined by a simple prick on ear lobe.
IN VIVO EFFICACY OF THROMBINASE IN RABBIT JUGULAR VEIN THROMBOSIS MODEL (BOLUS ADMINISTRATION) (n=6)

The in vivo efficacy of Thrombinase was tested in rabbit followed by intravenous bolus administration of Thrombinase in experimentally induced Jugular venous thrombosis. Its effect on haematological and coagulation were also studied.

Procedure

Animals were anaesthetized by intramuscular injection of Ketamine (50mg / kg of body weight). After anesthesia, an occlusive thrombus was induced with help of Thromboplastin (50μl) in the separated segment of Jugular vein as described in detail above (Collen et al., 1983). Doppler flow patterns before and after the thrombus formation was recorded. Thrombinase was administered as a bolus, at a dose of 0.45mg over a period of one minute in contralateral ear lobe vein. Blood samples were collected (in 3.2% sodium citrate) from the contralateral marginal ear lobe veins before the clot induction and 30, 120, 240 minutes after the clot lysis. A sample of blood was used to measure haematological parameters. Another blood sample was centrifuged and plasma was separated. This plasma was used for the measurement of coagulation parameters. Coagulation time was determined by capillary method.
COMPARATIVE THROMBOLYTIC EFFICACY OF THROMBINASE AND STREPTOKINASE IN DOG THROMBOSIS MODELS (n=4)

The thrombolytic efficacy of Thrombinase was also studied along with a known thrombolytic drug, streptokinase in dog carotid artery and jugular vein thrombosis model.

Materials

Anesthetization agent used: Pentothal sodium (Abbott Laboratories India Ltd., India)

Thromboplastin (Biopool international, USA): Thromboplastin was used to induce clot within vein and artery.

Streptokinase

Streptokinase (SK) was purchased from Hoechst Roussel Laboratories Limited, India (Brand name: streptase). The lyophilized powder was reconstituted in sterile saline to obtain a dose of 1,50,000 units / ml.

Experimental Procedure

The dogs were anaesthetized with Pentothal sodium at a dose of 40mg /Kg body weight (I.V.) and placed on the Brodie’s operation table on its spine position.

Tracheal Cannulation: An incision was made over the mid line of the neck and trachea was exposed. A ' T ' metal tube was inserted up to three
rings of the trachea and tied with cotton thread. This allows the animal to respire easily.

**Recording of blood pressure and respiration**

Tracheal cannula arrangement was convenient to record the respiration through rubber tubing attached to a Marry's tambour connected with a writer. The writer was allowed freely to write on the smoked paper of kymograph.

The two carotid arteries with the accompanying vagus nerve were separated from the protective enclosed carotid sheath. The carotid artery on one side was cannulated for recording blood pressure with a 'U' shaped mercury manometer and floating writer which was connected to the kymograph. The dissection and setting of the recording instruments was done as described by Ravindra Rao and Muralidhar (1981).

**Induction of venous thrombosis**

After dog was anaesthetized, the right and left jugular veins were exposed to a length of approximately 5 to 6 cm. One of the jugular veins was clamped (at 1cm distance) with two bulldog clamps to form a separated venous segment. A needle with thread was introduced at the site of separated venous segment. Then, the needle was passed into the vascular lumen for approximately 1cm distance and by rotating the needle the intima of the vessel was damaged. After this, the needle was taken out of the vessel through the vascular wall. The introduced cotton thread was left inside the vessel. This helps to avoid the emboli formation in larger blood vessels while inducing
Plate 2 - Dog jugular vein Thrombosis model

Dog with experimental setup

Exposed left jugular vein

After clot induction

After clot lysis
clot formation (Larsen et al., 1991). Thromboplastin (100 µl) was injected into the clamped segment of the vein to hasten the process of thrombus formation (Colucci et al., 1998). The clot was allowed to age for 30 minutes. Then, the clamps on either side of the thrombus were removed. Cessation of blood flow was confirmed by doppler flow meter (Markland et al., 1994).

**Induction of arterial thrombosis**

The same procedure was repeated in arterial thrombus formation, after exposing the right carotid artery. The clots were allowed to age for 45 minutes. (Jorgensen et al., 1971).

**Infusion of Thrombinase and Streptokinase**

Thrombinase and SK was administered (dose of 4.5 mg of protein / 3ml) at a flow rate of 3ml / hr with the help of the slow infusion peristaltic pump.

Saline was administered in the control group of both arterial and venous thrombosis at a flow rate of 3ml / hr with the help of the slow infusion peristaltic pump.

**GROUPS STUDIED**

The dogs used for experiments were divided into six groups (n = 4),

**Group I** - Venous thrombosis was induced. Saline (3ml/hr) was administered and this group forms the strict
control which helps to understand, whether the endogenous action of plasminogen can dissolve the clot formed.

Group II - Arterial thrombosis was induced. Saline (3ml/hr) was administered and this forms the strict control, which helps to understand, whether the endogenous action of plasminogen can dissolve the clot formed.

Group III - Venous thrombosis was induced. The Thrombinase was administered after the complete occlusion of the vein (confirmed by the doppler flow meter).

Group IV - Arterial thrombosis was induced. The Thrombinase was administered after the complete occlusion of the artery (confirmed by the doppler flow meter).

Group V - Venous thrombosis was induced following which streptokinase was administered.

Group VI - Arterial thrombosis was induced following which streptokinase was administered.
EFFECT OF THROMBINASE ON HAEMATOLOGICAL AND COAGULATION PROFILE IN NORMAL HEALTHY RABBITS (n = 6)

Thrombinase as a proteolytic enzyme its effect on haematological and coagulation profile in rabbits after the intravenous administration of Thrombinase was studied.

Randomly selected (three male and three female) animals were used for this study. Animals were placed in animal restraining cage, to collect blood samples from marginal ear lobe vein before (Control) and 30 minutes after the drug administration (Test). Thrombinase was administered as bolus in one of the ear lobe vein at the dose of 1mg of protein/ Kg of body wt. intravenously.

Then, blood samples were collected from contralateral ear lobe vein in a syringe containing 3.2% citrate. The haematological parameters were then studied.

HAEMATOLOGICAL PARAMETERS

Total Red Blood Cell Count - determined by using hemocytometer and hayem’s diluting fluid

Total Leukocyte Count (TC)  – counted in a hemocytometer using Turk's fluid.
Differential Leukocyte Count (DC) - A blood film stained with Leishman's stain, was examined under immersion oil and the percentage distribution of these cells was then determined.

Haemoglobin concentration (Hb) - Using Sahil's haemoglobinometer, the red blood cells are converted into acid haematin by adding N/10 HCl. The brown color so developed was matched against standard comparator and results were expressed as gm/100ml of blood.

Platelets count – Using Ammonium oxalate 1% (known as Brecher and Cronkite solution), RBC pipette and hemocytometer (Lewis et al., 2001) platelets were counted.

Packed Cell Volume (PCV) - A sample of blood with anticoagulant was centrifuged in haematocrit tube. The RBCs were packed towards the bottom of the tube by the centrifugal force and reading of packed cells was taken as percentage.

COAGULATION PARAMETERS

Another blood sample (with 3.2%citrated) was taken and centrifuged. The plasma was separated and used to estimate coagulation profiles.

Prothrombin Time (PT)

Prothrombin is factor II. The variation in plasma prothrombin concentration affects the normal coagulation profile. Determination of Prothrombin Time is helpful in assessing the action of fibrinolytic drug.
Principle

Tissue Thromboplastin, in the presence of calcium activates the extrinsic pathway of blood coagulation mechanism. When Tissue Thromboplastin reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time.

Reagent

It is a ready to use liquid calcified Thromboplastin reagent, derived from rabbit brain (UNIPLASTIN, Tulip Diagnostics (P) LTD, India).

Test procedure

1. The reagent was prewarmed to 37°C before using in test procedure.

2. The citrated blood was centrifuged at 3000 rpm for 20 minutes to prepare platelet poor plasma (PPP) (Lewis et al., 2001a). In a tube, 100μl of PPP was taken and incubated in a water bath for 3 to 5 minutes at 37°C.

3. Followed by this, 200μl of reagent was added forcibly into the tube and simultaneously a stopwatch was started. The tube was shaken gently to mix contents.

4. The stopwatch was stopped as soon as the first fibrin strand is visible and the time was noted. The test was repeated in duplicates and the average was measured as Prothrombin Time (PT).
Activated Partial Thromboplastin Time (APTT)

Activated partial thromboplastin time determination helps in the diagnosing the defect in intrinsic pathway of blood coagulation. The abnormality of APTT indicates the deficiency of factors VIII, IX, XI and XII.

Principle

Cephaloplastin activates the intrinsic pathway of coagulation in the presence of calcium ions.

Reagent

It is an activated cephaloplastin reagent for the determination of APTT. It is a phospholipid preparation derived from rabbit brain with ellagic acid as an activator. (Liqucelin-E, Tulip Diagnostics (P) LTD, India)

Test procedure

1. The reagent was prewarmed to 37°C before the test procedure. 100μl of test plasma was taken in a tube, followed by addition of 100μl of reagent. The test plasma was briefly mixed with the reagent and incubated at 37°C for 5 minutes.

2. After incubation, 100μl of prewarmed (37°C) Calcium chloride was added forcefully. The tube was tilted back and forth until a gel clot was formed. Time for gel formation was recorded.
QUANTITATIVE ESTIMATION OF FIBRINOGEN

Fibrinogen (factor I) is a high molecular glycoprotein synthesised in liver, which is converted to fibrin by the action of thrombin and is one of the key components of clot formation.

Reagents

FIBROQUANT kit (Tulip Diagnostics (P) LTD, India) contains:

1. Thrombin reagent, a lyophilized preparation from bovine source (50 NIH units/ vial).
2. Fibrinogen calibrator, a lyophilized preparation of human plasma equivalent to stated amount of fibrinogen on mg basis.
3. Owrens buffer, (pH 7.35).

The addition of thrombin coagulates fresh citrated plasma. The coagulation time is proportional to the fibrinogen concentration. This allows the estimation of plasma fibrinogen by functional clotting assay. Using fibrinogen calibrator, a standard calibrated graph (time (in seconds) versus mg/dl) was prepared.

Test Procedure

1. 200 µl of test plasma was taken in a test tube at 37°C.

2. 100 µl of Thrombin reagent was added to the test plasma and tilted gently back and forth. The time was recorded at the first sight of fibrin web.
From the standard calibrated curve (Time versus mg/dl) the fibrinogen concentration corresponding to the recorded time (seconds) was determined.

PHARMACOKINETIC STUDIES OF THROMBINASE

The following methods were designed to study the pharmacokinetic properties of the drug,

1. **Half-life of Thrombinase** (based on the measurement of its thrombolytic activity) — *which is essential to the determine the effective dose of the drug administration*

2. **Plasma clearance of $^{125}$I labeled Thrombinase** - *which is essential to decide the dose and duration of the drug administration*

3. **Organ distribution of $^{125}$I labeled Thrombinase** - *To understand the presence of drug in the body after it disappears from the plasma. This gives an insight into the distribution pattern of the drug*

4. **Excretion study of $^{125}$I labeled Thrombinase** — *To know the mode of excretion, the bile, fecal matter and urine were collected and observed for the radioactivity*

HALF-LIFE STUDY OF THROMBINASE (BASED ON THROMBOLYTIC ACTIVITY)

In this study, half-life of Thrombinase based on thrombolytic activity was evaluated in rats and rabbits after a bolus injection of Thrombinase in the
circulation. Half life is the time when the thrombolytic activity of the drug becomes half its initial activity. (Rodney, 2003)

**Method adapted for rats (n=12)**

Rats were anaesthetized with Thiopentone sodium (40mg / Kg body wt/ i.p). A small incision was made in the ventral aspect of neck and both the right and left jugular veins were exposed. A sample of blood was collected from each animal from jugular vein before the administration of Thrombinase. Following this, a bolus injection of Thrombinase (2, 70, 000 Thrombinase Units (TU) /270μg of protein in 300 μl of saline) was given into the right jugular vein. The blood samples were then collected from left jugular vein at the interval of 5, 10, 20, 30, 50 and 60 minutes in 3.2% sodium citrate solution. In order to avoid blood volume depletion induced changes, three samples were taken for each animal.

**Method adopted for rabbits (n=6)**

The half-life may vary with species and animal size. Hence, the study was repeated in rabbits. Rabbits were anaesthetized with Ketamine (Themis chemicals Ltd, India) (50mg / Kg of body wt/ I.M). A sample of blood was collected from each animal from the right ear lobe vein before the administration of Thrombinase. Followed by this, each animal was injected (I.V) with Thrombinase [17, 41, 392 T.U /1.74mg of protein dissolved in 1 ml of saline] in left ear lobe vein. Blood samples were then collected from right ear lobe vein in vials containing sodium citrate [3.2%] at the interval of 5, 10, 20, 30, 40, 50 and 60 minutes.
The blood samples obtained from rat and rabbits were centrifuged and plasma was separated. The obtained plasma was tested for Thrombinase activity by using blood clot assay (Toombs, 2001) as described earlier in in vitro studies.

**LABELING OF THROMBINASE**

Labeling of Thrombinase is essential, to study its plasma elimination, organ distribution and excretion. Elimination and distribution studies give idea about the fate of the enzyme and the preference of organ uptake. Radioactive iodine is the most readily available and convenient label for proteins. The substitution of 5-10 atoms of iodine in the molecule of protein takes place predominantly in the tyrosine ring and is associated with little or no alteration of the physical properties of the proteins.

**Lactoperoxidase method**

**Principle**

Thrombinase was labeled with $^{125}\text{I}$ by lactoperoxidase method according to Summaria *et al.*, (1977). Peroxidases have the ability to oxidize iodide. Enzymatic radiiodination of peptides using lactoperoxidase is a gentle, simple and rapid method. Unlike the strong oxidation agent chloramine - T, oxidation by lactoperoxidase is limited to iodide only and enzyme specificity prevents degradation of proteins leading to minimal loss of biological activity. Exposure of the protein and $^{125}\text{I}$ for defined period to oxidizing agents lactoperoxidase and hydrogen peroxidase (H$_2$O$_2$) facilitates
the incorporation of $^{125}\text{i}$ atoms into one or more tyrosine residues in the peptide.

**Reagents**

All the reagents used in the entire study were prepared with double distilled water (H$_2$O) only, until otherwise specified.

1. $^{125}\text{i}$ carrier free sodium iodide with radioactivity of 0.5 mCi/5μl

2. PBS (0.05M) (pH 7.4)

Sodium dihydrogen phosphate - 710 mg (Solution A) in 50 ml and disodium hydrogen phosphate - 600mg in 50ml (Solution B).

50 ml of solution A and 20 ml of solution B were mixed together. With this, 900mg of sodium chloride was added and pH was adjusted to 7.4. Then the solution was made up to 100ml with double distilled water.

3. Sodium acetate buffer (0.1 M) (pH 5.6):

82 mg of anhydrous sodium acetate was dissolved in 9 ml distilled water, pH adjusted to 5.6 and the solution was made up to 10ml with distilled water.

4. Hydrogen peroxide (H$_2$O$_2$): 1μl of the H$_2$O$_2$ was diluted in 0.9 ml distilled water to prepare the 1:10,000 dilution of 30% solution. Prepared just before use.

5. Lactoperoxidase (Sigma Chemical co, USA): 4μg of lactoperoxidase in 4μl of PBS (0.05M) (pH 7.4). This was prepared just before use.
6. Thrombinase: 25μg/5μl of 0.05 M PBS (pH 7.4).

7. Sodium metabisulphate solution: 24 mg Sodium metabisulphate in 5ml of 0.05M PBS (pH 7.4)

8. Potassium Iodide and sucrose solution: 100mg of potassium iodide and 800 mg of sucrose in 10ml of 0.05 M PBS (pH 7.4)

**Preparation of Sephadex PD 10 column**

Pre packed sephadex PD 10 column (Amersham pharmacia) was equilibrated with 25ml of 0.05M PBS (pH 7.4)

**Iodination procedure:** Iodination was performed at room temperature (about 22°C). The following steps were performed in sequence.

1. 5 μl of carrier free of $^{125}$I (0.5mCi) was taken in a reaction vial.

2. 25 μgm of Thrombinase in 5μl of 0.05M PBS (pH 7.4) and 20μl sodium acetate buffer was added to the reaction tube and mixed gently.

3. The iodination reaction was initiated by adding 4μg/ 4μl of lactoperoxidase and 5 μl of H$_2$O$_2$. (1:10000 dilution). The contents were mixed gently and incubated for 60 seconds.

4. At the end of incubation, the reaction was arrested by addition of 50μl of sodium meta bisulphate, 100μl of potassium iodide and sucrose solution and mixed well.
5. The reaction mixture was immediately transferred to equilibrated sephadex PD 10 column to remove unbound iodide and eluted. The elutant (fraction) was collected in 30 micro centrifuge tubes (10 drops of elutant in each tube).

10μl of elutant from each collected fraction was used to measure the radioactivity, in LKB microprocessor based gamma counter for 60 seconds. The radioactivity of the fractions when plotted in a graph paper, two peaks were detected (Figure - V). The first peak corresponds to protein peak and the second to salt peak. The fraction corresponding to the protein peak was taken for animal studies as it contained least damaged labeled protein. The residual radioactivity in the iodine vial and reaction vial was counted and taken as the residual count. The specific activity of the labeled protein was also calculated.

ELECTROPHORESIS AND RADIO AUTOGRAPHY OF IODINATED PROTEIN

In analytical studies, the incorporation of radioactive iodide into the protein was established by subjecting them to SDS-PAGE electrophoresis and staining with coomasie blue. Then the strips were exposed to radio autography.

Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Laemmli System (1970) is one of the widely used techniques for the analysis of proteins based on their molecular weights with good
Figure - V Radioiodination of Thrombinase (Lactoperoxidase method)
resolution. This system is a discontinuous SDS system and consists of a relatively higher molarity tris- HCl buffer pH 8.8 in the gel and a low molarity tris -glycine buffer pH 8.3 in the electrode compartments. Resolution is increased considerably by a stacking gel containing a pH 6.8 low molarity tris -HCl buffer.

**Principle**

Proteins in the sample rapidly migrate into the stacking gel (negatively charged at pH 8.3 where the sieving effect of the stacking gel is minimal), and concentrate into a narrow band, as the neutral stacking gel buffer reduces their electrophoretic mobility. The concentrated band of protein migrates through the stacking gel and enters the pH 8.8 separating gel. Once again the proteins become more highly charged and they separate according to their charge as they migrate through the gel. By comparing the electrophoretic mobility of a given protein with the mobility of appropriate standard proteins, its molecular weight can be determined. In general, the slab gel system is preferred because it allows multiple samples with more precise alignment of different samples.

**Materials**

1. **Slab gel electrophoretic apparatus** – complete system (Bio tech, India)
2. **Acrylamide stock** – (40%) (100ml)

Acrylamide - 40gm, N, N methylene bisacrylamide - 1.066 gm were dissolved in 60 ml of double distilled water and its volume was adjusted to
100 ml. Mixed bed resin (1%) was added and left in shaker for 1 h. This was filtered over Whatman No.1 filter paper and stored at 4 °C in a brown bottle.

3. Sodium Dodecyl Sulphate (SDS) -10% (100x)

10 gm of SDS was dissolved in 100 ml of double distilled water. The solution should be clear and colourless.

4. Stacking gel buffer (4x) (pH 6.8) (0.5M)

Tris - 6.0 gm was dissolved in 40 ml double distilled water, titrated to pH 6.8 with 1M HCl and the final volume was adjusted to 100 ml with distilled water. The solution is filtered through Whatman No.1 filter paper and stored at 4°C.

5. Separating gel buffer (8x) (pH 8.8) (3M)

Tris -36.3 gm was dissolved 48 ml of distilled water, the pH was adjusted to 8.8 with to 1M HCl. The final volume made up to 100 ml. The solution is filtered through Whatman No.1 filter paper and stored at 4°C.

6. SDS Sample buffer (4X) (10 ml)

Glycerol (40%) 4.0 ml
Stacking gel buffer (4x) 4.0ml
10% SDS stock (100x) 2.0ml
Bromophenol blue (40mg/ml) 100μl (Aqueous as tracking dye)
Mercaptoethanol 400μl
Distilled water 600μl
7. **Running buffer (1000ml) (pH 8.3)**

Tris -3.02 gm, Glycine -14.40 gm and SDS -1 gm were dissolved in distilled water and volume was made up to 1 liter. The solution is stored at 4°C.

8. **Ammonium persulphate solution (75 mg/ ml) (100x)**

0.75gm-ammonium persulphate was dissolved in 1ml of double distilled water. Prepared freshly just before use.

9. **Overlay solution (10ml)**

5ml Butanol in 5ml Distilled water

10. **Coomassie brilliant blue staining reagent**

200 gm of coomassie brilliant blue R-250 was dissolved in 45 ml of methanol, and then 10 ml of glacial acetic acid was added to it. The volume was made up to 100 ml with distilled water.

11. **Destaining solution**

45 ml of methanol and 10 ml of glacial acetic acid were mixed and the volume was adjusted to 100ml with distilled water.

**Method**

1. The glass plates were cleaned thoroughly with detergent, distilled water and ethanol.
1. Two glass plates were placed together, with the 3 spacers between them (along the side edges and along the bottom edge and the whole assembly was fixed tightly with clamps).

2. The two side edges and the bottom edge were sealed with 2% agar and the assembly is kept vertically on a flat surface.

3. The separating gel 10% (for 10ml) was prepared by mixing

Separating gel buffer - 1.25 ml  
Acrylamide (40%) - 2.5 ml  
Ammonium Persulphate - 100 μl  
10% SDS - 100μl  
Double distilled water - 6.05 ml

4. The solutions were mixed in a 100ml conical flask and deaerated. Then, TEMED 8 μl was added, mixed, and poured immediately between the glass plates, up to about 1.5 cm below the notch.

5. The gel was overlaid with overlaying solution in order to achieve an even surface. The polymerization was complete in 30-60 minutes at room temperature.

6. The upper water layer and unpolymerized gel were poured out. The upper layer of gel was rinsed with water twice to ensure the removal of unpolymerized gel.
8. Stacking gel was prepared as below

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel buffer</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Acrylamide (40%)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>50μl</td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>50μl</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>3.15 ml</td>
</tr>
<tr>
<td>Temed</td>
<td>4 μl</td>
</tr>
</tbody>
</table>

The solution was deaerated before adding TEMED.

10. The stacking gel was poured and a 5 well comb was inserted into the stacking gel. Care was taken to prevent trapping of air bubbles.

11. The stacking gel was allowed to polymerize for 15 to 20 minutes.

12. The comb was carefully removed by sliding vertically upwards.

13. The slots were rinsed with distilled water 3 to 4 times to washout the unpolymerized gel.

14. The clamps and the bottom spacer were removed and the bottom edge was cleaned with a tissue paper. The gel assembly was fixed to the electrophoresis chamber with the help of clamps. Any possible leakage of buffer from the upper chamber was prevented.

15. The upper and lower chambers were filled with running buffer. The air bubbles trapped at the bottom edge of the gel between the glass plates were removed using a syringe with a bent needle.
16. The samples (5 to 50 µl) and molecular weight standards (10µl) were dispensed in the slots with the help of a micro syringe or micropipette.

17. The electrodes were connected to the power supply and electrophoresis was run at a constant voltage of 150 V till the sample reached separating gel. The current was then increased from 15 to 25 mA.

18. The electrophoresis was run till the tracking dye reached the end of the separating gel. After completion of the run, power supply was disconnected. The gel assembly was removed from the electrophoresis chamber. The side spacers and the notched glass plate were removed with the help of a spatula.

19. The gel was stained with coomassie blue staining reagent.

**Staining Method**

The gel was placed in rectangle glass tray with Coomassie blue staining reagent till the gel was stained (20 to 30 minutes). After staining, gel was placed into destaining solution for about 1 hr. The destaining solution was changed frequently.

The gel was preserved by following method: A glass plate was washed thoroughly and a wet glassy paper was spread over the glass plate above which the gel was placed. Again a wet glassy paper was spread over the gel without entrapping air bubbles and folded to the side of the glass plate. The gel was allowed to dry for a day and preserved.
SDS-PAGE OF $^{125}$I LABELED THROMBINASE

Lane 1 - Medium molecular weight marker
Lane 2 - Unlabelled Thrombinase
Lane 3 - $^{125}$I labeled Thrombinase (Fraction 9)
Lane 4 - Fraction 10
Lane 5 - Free iodine (Fraction 21)

RADIOAUTOGRAPHY OF $^{125}$I LABELED THROMBINASE SDS-PAGE

Lane 1 - Medium molecular weight marker
Lane 2 - Unlabeled Thrombinase
Lane 3 - $^{125}$I labeled Thrombinase (fraction 9)
Lane 4 - fraction 10
Lane 5 - Free iodine (fraction 21)
Radioautography

Association of iodine label with specific protein after electrophoresis was demonstrated by using radioautography as described by Moran et al. (1984). The strips were subjected to radioautography against X-ray film. The exposure time was varied from 7 to 10 days. A direct relation between the position of stained protein bands and radioactive induced bands was observed.

Once the radiolabeling of Thrombinase was confirmed, $^{125}\text{I}$ labeled Thrombinase was used to study the pharmacokinetic parameters of Thrombinase.

In all experiments involving $^{125}\text{I}$ labeled Thrombinase, thyroidal uptake of radioiodide ($^{125}\text{I}$ labeled Thrombinase) was blocked by intravenous administration of potassium iodide (0.5 ml of 2% solution) just before the experiment. The radioactivity was measured in LKB microprocessor based gamma counter for 60 seconds.

**PLASMA CLEARANCE OF $^{125}\text{I}$ LABELED THROMBINASE IN RABBITS ($n=4$)**

Animals were anaesthetized with Ketamine (50mg /Kg of b.wt / IM). Followed by this, $^{125}\text{I}$ labeled Thrombinase with a known radioactivity (23, 42, 930 cpm / 228 pico gm$^{-1}$) was administered as bolus in the marginal vein of the ear lobe. To measure the plasma concentration of $^{125}\text{I}$ labeled Thrombinase, blood samples were collected (3.2% sodium citrate at 1:9 dilution) in the following time points 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80,
90, 105, 120, 150, 180, 210 and 240 minutes from the contralateral ear lobe vein. Each time 500 µl of blood was withdrawn to prevent significant changes in blood volume. Then, the blood samples were centrifuged at 4°C for 15 minutes at the speed of 6000 rpm. 300 µl of plasma was used to measure the radioactivity in each sample. The radioactivity for one ml of blood was calculated. The plasma concentration was presented as specific activity of $^{125}$I labeled Thrombinase which was injected (Aoki et al., 2001).

**ORGAN DISTRIBUTION OF $^{125}$I LABELED THROMBINASE**

In this study, the distribution of radioactivity in the tissues of rats and rabbits were examined after a single IV. administration of $^{125}$I labeled Thrombinase at 10, 30 and 90 minute time points.

**Method adapted for rat**

Six animals were used for each 10, 30 and 90 minutes duration. Rats were anaesthetized with ether and a small incision was made in ventral aspect of the neck, both the jugular veins were exposed. 0.5 ml of testing solution containing 3, 50,000 - 4, 00,000 cpm of $^{125}$I labeled Thrombinase (10 µl $^{125}$I labeled Thrombinase + 490 µl Saline) was administered through jugular vein in all the animals. Animals were sacrificed at each time point by giving excess dose of anesthetic ether (Oikawa et al., 2000).

**Method adapted for rabbit**

Three animals were used for this study in 10, 30 and 90 minutes time points. 1 ml of testing solution containing 13, 58, 028 cpm (35 µl $^{125}$I labeled
Thrombinase + 965 μl Saline) was administered through ear lobe vein of all the animals. Animals were sacrificed at each time point by giving excess dose of anesthetic ether (Lijnen et al., 1991).

To determine tissue distribution of $^{125}$I labeled Thrombinase in rats the different tissues including blood, brain, heart, lung, liver, spleen, and kidney were removed at 10 or 30 or 90 minutes after the dosing. Similarly in rabbits, different tissues including blood, brain, heart, lung, liver, gallbladder, spleen, kidney, urine were removed at 10 or 30 or 90 minutes after the dosing. Whole organ wet weight was measured and organ with a known weight was taken. PBS was added (volume of 1ml/gm tissue taken) and homogenized. Radioactivity in 500μl of homogenized solution was measured. The radioactivity of whole organ was expressed in % radioactivity.

EXCRETION STUDY OF $^{125}$I LABELED THROMBINASE IN RABBITS (n=3) .

**Materials:** Rabbit metabolic cage (Vishnu scientific, India), sterile siliconized conical flask to collect urine and feces sample

**Procedure:** $^{125}$I labeled Thrombinase with 11, 94, 685 cpm/ml was added to saline and administered intravenously to each animal. After the intravenous administration of $^{125}$I labeled Thrombinase the radioactivity excreted in the urine and feces were measured. The animals were housed in metabolic cage two days before the experiment and accustomed to access water and food. For the excretion study,
$^{125}$I labeled Thrombinase was administered in ear lobe vein of rabbit. Animals were restrained back into a metabolic cage and they excreted urine and feces freely. $^{125}$I labeled Thrombinase activity in the urine and feces samples was measured at 6, 12, 24 hours on the day of injection and every 24 hrs for 7 days (to monitor the complete elimination of the drug). The radioactivity for 500μl urine was measured for each time point. Since the total urine output for that duration is also known, the radioactivity of the whole volume of the sample was calculated. It is expressed as percentage of radioactivity considering the injected amount as 100%. The feces sample was weighed and homogenized with saline. 500μl volume of homogenate feces was measured for its radioactivity (Aoki et al., 2001). On the seventh day the animals were sacrificed and organs were examined for radioactivity.

**ECG PATTERN OF RATS FOLLOWED BY INTRAVENOUS BOLUS ADMINISTRATION OF THROMBINASE (n=6)**

This study was designed to find whether the intravenous administration of Thrombinase, a thrombolytic enzyme, does have any effect on the rat myocardium.

**Procedure**

ECG recording in rats was done according to the procedure described by Sreepriya et al (1998) using needle electrode inserted subcutaneously in the four limbs of the ether-anesthetized animal fixed to the board. ECG was recorded in standard Lead II using Cardiart 108T, BPL India instrument with a sensitivity of 1 cm deflection /mV and 50 mm / second as paper speed. All
the animals received Thrombinase (1mg / Kg b. wt) intravenously. ECG was recorded at 30 minutes before the administration of Thrombinase, 10 minutes, 30 minutes and 2hrs after the administration of Thrombinase.

The following parameters were calculated from the ECG recordings: Heart rate [HR], PR and QT intervals, QRS duration and amplitude of R wave. These parameters can reveal the rate, rhythm and conduction problem that may be encountered after the drug administration.

PYROGEN TEST FOR THROMBINASE IN RABBITS (n=3)

The test involves the measurement of the rise in body temperature of rabbits following the intravenous injection of sterile solution of the substance being examined, to evaluate the presence of substances classified as pyrogens. The pyrogen can act at the thermoregulatory centers which are acting as thermostat in the biological system. The procedure adapted was as given in Indian Pharmacopoeia (1996).

Recording of Temperature

Materials

All glasswares were washed and sterilized in hot air oven at 200°C for 1 hour. All the diluents, syringes and solutions for washing and rinsing of devices were kept sterile and pyrogen -free. The temperature was measured with Biopack (an electronic device with probe) (Biomonitor, India).
Procedure

Rabbits were restrained in restraining boxes with loose-fitting neck stocks and rest of their body remained relatively free. The animals were able to sit comfortably in restraining box in normal position during the experimental procedure. Animals were withheld for food and water for over night and until the test was completed. A temperature-sensing probe was inserted into the rectum of test rabbit to a depth of 5cm to record its temperature. The device was calibrated to an accuracy of 0.1°C and reading was obtained within 5 minutes. The depth of insertion of probe was maintained constant in all the tests. The temperature was recorded in the animals 90 minutes before the injection of test substance and every 30 minutes for 3 hours after the injection of the test substance.

Temperature of each rabbit was recorded, at an interval of 30 minutes for one and half hour before the administration of Thrombinase. The average of the readings was taken as the Mean initial temperature of the individual.

One mg powder of Thrombinase was dissolved in 1ml of pyrogen free saline. Test substance, Thrombinase was slowly administered through the marginal vein of the ear lobe of each rabbit over a period not exceeding 4 minutes. Following the injection, temperature of the animal was recorded every 30 minutes for three hours. The mean of temperature recorded in 3 hours after the administration of Thrombinase is called as mean maximum temperature.
The temperature raise in individual rabbit, i.e., the difference between mean initial temperature and mean maximum temperature was noted.

The test was conducted in three animals. The mean of the temperature raise of the three individual rabbits was calculated as **summed response**.

**INTRADERMAL SKIN TEST REACTIVITY TO THROMBINASE IN RABBITS (n =4)**

Skin tests are usually employed to assess the state of hypersensitivity. Thrombinase as a protein, when infused or injected as bolus in vivo, may induce hypersensitivity reaction. To rule out this, the intradermal skin test was conducted in rabbits.

**Method**

Rabbits were restrained by using restraining cage. Hair on the back of lateral aspect was shaved carefully at three different sites. 50μl saline was injected intradermally at one site, 50μl of histamine (1mM) at another site and 50μl of Thrombinase (50μgm) was injected in the third site using a syringe with 27-gauge needle. Skin reactions were evaluated at 15 and 30 minutes. 15 minutes after the injection, the diameter of wheal at each site was measured in centimeters. Each wheal was subjectively graded from 0 to +4 on the basis of size, erythema and turgidity. Reactions were noted for saline, histamine and drug injected sites. The presence or absence of intradermal reactivity was compared in all the animals with reference to saline, histamine and Thrombinase (Marsella, 2000).