Discussion
EFFICACY STUDIES OF THROMBINASE

In view of potential limitations of existing thrombolytic agents, alternative intervention having potent thrombolytic activity on their own or synergistically with plasminogen activators need to be evaluated. Most of thrombolytic drugs apart from their thrombolytic action, act on circulating fibrinogen and other clotting factors which lead to a haemorrhagic state. Hence, the thrombolytic efficacy of Thrombinase and its effect on coagulation mechanism, as a new thrombolytic drug was tested in rabbit and dog thrombosis models. The results of this study are the first fully documented in vivo thrombolytic potential of Thrombinase.

IN VITRO EFFICACY OF THROMBINASE

It is essential for any drug, to be tested for its in vitro activity, before evaluating its in vivo efficacy. In vitro studies showed that increase in Thrombinase concentration increases thrombolytic activity. Thus by adjusting the concentration of Thrombinase depending upon the clot location and size in in vivo conditions, an expected rapid lysis of the clot can be achieved. According to Linjen et al (1991) systemic infusion of staphylokinase resulted in $27 \pm 1\%$ of clot lysis with a dose of $0.016 \text{ mg/ Kg b.w}$ and $91 \pm 3 \%$ clot lysis with $0.25 \text{ mgm / Kg b.w}$. Streptokinase causes $18 \pm 5 \%$ of clot lysis with $0.032 \text{ mgm/Kg b.w}$ and $69 \pm 7\%$ with $0.5 \text{ mgm/ Kg b.w}$. Hence Thrombinase can be used in different dosages according to the requirement.
Coagulation is a sequence of reaction by which fibrinogen is converted to fibrin by the enzyme thrombin. The fibrin gel is then converted to stable clot by factor XIII. The fibrin network is acted by the thrombolytic enzymes, to release the cross linked fibrin degradation products (XL FDP). The cross linked fibrin degradation products include D dimer, D dimer E and high molecular weight derivatives. D dimer comprising of two D fragments cross linked together, is the smallest plasmin resistant molecular unit present within the XL FDP. The reagent used in this experiment will be able to detect all forms of the fibrin degradation products.

In vitro experiment with Thrombinase and streptokinase in human clot showed agglutination with FDP antibodies and there by confirming the presence of fibrin degradation products. This indicates, like streptokinase which is a known thrombolytic drug, Thrombinase also acts over the fibrin in the thrombus.

IN VIVO EFFICACY OF THROMBINASE

Thrombinase was tested for its in vivo efficacy by local injection, immediately proximal to the occlusive platelet rich arterial and venous thrombus in dog model. This study, although limited to a local application of Thrombinase, demonstrates that the local administration of the enzyme lyses a carotid arterial thrombus rapidly and with no observable untoward systemic effects. The local application Thrombinase has certain limitations, hence this drug, Thrombinase was administered systemically to understand its efficacy in rabbit thrombosis model.
Several different animal models have been described for in vivo evaluation of thrombolytic drugs. The utility of animal model lies with ability to predict or reflect the human experience. To investigate the thrombolytic efficacy of Thrombinase, a simple classical rabbit Jugular vein thrombosis model described by Collen et al (1983) was used. The advantage of this model is, it has a minimal surgical intervention with reproducible results. In this study, a standard drug infusion time of 1 hour was maintained. It is helpful to complete the experiment in time and prevents the animal to be kept under prolonged anesthesia. The animal under prolonged anaesthesia may affect the vital parameters like oxygenation, blood pH, respiration and probably this may interfere with thrombolytic efficacy of a drug (Collen et al, 1983). The same type of animal model was used for bolus and slow infusion of Thrombinase study, to find the effective mode of drug administration.

Effective dose of Thrombinase

When Thrombinase was infused systemically, the base line (100%) blood flow was established in $10.7 \pm 0.29$ minutes. It required only $0.21 \pm 0.01$ mg Thrombinase/Kg of body weight. The rapid thrombolytic efficacy of Thrombinase was agreeable with other thrombolytic drugs reported like, Streptokinase and Staphylokinase (Linjen et al, 1991).

Based on this, a range of bolus dose of Thrombinase at 0.1, 0.2, 0.5 and $0.9$ mg/Kg of body weight was tried in individual animals. In this study, the effective dose was found to be $0.23$ mg/Kg of body weight for bolus administration of Thrombinase.
As indicated by the results that the bolus injection of Thrombinase (0.23 mg/Kg) initiates the blood flow of the occluded vessels earlier than slow infusion of Thrombinase. The reperfusion of blood vessels (20% of the basal blood flow) occurred at 8 ± 0.34 minutes for bolus injection and for slow infusion it was at 9 ± 0.01 minutes. When we consider the complete (100% of basal blood flow) clot lysing capacity, the slow infusion is better than bolus administration and it was supported by the observation, as continuous infusion has an advantage by re-establishing the complete base line blood flow within 11 minutes. The bolus injection of Thrombinase requires 16 minutes to restore a complete base line blood flow. This is because, during bolus injection the concentration of drug at a given time, at the site of clot induced may be high. The increased concentration of the drug at the site of clot favours an effective clot lysis and reperfusion (20% of basal blood flow) of the occluded blood vessel. The increased concentration of Thrombinase in circulation might have been removed or inactivated by its inactivator as time progresses. This could be the reason for its delay in re-establishing the complete (100%) blood flow patency. Though in continuous infusion also Thrombinase may be removed or inactivated by its inactivator as time progresses, there must be a reasonable concentration of drug circulating in the blood at a given instant. This would be helpful in early regaining of complete blood flow (100%) in occluded vessel. However, the significant difference in the effectiveness of the drug between bolus injection and slow infusion was observed.

The result outcomes of Thrombinase with bolus administration and slow infusion was agreeable to results of t-PA. Clozel et al (1989) studied the time
course of thrombolysis induced by intravenous bolus injection or infusion of tissue plasminogen activator in rabbit jugular vein thrombosis model. They observed decreased rate of lysis as time progresses after a bolus injection and correlated this decrease in activity due to the decreased concentration of the drug in the plasma (Mattsson et al, 1983 and Beebe and Aronson, 1986). In contrast during the slow infusion, the plasma concentration of t-PA was stable because the new t-PA was continuously administered. All these observation clearly indicates that thrombolytic action of the drug depends on the its dose and mode of administration (bolus or slow infusion). The thrombolytic efficacy study of Thrombinase suggested that it could be administered as bolus in the beginning of the therapy and could be followed by a slow infusion.

**Thrombolytic efficacy on arterial thrombosis**

The dog has been widely used by earlier workers for the study of thrombosis and thrombolysis, it is believed to give results more applicable to human than does the rabbit particularly with reference to plasminogen activators as fibrinolytic agents (Dupe et al., 1984). However the use of urokinase which is active in cat, man and dog (Duckert, 1978) is poorly active in rabbit while the streptokinase is highly active in rabbit. Hence, Thrombinase, being a new thrombolytic drug, it is essential to evaluate the action of this drug in different animal models. Markland et al (1994) and Collen et al (1993) suggested that the study in carotid artery is better than coronary artery, as it has the advantage of greater yield of experimental data and also allows the animal to avail its own internal control, thereby facilitating the better interpretation of results.
Arterial thrombi are formed as platelet aggregates and are then transformed into fibrin masses in the course of hours or days. The platelet component of mixed thrombi is more resistant to dissolution than fibrin (Jorgensen et al, 1971). In the present study comparative thrombolytic efficiency of Thrombinase was tested along with Streptokinase towards the venous whole blood clot and the platelet rich arterial thrombus model in dogs.

When the efficacy of Thrombinase and Streptokinase in jugular vein thrombosis model of dogs was compared, there was no significant difference in time and drug required for clot lysis. The time required for clot lysis was (9.25 ± 0.75 minutes) in Thrombinase and Streptokinase (9.50 ± 0.87 minutes). It is almost similar. The quantity of drug required for the complete dissolution of clot was also found to be similar.

When the efficacy of Thrombinase and Streptokinase in dog's carotid artery thrombosis model was compared, Thrombinase requirement was significantly lower, compared to Streptokinase. Thrombinase also required significantly shorter reperfusion time (11.00 ± 0.14 minutes), compared to Streptokinase (17.75 ± 0.85 minutes).

When the efficacy of Thrombinase in arterial and venous thrombosis were compared, the time and dose required for clot lysis showed no significant difference. These results indicating again that Thrombinase, can be a drug of choice irrespective of the type of clot whether it is in artery or in the vein.

These results are agreeable with the results of effectiveness of APSAC (Anisoylated Plasminogen Streptokinase Activator Complex) and Recombinant
Fibrolase in dog carotid artery thrombosis model (Markland et al., 1994). In this study, the infusion of APSAC alone immediately proximal to the occlusive thrombus, lysed the clot within shorter duration (a mean time of $17 \pm 3$ minutes). Despite the efficacy of APSAC in achieving thrombolysis, reocclusion of the carotid artery occurred in all animals within an additional $40 \pm 5$ minutes. The present preliminary study of Thrombinase in dogs indicates that Thrombinase initiates only the rapid thrombolysis and there is no reocclusion of the injured arterial endothelium within the observation period (4 hours). Thus, Thrombinase act as an effective clot lytic agent and also prevents further reocclusion of the same vessel.

Currently, the main clinical use of available thrombolytic drugs is for acute myocardial infarction. Acute myocardial infarction is due to the appearance of thrombus inside the coronary artery. Many clinical studies shown that the delay of reopening of coronary arteries after myocardial infarction is an important factor for further clinical outcomes (Laffel and Braunwald, 1984). Therefore, the initial rate of thrombolysis might be a very important factor for the success of thrombolytic treatment. The present study showed not only the thrombolytic efficiency of Thrombinase but also indicates the early reperfusion capability in arterial thrombus.

EFFECT OF THROMBINASE ON COAGULATION AND HAEMATOLOGICAL PROFILE

The ultimate objective of thrombolytic therapy is to digest a fibrin thrombus without inducing generalized proteolytic state resulting in the
digestion of fibrinogen. According to Linjen and Collen (1991) UK, SK and APSAC activate circulating plasminogen as well as fibrin bound plasminogen within the thrombus. This leads to widespread systemic activation of fibrinolytic system to generation of free plasmin that degrades several plasma proteins including fibrinogen and factors V and VIII. This results in the haemorrhage. Larsen et al (1991) who evaluated the fibrinolytic and haemostatic effect of variant forms of t-PA after a bolus intravenous injection in rabbit jugular vein thrombosis model. In their study, at dose of 0.5 mg/Kg variant forms of t-PA agents produced a significant decrease in fibrinogen level, indicating some systemic activation. The depletion of fibrinogen was significantly great in variant forms of t-PA than normal t-PA (Activase) which was attributed to the slow plasma clearance of variant forms of t-PA. Similarly, rt-PA, activates the fibrinolytic system and is effective in restoring blood flow in occluded arteries and veins was associated with bleeding complication (Abel,1992). This may not be true with all thrombolytic drugs.

According to the results, the slow infusion and bolus injection of Thrombinase in rabbits could induce thrombolysis without causing peripheral consumption of coagulation factors. There was no significant change in fibrinogen level, in 20 minutes after the slow infusion and 240 minutes after the bolus administration of Thrombinase. These indicate that Thrombinase may not be acting like plasminogen activators where the fibrinogen is one of the substrate. There was no significant change in PT, APTT and coagulation time in the given dosage.
It is agreeable with results observed by Clozel et al (1989) who reported that followed by a bolus administration of t-PA at dose of 0.05 mg to 0.4 mg/Kg/min, plasma fibrinogen and plasminogen levels did not change significantly up to 240 minutes. Only at the highest dose (1mg/Kg) degradation of fibrinogen was significant with a decrease of 26% at 120 minutes and 31% at 240 minutes. Nowark and Gurewich showed (1974) that significant fibrinogenolysis did not occur even at the highest doses of SK and demonstrated the remarkable specificity of SK towards rabbit fibrin.

In conclusion, the coagulation status of the animal was unaffected by the systemic administration of Thrombinase. There was no change in fibrinogen, PT, APTT and coagulation time indicating that the Thrombinase appear to be very specific about its substrate as fibrin alone. Thus, fibrin specificity of Thrombinase can be considered to be of great importance in the development of effective and safe thrombolytic agent. The observation on Thrombinase has added advantage that it did not initiate any bleeding complication by degrading the fibrinogen and other clotting factors.

Moreover, slow infusion and bolus administration of Thrombinase in rabbit did not change circulating number of RBC, WBC and platelets. The haemoglobin and haematocrit values also remain constant during observation period. No evidence of haemorrhage or alteration to the haemostatic system were observed in these studies. These results demonstrate the favorable therapeutic potential of Thrombinase.
EFFECT OF THROMBINASE ON PHYSIOLOGICAL PARAMETERS

Markland et al, (1994) when administered fibrolase, at the end of the experiment, mean arterial pressure decreased below that of initial control value. Hence, it become unavoidable to measure these parameters, when Thrombinase was infused in vivo. No changes in blood pressure, respiratory rate and rectal temperature in dogs followed by Thrombinase infusion was observed. No alteration in functional parameters is an added advantage to this new thrombolytic enzyme, Thrombinase.

PHARMACOKINETIC STUDIES OF THROMBINASE

Pharmacokinetics is used to describe the study of the absorption, distribution, metabolism and excretion of the drugs and their relations, in other words “what the body does to the drug” (Hardman and Limbird, 2001a). As Thrombinase has to be administered systemically for its therapeutics, it is essential to understand the fate of this drug, half-life, plasma clearance, organ distribution and its excretion.

HALF - LIFE STUDY OF THROMBINASE IN RATS AND RABBITS (BASED ON THROMBOLYTIC ACTIVITY)

In vivo administration of the enzyme activity is subjected to inactivation either by the action of proteolytic enzymes in the circulation or removal of the peptide by organs like liver spleen etc. Hence, half-life based on biological activity is essential to evaluate the duration of efficacy of the drug in circulation and there by determining the dosage of the drug during therapeutics. In a
biological system, the time taken by a drug to become half in its initial biological activity gives the half-life, based on thrombolytic activity. The thrombolytic activity of Thrombinase was determined by Blood clot assay method (Toombs, 2001). The half life of Thrombinase based on its thrombolytic activity showed difference between rabbit (14 minutes) and rats (24 minutes). The variation in half-life of Thrombinase may be due to the species variation and the total blood volume involved.

The half-life of Thrombinase is in agreement with the half-life of the other available thrombolytic agents like streptokinase and urokinase, which shows similar half-life of 18 minutes and 14 minutes respectively. The drugs like rt-PA has only 2-6min as half-life and pro-UK 8-10 min (Bell 2002). Some of the new thrombolytic agents have shown promise in animal models of venous or arterial thrombosis and shown prolonged half-life when modified slightly without alteration in their biological activity. Monteplase, a modified tissue type-plasminogen activator (t-PA), has a prolonged half-life (based on biological activity) of more than 20 min, as compared to 4 min for native t-PA (Verstraete, 1999).

PLASMA CLEARANCE OF $^{125}$I LABELED THROMBINASE

Clearance is one of the most important concepts, to be considered when a rational regimen for long term or short-term administration of drug to be designed. It is extremely useful in clinical pharmacokinetics because clearance of given drug is usually constant over the range of concentrations encountered clinically. Clearance is expressed as the ml of plasma cleared of the substance
The plasma clearance rate of Thrombinase was determined by measuring plasma radioactivity in rabbits following a bolus intravenous administration.

The analysis pharmacokinetic parameters of Thrombinase demonstrated that Thrombinase had a slow rate of plasma clearance (0.03 ml/min) from the circulation. This rate of clearance was slower than other available thrombolytic drugs. The clearance rate of \(^{125}\)I t-PA was reported to be 3.2 ml/min, (Borger et al, 2000). The plasma clearance of Wild type t-PA was 44.5 ml/min, and 17.1 ml/min was for Gln 17 t-PA. The plasma clearance study of streptokinase and staphylokinase showed to have rapid plasma clearance in rabbits. The clearance was 14 and 15 ml/minute (Linjen et al, 1991). The drug molecules spend different times within the body as not all of them cleared simultaneously. The mean residence time is the average time taken by individual drug molecule before elimination (Venkateswarlu, 2004). The mean residence time was 22.9/ min for Gln 17 t-PA and 4.8 minutes for WT t-PA (Aoki et al, 2001). The mean residence time of \(^{125}\)I labeled Thrombinase was 150.7 minutes.

The plasma clearance half-life of Thrombinase was 106 minutes. This is the time required for one-half of the total amount of a particular substance in a biological system has to be consumed or broken down by biological processes, when the rate of removal is approximately exponential. The therapeutic response of the drug normally depends upon the adequate concentration of the drug at the site of action. When a drug administered into systemic circulation, a dynamic equilibrium exists between the concentration of the drug in the blood and its site of action. The concentration of the drug at the site of action can be
predicted from the blood concentration of the drug. *The slower rate of clearance, prolonged plasma elimination half-life of Thrombinase may be more helpful to determine the dose and frequency of drug administration. It will also prevent repeated multiple drug administrations.*

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

When a drug administered intravenously, the drug that is only in unbound (free form) form can dissolves in plasma, passes out of the plasma through capillary endothelium, and reaches other body fluids and tissues. The distribution of radio labeled Thrombinase indicates that it enters in different organs in different manner. This variation may be due to the nature of capillary membrane of the organ that varies in their permeability, which is also organ specific. It also depends upon the rate of blood flow to the organ. The organ that receives more blood supply equilibrated earlier than the organ that has lesser blood supply.

The pattern of organ distribution and concentration of Thrombinase depends on the species (rabbit/ rat) variation. In rabbit, the organ distribution showed a highest concentration in all the organs at 10 minutes and a steady decline at 30 minutes and 90 minutes whereas in rats the liver and blood alone showed the highest level at 10 minutes. At 30 minutes, the organs like brain, heart, and lung showed the highest concentration. Similar findings have been reported by Aoki *et al* (2001) who have worked with Gln 117 t-PA in rats reported the highest radioactivity in blood liver and adrenal gland in 5 minutes.
Organ distribution study of labeled Thrombinase in rabbit clearly showed that the higher concentration of Thrombinase in the liver at 10, 30 and 90 minutes when compared to the other organs studied. This indicates that the liver may be the major site of uptake of $^{125}$I Thrombinase. The liver concentration of $^{125}$I-Thrombinase was 3.5 times higher than that of the blood at 10 minutes. Oikawa et al, (2000) also reported that recombinant wild type labeled tissue plasminogen activator ($^{125}$I) (rwt-PA) showed 2.9 times higher concentration in liver at 5 minutes than in blood. He also reported that the concentration of $^{125}$I pamiteplase was highest in the blood followed by, liver and kidneys. It is relevant to point out that these results are contrary with pharmacokinetic studies of Streptokinase and Staphylokinase in rabbits and hamsters reported by Lijnen, et al (1991). At 5 minutes after the injection of $^{125}$I labeled Streptokinase and Staphylokinase, major part of the radioactivity was found in muscle and kidney. Further, they reported a less important role of liver for the clearance of staphylokinase and streptokinase. This indicates that not all the drug gets distributed in same pattern and also it depends on different time interval.

The rapid decline in the concentration of labeled Thrombinase indicates that this drug may not be bound to any other plasma proteins. These data also indicate that as soon as the injection, Thrombinase gets distributed among the organs and leaves the blood circulation. Thus, Thrombinase disappeared exponentially from plasma, distributed to tissues, extensively present in liver, and finally excreted in the urine through kidney. The major organ involved in clearance may be liver and kidney.
EXCRETION STUDY OF $^{125}$I LABELED THROMBINASE IN RABBITS

Elimination includes biotransformation (metabolism) and excretion. Metabolism of the drug leads to modification of the drug into its metabolites or its inactive form. Excretion study is helpful to understand whether the drug totally eliminated from body or it is distributed in the organ and have any temporary storage depot due to binding with tissue Thrombinase as a protein, the proteolytic enzyme of blood can act upon and metabolized as free amino acid and free labeled iodine, as it is in $^{125}$I labeled Thrombinase. The excretion studies of $^{125}$I labeled Thrombinase in rabbit shows that the main route of excretion was urine and the fecal excretion was minimal. This may be because the route of administration is intravenous and the hydrophilic nature of drug. This is further supported with the concentration of $^{125}$I Thrombinase in urine is significantly high at 90 minutes in rabbit organ distribution study. The distribution of labeled protein, in gall bladder and bile was less when compared to kidney and urine. Since the drug has elimination half-life of 106 minutes, most of radioactivity excreted in 12 to 24 hours from the body.

These results are similar to the report of excretion of Wild Type t-PA and Gln 117 t-PA mutant type. After intravenous administration of $^{125}$I Gln 117 t-PA in rats, 88.5, and 5.5% of the dose were excreted in urine and feces, respectively, within 288 h. The urinary and fecal excretion profile of Gln 117 t-PA was similar to that of Wild Type t-PA. Further he also reported the presence of radioactivity in thyroid gland at the end experiment (Aoki et al, 2001).
Drug induced undesirable effects on body systems

When a drug is evaluated for its efficiency, it should also be analysed for undue reactions and side effects, which is more essential to be considered for its therapeutics. Bleeding is the most frequent adverse reaction associated with thrombolytic therapy. In addition, undesirable effects that have been observed in association with thrombolytic therapy include fever and allergic reactions. Hence this study was also focused to study the changes in hematological parameters, ECG, pyrogenicity, and hypersensitivity reactions. The results clearly indicated that this drug Thrombinase got no undue reactions in the haematological parameters. Particularly no change in the clotting profile indicates that the clotting factors are not utilized and exhausted by the Thrombinase. Bolus administration of Thrombinase did not alter the cardiac electrical activity and interfere with its performance. No immediate hypersensitive reaction observed indicate that Thrombinase may not activate the IgE mediated reactions.