Review of Literature
The physiological systems that control blood fluidity are complex. Blood must remain fluid within the vasculature system; however it forms the clot quickly when exposed to nonendothelial surfaces at the site of vascular injury. When intravascular thrombi do occur in a system, thrombolysis is activated to restore fluidity. In normal situation a delicate balance exists between thrombosis and haemorrhage. This allows the physiological fibrinolysis without affecting the normal fibrinogen level by fibrinogenolysis. (Hardman and Limbird, 2001)

In the history of blood coagulation as well as for phylogenesis, the extrinsic system was developed before the intrinsic system (Milstone, 1952). Though the relation between the blood and injured tissue had been known for at least about 165 years (Thackrah, 1819), the intrinsic pathway was first described by Lister, only in 1863. Coagulation is cascade system as first described in 1964 by McFarlane, Davie and Ratnoff.

For many years the two systems were thought to be separate. After the discovery of the various coagulation factors the concept of the intrinsic cascade and the extrinsic activator complex as two separate pathways, converging at the level of activation of factor X, was established. In spite of presence of clotting factors available in the blood the triggering is not normally present in the blood.
HAEMOSTASIS AND BLOOD COAGULATION

The term haemostasis means prevention of blood loss. Whenever a vessel is severed or ruptured, haemostasis is achieved by several mechanisms viz,

1. Vascular spasm
2. Formation of platelet plug
3. Formation of blood clot as a result of blood coagulation and
4. Eventual growth of fibrous tissue into the blood clot to close the hole in vessel permanently.

BLOOD COAGULATION

More than 50 important substances that affect blood coagulation have been found in the blood and in the tissue, some that promote coagulation, called procoagulants, and others that inhibit coagulation called anticoagulants. The ability of blood to coagulate inside the blood vessel depends upon the balance between the two groups of substance. In the blood anticoagulants normally predominate and also aided by the smoothness of vessel wall, so that the blood does not coagulate. But when vessel is ruptured, loss of smoothness activates the procoagulants which override the anticoagulants, and then a clot does develop.

The classical work established more specifically the ability of a triggered tissue substance to cause the rapid clotting of blood. When the trigger is present outside the blood involving a tissue factor, it is designated as
extrinsic pathway for blood clotting. In contrast, the intrinsic system has been thought of depending only on substances normally present within in the blood vessel (blood trauma by exposure to collagen) for its activation and progress towards thrombin formation named intrinsic pathway for blood clotting (Guyton and Hall, 2000) (Figure II)

GENERAL MECHANISM OF BLOOD COAGULATION

Coagulation takes place in three essential steps:

1. In response to rupture of the vessel or damage to blood itself, a complex cascade of chemical reactions leading to the formation of a complex activator substance collectively called prothrombin activator.

2. The prothrombin activator catalyzes the conversion of prothrombin into thrombin

3. The thrombin acts as an enzyme to convert fibrinogen into fibrin fibers that enmesh the platelets, blood cells, and plasma to form the clot.

THROMBOSIS

Thrombosis can be defined as haemeostasis in wrong place, involving either both the mechanisms or one which is closely related (Macfarlane, 1977). Virchow (1856) identified components of thrombus formation which include blood vessel wall abnormalities, the blood flow pattern and the
Figure – II  Mechanism of coagulation and fibrinolysis
change in blood contents like increased platelet content. All forms of thrombosis contain fibrin, platelets, white cells and red cells. Thus the whole process involved with development of stickiness of platelet followed by blood coagulation leading to thrombosis.

**FIBRINOLYTIC SYSTEM**

**Fibrinolysis**

Many proteolytic enzymes are capable of digesting fibrin and the term 'Fibrinolysis', by convention, means the specific process of fibrin degradation caused by the splitting of a limited number of peptide bonds. In essence, this fibrinolytic system consists of a number of elements which either promote or inhibit the conversion of polypeptide proenzyme, plasminogen to plasmin.

**Concept of thrombolysis**

Wiman and Collen (1978) have proposed a molecular model for physiological fibrinolysis which helps to explain the restricted action of plasmin in vivo. They stated that the system is regulated at two levels:

1. Localized plasminogen activation at the fibrin surface and
2. Sequestration of the formed plasmin by circulating antiplasmin.

The tissue plasminogen activator (t-PA) within a thrombus during activation, is a single chain structure, converted to two chain t-PA form. In this two chain form it is more reactive with plasminogen and the generation of plasmin is accelerated in the environment of the thrombus. Outside the thrombus the two chain t-PA is rapidly inactivated by the fast acting t-PA
inhibitor (which is less active against single chain t-PA) and the thrombolysis reaction is limited to the thrombus and not allowed to spread out into the general circulation.

Basic components of fibrinolytic system

Although the coagulation system serves to aid haemostasis and prevent blood loss at the site of vascular injury, there are also several processes that occur to limit the extension of the blood clot and resolve the obstruction to blood flow. The fibrinolytic system removes the fibrin clot once it has achieved its haemostatic function.

The major components of fibrinolytic system and their properties are listed in Table - I. Biochemical defects, excessive fibrinolytic activity or impaired fibrinolysis leads to clinical disorders which manifest as either bleeding or thrombosis.

Plasminogen

Plasminogen is a single chain globulin composed of 791 amino acid residues and about 2% carbohydrate. It is synthesized mainly in liver. The bone marrow, eosinophils and kidney have all been also suggested to play a role (Barnhart and Riddel, 1963). It is also present in a wide variety of tissues and body fluids, including saliva, lacrimal gland secretion, seminal vesicle and prostate secretions. Plasminogen circulates in plasma where it achieves a concentration of approximately 200μg/mL. It has a half life of approximately
Table I Components of the plasma fibrinolytic system

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Weight (x10^3)</th>
<th>Chains Number</th>
<th>Plasma Concentration</th>
<th>Plasma Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen</td>
<td>88 (Glu, 79 (Lys)</td>
<td>1</td>
<td>2 μM (0.2 mg/ml)</td>
<td>2.2 days</td>
</tr>
<tr>
<td>Plasmin</td>
<td>88 (Glu, 79 (Lys)</td>
<td>2</td>
<td></td>
<td>0.1 sec</td>
</tr>
<tr>
<td>Endogenous Plasminogen Activators</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-PA</td>
<td>70</td>
<td>1 or 2</td>
<td>70 pM (5 ng/ml)</td>
<td>6 min</td>
</tr>
<tr>
<td>scu-PA</td>
<td>54</td>
<td>1</td>
<td>74 pM (4 ng/ml)</td>
<td>3-6 min</td>
</tr>
<tr>
<td>tcu-PA</td>
<td>33</td>
<td>2</td>
<td></td>
<td>3-6 min</td>
</tr>
<tr>
<td>Exogenous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptokinase (SK)</td>
<td>48</td>
<td>1</td>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>APSAC</td>
<td>131</td>
<td></td>
<td>Bimolecular complex</td>
<td>70 min</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2-antiplasmin</td>
<td>58</td>
<td>1</td>
<td>1 μM (69 μg/ml)</td>
<td>3 days</td>
</tr>
<tr>
<td>PAI-1</td>
<td>50</td>
<td>1</td>
<td>0.5 nM (25 ng/ml)</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Richard et al, 1999
2 days. However, under diseased conditions where the fibrinolytic system is activated, half life is shortened dramatically.

**Plasmin**

The plasma proteins contain a euglobulin called plasminogen or profibrinolysin that, when activated becomes a substance called plasmin or fibrinolysin. Plasmin is non-specific proteolytic enzyme that resembles trypsin. It is capable of digesting fibrin fibers as well as other protein coagulant such as fibrinogen, Factor V, Factor VIII, prothrombin, and Factor XII. Therefore, whenever plasmin is formed, it causes lysis of the clot by destroying many of the clotting factors, thereby sometimes even causing hypercoagulability of the blood.

**Plasminogen activators**

It is essential to understand the biologically available fibrinolytic system activator to understand whether Thrombinase action depends on these systems.

The process of plasminogen activation can occur through three distinct pathways including (Fig. III),

1. **The intrinsic activator system**

   - analogous to the contact system of blood coagulation,
Figure - III Plasminogen activation pathways

Fibrin formation and stabilization

plasminogen

<table>
<thead>
<tr>
<th>Intrinsic activation pathway</th>
<th>Extrinsic activation pathway</th>
<th>Exogenous activation pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xla</td>
<td>t-PA</td>
<td>streptokinase or APSAC</td>
</tr>
<tr>
<td>Factor Xlla</td>
<td>u-PA</td>
<td></td>
</tr>
<tr>
<td>kallikrein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMW kininogen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

fibrin(ogen) degradation
clot lysis

Richard et al, 1999
2. The extrinsic activator system

- tissue–type plasminogen activator (t-PA) and
- tissue–type urokinase activator (u-PA)

3. An exogenous activator system

- involving pharmacological agents- thrombolytic drugs like streptokinase

The pathways activated in vivo appears to be the extrinsic pathway, however, both the intrinsic as well as exogenous –activated system could play an important role in human disease.

Intrinsic activator system

The body has evolved a mechanism to recognize host invasion by foreign substances. Many of these foreign substances contain negatively charged surfaces that allow the activation of intrinsic (contact) pathway consisting of Hageman factor (factor XII), high molecular weight kininogen and factor XI. Plasminogen can interact with this intrinsic pathway of blood coagulation to generate plasmin. It has been estimated that this pathway contributes only 15% of the total fibrinolytic activity in human plasma (Kluft et al., 1987).

Extrinsic activator system

There are two dominant extrinsic fibrinolytic activator systems in the body,
1. Tissue type plasminogen activator (t-PA), and
2. Urokinase tissue type plasminogen activator (u-PA)

These activators have unique structure and properties that affect the specificity and rate of plasmin generation.

**Tissue Type Plasminogen Activator (t-PA)**

The tissue type plasminogen activator (t-PA) molecule is serine protease with a molecular weight of 70,000. The t-PA molecule is predominantly an endothelial cell enzyme and factors that regulate its secretion and release from the endothelium are quiet important for the initiation of fibrinolytic process. Many of the factors that cause the release of t-PA are important mediators of blood clotting or inflammation. These include thrombin, histamine, acetylcholine, bradykinin, epinephrine, interleukins, shears stress, and vasoocclusion (Collen et al., 1989). t-PA is present extremely at low levels in normal human plasma. Concentration reported to be 0.1nm/L and half-life in plasma is quiet short, about 6 minutes. In plasma t-PA circulates as a complex with its natural inhibitors, plasminogen activator inhibitor 1(PAI-1), with less than 5% of t-PA in the free active form. The t-PA molecule and t-PA/PAI-1 complex are cleared from plasma by two specific cell receptor systems in the liver that rapidly internalize and degrade the protein.

Although this single chain t-PA has low catalytic activity, it can be converted into more efficient plasminogen activator by cleavage of peptide bond which converts it t-PA from a single chain in to a 2-chain form. Tissue
plasminogen activator manifests its full fibrinolytic potential only when bound to fibrin.

**Urokinase Tissue Type Plasminogen Activator (u-PA)**

The other major extrinsic activator is urokinase-type plasminogen activator (u-PA). The presence of a fibrinolytic activity in urine was discovered in 1913 by Johansson. It was subsequently detected in the media of cultured human cells, endothelial cells, malignant cell lines, tumor cells and also in plasma. u-PA is a serine proteinase and is synthesized as a single chain molecule called prourokinase or single chain u-PA (scu-PA). The half life of the scu-PA is quiet short, about 5 minutes, and metabolism occurs in both liver and kidney. Scu-PA has got a very low level proteolytic activity. A second enzymatic form of urokinase known as low molecular weight two-chain u-PA is formed by second cleavage. This cleavage produces efficient enzyme and this low molecular weight is used for thrombolytic therapy clinically (Stump *et al.*, 1986).

**An exogenous activator system**

Several bacterial proteins have been identified as activators of fibrinolysis. This includes Streptokinase, Staphylokinase and an exogenous activator called Anisoylated Plasminogen Streptokinase Activator Complex (APSAC) which possesses a non-covalent complex of modified forms of plasminogen and streptokinase (Smith *et al.*, 1981).
Plasma and tissue inhibitors of the fibrinolytic mechanism

A wide variety of natural inhibitors to fibrinolysis exist in plasma, blood cells, tissues and extra cellular matrices. The natural inhibitors can act to either inhibit plasmin directly or block the conversion of plasminogen to plasmin.

In human plasma, approximately 10% of several proteins (by weight) are proteinase inhibitors. Several of these inhibitors are capable of inactivating the serine proteinases. When plasmin is generated, $\alpha_2$-anti plasmin forms a 1:1 complex with plasmin and thus plays a major role in inactivating plasmin. In addition, plasmin can also be inactivated by other serine proteinase inhibitors such as, $\alpha_2$- antitrypsin, $\alpha_2$- macroglobulin, anti thrombin III, and C-1 esterase inhibitor. The efficiency by which $\alpha_2$- macroglobulin forms an irreversible complex with plasmin molecule is only 10% as that of $\alpha_2$- anti plasmin (Aoki et al., 1978). The physiologic significance of these inhibitors is less established and at most, they regulate plasmin activity only after $\alpha_2$- anti plasmin levels are completely depleted.

$\alpha_2$-Anti Plasmin

The complete structure of the $\alpha_2$-anti plasmin molecule has been defined. It is 58,000 molecular weight protein with 452 amino acid residues. The protein is synthesized and secreted mostly by hepatocytes and also present within the $\alpha$-granules of platelets. $\alpha_2$-anti plasmin circulates in plasma at a concentration of approximately 1$\mu$mol/ L, which is one half of the plasma concentration of the plasminogen. The $\alpha_2$- anti plasmin molecule circulates in
blood for about 2.5 days and is present at higher levels than any of the other plasmin inhibitor. Several molecules inhibit fibrinolysis including lysine, 6-aminohexanoic acid, and fibrinogen, can interfere with ability of $\alpha_2$-anti plasmin to complex with plasmin (Sakata and Aoki, 1980).

**Mechanism of thrombolysis**

When plasminogen is activated, it forms a serine protease by name plasmin. Plasmin has broad substrate specificity but renders relatively specific for fibrin in vivo. It means that in a pure system, plasmin is an aggressive non-specific protease but normally in vivo plasmin activity is restricted and plasmin acts largely as a fibrinolytic enzyme. The regulation of this restriction is not fully understood and the following mechanisms are suggested.

**Physiological regulation of fibrinolysis**

Fibrin formation is the initial stimulus for fibrinolysis and the clot itself provides important control mechanisms. Regulation of fibrinolytic activity is achieved by complex interactions between the blood components and endothelial cells. During clotting, plasma plasminogen and t-PA both are bound with fibrin and resulting enzymatic activity of fibrin bound t-PA contributes to the formation of plasmin in the clot. $\alpha_2$-anti plasmin is not efficient inhibitor of fibrin bound plasmin. This property limits fibrin degradation to the thrombus. Platelets within the haemostatic plug can release plasminogen activator inhibitor I (PAI-I) and $\alpha_2$-anti plasmin and in this manner modulate plasmin activity within the clot. Thus balance of factors
in plasma and within the clot itself influences the rate of fibrinolysis (Richard et al., 1999).

IN VITRO EVALUATION OF THROMBOLYTIC AGENTS

Three groups of experiments were conducted in vitro, to determine the relative rates of thrombolysis produced by current clinically-relevant concentrations of streptokinase (SK), urokinase (UK), tissue-type plasminogen activator (t-PA), and plasmin. In one group, to find out the activity of these thrombolytic agents, the freshly prepared clot weight was quantified before and 2-hour after fibrinolysis. In another group, radio-labeled fibrinogen clots were used, and the amount of lysis was determined by measuring radioactivity in residual clot and supernatant fluid. Results indicate that SK, UK, plasmin, and t-PA in saline, all produced roughly equivalent rates of lysis. The efficacy of fibrinolytic agents was significantly enhanced by admixing clot and agent, using intrathrombic injections or clot maceration which indicates that the modification of these substances occur within the thrombus (Bookstein and Saldinger, 1985).

EXPERIMENTAL INDUCTION OF THROMBOSIS IN ANIMALS

Coagulation within the vessels may be brought about experimentally by many methods according to Taylor (1961),

a. By the injection of thrombin into the blood stream or

b. By repeated injection of a tissue extract (particularly) of lung, thymus or lymph glands (source of thromboplastin) or
c. By causing injury to the vessel wall by chemical, mechanical and infective agents, a roughened surface is exposed to the blood stream. Thromboplastin is also liberated from the injured vascular wall.

Induction of thrombus followed by lysis of the clot with thrombolytic drugs has been evaluated in rats, guinea pigs, cats and primates. It has been suggested on the basis of animal experiments that the primary event in venous thrombogenesis is, the local generation of thrombin in presence of vascular stasis (Hume et al., 1970). The efficacy of activated clotting factors in producing thrombi in the presence of stasis was shown by Gitel et al (1977) who injected clotting factor IX in to the isolated venous segment of rabbits and observed clot within minutes. However it is important to note that stasis alone had no effect, nor did the infusion of activated clotting factors alone, at the dose they used. It is the combination of stasis and hypercoagulability acting together is a potent thrombogenic stimulus experimentally (Wessler, 1962).

EVALUATION OF THROMBOLYTIC DRUGS IN RABBIT THROMBOSIS MODEL

Most of the workers used either rabbits or dogs for thrombolytic studies and they have been reviewed by Henry (1971). Johnson and Tillet (1952) induced thrombi in the ear vein of the rabbit by means of sodium morrhuate and studied the effect of streptokinase (SK). The measurement of thrombolytic activity was qualitative only; it depends on the visual changes occurring in the vein after the treatment. Ambrus et al (1957) described a
model to evaluate SK, using a $^{131}$I label clot produced by sodium morrhuate in the marginal ear vein of the rabbit, but the measurement was only semi-quantitative. Hirsh et al (1968) described an extra-carboreal model of thrombosis, in which an arterial (Carotid) and venous (Jugular) shunt was used and a clot was induced in it by infusion of thrombin and labeled fibrinogen. Followed by this, the activities of both SK and UK were studied by analyzing the residual thrombus weights of treated and controlled animals. The results showed good activities for SK but again the results were only semi-quantitative and no dose response was studied.

A new quantitative model for the examination of potential thrombolytic agents was described by Dupe et al (1981). A thrombus was formed in the inferior vena cava of the rabbit, using $^{125}$I-labeled fibrinogen mixed with a standard amount of thromboplastin. The thrombus was anchored securely by means of woolen thread inserted longitudinally into the lumen of the vein. In animal given with thrombolytic agents, lysis could be followed continuously by measuring the rise in blood radioactivity. Total lysis was determined by counting the radiolabel in the residual clot after the treatment. The model has been used to study the thrombolytic activity of plasmin and streptokinase-human plasminogen activator complexes.

Collen et al (1983) developed a simple venous thrombosis model in rabbits. A thrombus was formed in an isolated segment of the jugular vein by using a mixture of $^{125}$I-labeled fibrinogen, whole rabbit blood, and thrombin. In order to immobilize the thrombus during lysis, it was formed around a woolen thread introduced longitudinally in the lumen of vein. The extent of
thrombolysis was measured as the difference between the radioactivities introduced in the clot and recovered in the vein segment at the end of the experiment.

Lijnen et al (1991) studied the comparative fibrinolytic properties of Staphylokinase and Streptokinase in rabbit jugular vein thrombosis model using $^{125}$I- Fibrin-labeled rabbit blood clots. The extent of clot lysis was examined as the difference between the radioactivity initially present in the clot and the residual radioactivity in the jugular vein after drug administration.

Lyle et al (1998) developed a model of FeCl$_3$- induced thrombosis in the rabbit allowing simultaneous assessment of efficacy against arterial and venous thrombosis. This is the modified model of Kurz et al (1990) in which, thrombosis was developed in rats by application of ferric chloride (FeCl$_3$) on the external jugular vein. The interval between application of the ferric chloride and zero flow velocity, detected by probe and was termed as the time of occlusion.

Colucci et al (1998) modified the rabbit jugular vein experimental thrombosis model described by Collen et al (1983). In their experiment, to produce thrombus, freshly collected blood from femoral vein was rapidly mixed with thrombin and thromboplastin, and then injected in the jugular vein segment. Clozel et al (1998) investigated the time course of thrombolysis induced by increasing doses of t-PA given either as a continuous infusion or as a bolus. For this purpose, they modified the model of Collen et al (1983)
by using an external gamma counter to follow the rate of thrombolysis continuously.

Jorgensen et al (1971) studied the effect of streptokinase on fibrin rich carotid artery thrombosis in rabbits. At different time intervals fibrinolysis tests like euglobulin lysis time and fibrin plate assay were performed. Markland et al (1989) performed in vivo evaluation of purified fibrolase in an acute rabbit renal arterial thrombosis model. Complete occlusion of the artery was confirmed fluoroscopically. Angiograms were repeated at intervals of 30 minutes, and the rate of lysis was observed. In another rabbit model, an occluding spring coil device was selectively introduced retrograde from the jugular vein into each iliac vein and thrombus formed within a few hours. Venographic studies were used to monitor clot lysis. Marder et al (2001) compared thrombolytic efficacy of plasmin, a new thrombolytic drug with tissue plasminogen activator (t-PA) in a rabbit abdominal aorta thrombosis model by using flow meter with probe.

STUDIES OF THROMBOLYTIC DRUGS IN RATS

Fibrinolytic protein isolated from the venom of Crotalus atrox (the western diamond back rattle snake) was tested for thrombolytic activity by Willis et al (1989). Fibrinogenolytic activity resulted in 60% decrease in rat’s plasma fibrinogen level. Histological examination of kidney, liver, heart and lung tissue showed neither necrosis nor haemorrhage.
STUDIES OF THROMBOLYTIC DRUGS IN DOGS

The dog has been used and believed to give results that are more applicable to man than does the rabbit, particularly with regard to the use of plasminogen activators as fibrinolytic agents. Urokinase, which is active in cat, man and dog, is poorly active in rabbit. Hence the species react close to human forms the better model. Klocking (1978) considers that thrombolytic system in the dog parallels that of man more closely than does that of many other laboratory animals. In practical terms, the dog is more useful for a variety of experimental measures like venography which can be used as an additional index of thrombosis and thrombolysis (which is not possible in rabbit and rats).

A wide variety of experimental models in the dog have been described, and reviewed by Henry (1971). Ambrus et al (1956) first prepared radioactive venous thrombi in the dog (using $^{125}$I fibrinogen) and their model afforded a semi-quantitative evaluation of streptokinase and plasmin. Sherry et al (1954) formed venous clots in the femoral artery of dogs by means of stasis plus thromboplastin injection and it was confirmed by a histological examination. Tsapogas and Flute (1964) using an injection of homologous serum into a segment of femoral artery evaluated the effect of SK by visual inspection, palpation and arteriography.

The efficacy of fibrolase, a direct acting fibrinolytic enzyme from southern copperhead snake venom, was investigated by Markland et al., (1994) in an occlusive thrombus formed in carotid artery of anaesthetized
dogs. Using a Doppler flow probe, blood flow was monitored continuously. Collen et al (1993) investigated the comparative immunogenicity and thrombolytic property of Streptokinase and Staphylokinase in baboons arterial and venous thrombosis model.

PHARMACOKINETIC STUDIES OF THROMBOLYTIC DRUGS

Tissue distribution, plasma clearance, and excretion

Since Thrombinase has to be introduced into in vivo system, it is essential to understand its plasma clearance at given time, their tissue distribution and mode of elimination. This study also helps to determine its dose and mode of administration.

Catabolism of streptokinase and polyethylene glycol derivatives of SK (PEG-SK) were studied in mice (Brucato and Pizzo, 1990). In this study, organ distribution was studied by measuring the radioactivity of each organ, after injecting radio labeled protein.

Lijnen et al (1991) studied the comparative pharmacokinetic and fibrinolytic properties of Staphylokinase and Streptokinase in hamsters and rabbits. The organ distribution of radioactivity was quantized in the blood, liver, lung-heart-spleen and kidney-urine. Clearance of $[^{125}\text{I}]$ t-PA from the circulation and its tissue distribution was determined in rats by Borgers et al (2000). Oikawa et al (2000) compared the pharmacokinetics of pamiteplase, a novel recombinant t-PA, in rats with the pharmacokinetics of recombinant wild - type tissue type plasminogen activator (rwt-PA). Aoki (2001) studied
the plasma concentration, distribution, metabolism, and excretion (urine and feces) of $[^{125}_{\text{I}}]$ Gln 117 t-PA, a mutant type of tissue type plasminogen activator in rats using a $\gamma$-counter.

**LABELING OF PROTEINS WITH RADIOACTIVE IODINE**

Proteins labeled with radioactive iodine have been extremely useful in biochemical studies. 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 to the physical properties of the proteins. A protein can be radioiodinated in several ways, which includes the chloramine-T method, the enzymatic method (lactoperoxidase method) and electrolytic method. Using $[^{125}_{\text{I}}]$ iodine, chloramine T oxidation method has enabled high specific labeling of hormones (Greenwood et al., 1963), bacteria flagellins and immunoglobulins by using carrier free protein. Moran et al (1984) described the radio labeling of Streptokinase with $[^{125}_{\text{I}}]$ by using chloramines-T. Oikawa et al (2000) prepared the $^{125}_{\text{I}}$ labeled pamiteplase, a recombinant t-PA, and $^{125}_{\text{I}}$-rwt-PA by using chloramine-T method with carrier free Na$^{125}_{\text{I}}$. Borgers et al (2000) described the chloramine T method for the t-PA labeling.

Enzymatic iodination of polypeptides with $^{125}_{\text{I}}$ to high specific activity was reported by Thorell and Johansson (1971). Summaria et al (1977) reported the method of labeling of protein for in vivo dissolution of human cross-linked plasma fibrin clots by the equimolar human –plasmin derived light chain streptokinase complex. Robbins et al (1983) iodinated proteins with Na$^{125}_{\text{I}}$ by a lactoperoxidase method and kinetic studies of the labeled
protein were carried out in intact healthy mongrel dogs radioactivity determined on each sample in whole blood, plasma, thrombin – clottable protein, thrombin non clottable supernatant, trichloro acetic acid (10% final concentration – perceptible plasma protein).

AVAILABLE THROMBOLYTIC DRUGS FOR THERAPY

Six Plasminogen activators (PAs) have been approved by the US Food and Drug administration (FDA) for use in major thrombotic diseases: streptokinase (SK), urokinase (UK), alteplase (t-PA), anistreplase (APSAC), reteplase (r-PA), and tenecteplase (TNK-t-PA). Those derived from a human protein (UK, alteplase, reteplase, saruplase, tenecteplase, lenoteplase) are essentially non antigenic, whereas those derived from a bacterial species such as SK from the streptococcus and staphylokinase from the staphylococcus present a potential for allergic response, although modified recombinant forms of SK and Staphylokinase have decreased antigenicity.

The half-life of each PA determines whether it can be administered as a bolus injection, short infusion, or continuous infusion. Among the approved agents, the most suitable for bolus injections are anistreplase (half-life approximately 40 min), reteplase, tenecteplase, and lanoteplase, and the least suitable is alteplase (half-life 5 min). Some agents induce a lesser plasma lytic state, notably tenecteplase, BAT-PA (from the salivary gland of Desmodus rotundus) and staphylokinase. All of the PAs are associated with a significant bleeding risk, roughly equivalent for all agents, except for the higher rate of intracranial haemorrhage (ICH) with t-PA or t-PA mutant derivatives.
Apart from these PAs, Plasmin, a thrombolytic agent, acts on fibrin when the agent is administered locally by catheter. The major advantage of plasmin would be protection from bleeding complications at remote site of vascular injury, since circulating plasmin can be neutralized by antiplasmin.

**Streptokinase**

Purified streptokinase (SK) has a molecular weight of 47,000 Dalton. SK was the first clinically used thrombolytic agent. SK is obtained from cultures of B- haemolytic streptococci. By itself, SK has no plasminogen activator activity, but after combining with plasminogen, a complex is formed that is capable of activation other plasminogen molecules to plasmin. It is not fibrin-selective and since it acts on fibrinogen, factors V, VIII, and other plasma proteins. Generation of fibrin(ogen) degradation products also contributes to the significant haemostatic defect of thrombolytic therapy. Although the lytic state predisposes patients to bleeding, the benefit of decreased blood viscosity that results from the lytic state may be clinically important. The half-life of SK is approximately 20 min. SK is used treat venous thromboembolism and myocardial infarction, as well as to reverse catheter-related thrombosis.

**Urokinase**

Urokinase (UK) molecular weight is 34,000. UK is not fibrin- selective and also produces lytic state. It is obtained from human fetal kidney cell cultures, and as a human protein. Hence its use is not associated with hypersensitivity or neutralization complications. The half-life of the UK is
approximately 20 minutes. UK is used to treat venous thromboembolism, myocardial infarction, and thrombolysis of clotted catheters. UK is more expensive than SK.

Tissue plasminogen activator (t-PA)

t-PA is naturally produced by vascular endothelium, and also produced by a human melanoma cell line. Currently t-PA is produced by recombinant technology as two chain species, with a molecular weight of 56,000. In vitro, t-PA is fibrin specific, because of its high affinity for fibrin, which forms a ternary complex with plasminogen. The bleeding complications with t-PA are similar to those of SK or UK. The half-life of t-PA is much shorter than that of SK or UK, approximately 5 minutes. t-PA is used to treat venous thromboembolism, acute myocardial infarction and recently has been approved for use in acute thrombotic stroke. t-PA is an expensive thrombolytic agent.

OTHER THROMBOLYTIC AGENTS

Modifications of a SK, UK and t-PA have been made an attempt to create more fibrin-selective agents with fewer bleeding complications. The advantage of Anisoylated Plasminogen -SK Activator Complex (APAC) over SK is its longer half-life of approximately 75 minutes, such that this drug can be given by bolus IV infusion. It shares the other disadvantages of SK, including hypersensitivity and induction of neutralizing antibodies, as well as production of lytic state.
Pro Urokinase (Pro-UK) or Single -Chain UK has improved fibrin selectivity and has been used clinically. t-PA has been modified as a single chain molecule. T-PA has only shorter half-life than two-chain t-PA. It has high fibrin specificity, but shorter half-life than two chain t-PA. Pro Urokinase (Pro-UK) or Single -Chain UK and Single chain t-PA are currently available only in the United States.

Monteplase is a mutant type of t-PA. It has a prolonged half-life of 23 minutes as compared to 4 minutes for native t-PA alteplase. TNK-t-PA (Tenecteplase) is recombinant t-PA mutant. TNK-t-PA showed an increased thrombolytic potency on platelet rich clots.

**DISADVANTAGES OF THE EXISTING THROMBOLYTIC DRUGS**

Despite the availability of fibrin specific thrombolytic agents, currently available therapy has number of limitations due to its adverse effects.

**Adverse effect of SK**

SK is a bacterial protein, it is antigenic, and allergic reactions occur in approximately 6% of patients. Anaphylaxis during SK usage occurs in approximately 0.1% of patients (Hull et al., 1982). SK must be given in high dose to overcome plasma antibodies that are directed against the protein. These antibodies result from prior streptococcal infections. Therefore, all patients receiving SK should be monitored for the associated lytic state. Its adverse reactions other than bleeding problem include allergic reactions, anaphylaxis, and fever.
Adverse effect of t-PA

Tissue plasminogen activator (t-PA), is a serine protease, manifests its complete fibrinolytic potential only when bound to fibrin. Clearance of t-PA is primarily by hepatic metabolism and the half-life of the protein is about 3 minutes. Even though t-PA is more fibrin specific, bleeding at the site of injury may be more severe. Besides, it is more expensive, than SK per therapeutic dose.

Adverse effect of UK

Current interest on Urokinase is limited. Like SK, it lacks in fibrin specificity and therefore readily induces a systemic lytic state. It is also very expensive than t-PA. (Hardman and Limbird 2001)

Recent studies suggest that the direct fibrinolytic enzyme, like plasmin, also can be used to dissolve thrombi, when the agent is administered locally by catheter. However significant percentages (20-30%) of patients with acute myocardial infarction are resistant to reperfusion within 90 minutes despite the use of most potent thrombolytic agents or combination. Besides it causes systemic fibrinogenolysis with accompanying bleeding encountered frequently. Further 10-30% of patients experience acute coronary reocclusion following thrombolytic therapy. There is also small but significant risk of neurological complications including stroke and intra cranial haemorrhage. Additionally, the rapidly acting plasma inhibitor of t-PA, for example Plasminogen Activator Inhibitor-1 (PAI-1) that is significantly increased in
myocardial infarction, along with other factors, predisposes patients to re-infarction (Markland, 1994).

**NEED FOR A NEW THROMBOLYTIC DRUG**

Thrombolytic agents, which are used clinically, rely on the activation of plasminogen to plasmin. Plasmin, in addition to degrading fibrin monomer also may lead to platelet activation and promote a thrombogenic state at the site of original occlusive lesion (Kerins et al., 1989 and Gram et al., 1993). It is essential to remember at this point that the clot formation may be as a result of insufficient plasminogen system in the patient. In such a case the above mentioned thrombolytic drugs usage are restricted, as these drugs are known to activate the in-vivo plasminogen system.

The inherent plasmin related deficiency created a need for an alternative thrombolytic agent that can be used clinically to enhance the incidence of re-canalization, preventing reocclusion, reducing the frequency of bleeding and further complications. In view of potential limitation of plasminogen activator-based thrombolytic agents, this drug was chosen for evaluating its efficacy in animal models. The drug was supplied by Malladi Drugs and pharmaceuticals Limited, Chennai which has been referred as **THROMBINASE**, it is a misnomer.

**THROMBINASE, A NEW FIBRINOLYTIC ENZYME**

Thrombinase is a unique blood clot dissolving fibrinolytic enzyme purified from the culture filtrate of *Bacilli* species. It is a protein in nature
with a molecular weight of 32 kDa, which is determined by Mass spectral analysis. The purity of Thrombinase was checked with HPLC using gel filtration column and PDA detector followed SDS-PAGE. No known protease with clot dissolving activity has this lowest molecular weight so far. Based on the *in vitro* studies, it has been concluded that Thrombinase possesses direct fibrinolytic activity. The choice of this drug, is having an added advantage, as the circulating fibrinogen levels are not affected and the specific substrate for this enzyme is only fibrin monomer. It does not activate plasminogen or protein C or does not require any blood borne components for it's activity, according to the unpublished data of Malladi Dugs and Pharmaceuticals Limited, India.

*Since the drug company has been applied for patent, the source, isolation and purification cannot be given in detail.*

*The current study is focused on evaluation of thrombolytic action of Thrombinase in experimental animal models.*