

Streptokinase: about of a thrombolytic patented in Cuba

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REVIEW

ABSTRACT

A failure of hemostasis of the organism could cause the formation of clots inside the circulatory system, causing disorders of the system that lead to such processes like the stroke or myocardial infarction. The treatment of these pathologies could require the application of thrombolytic agents like the urokinase (Uk), the tissue type plasminogen activator (tPA) or the streptokinase (Sk). This review approaches the position of Sk as clinically important and with good relationship cost-effectiveness in the treatment of the thrombolytic processes. The aspects discussed include mode of action, structure and relationship structure-function, structural modifications that could improve their function, sources of obtaining, recombinant streptokinase, production and purification of this protein and their current position in the market.

Key words: streptokinase, urokinase, tissue type plasminogen activator, thrombolytic agents

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RESUMEN

Estreptoquinasa: a propósito de un agente trombolítico patentado en Cuba. Las alteraciones de la hemostasis del organismo pueden provocar la formación de coágulos en el sistema circulatorio, que ocasionarían desórdenes que llevan a procesos tales como el infarto cerebral o del miocardio. El tratamiento de estas afecciones puede requerir la aplicación de agentes fibrinolíticos como la uroquinasa (Uk), el activador tisular del plasminógeno (tPA) o la estreptoquinasa (Sk). Este trabajo trata, específicamente, la posición de la Sk como un agente clínicamente importante y con una buena relación costo-efectividad en el tratamiento de los procesos trombolíticos. Los aspectos que se discuten incluyen: modo de acción, estructura y relación estructura-función, modificaciones estructurales que pueden mejorar su función, fuentes de obtención, estreptoquinasa recombinante, producción y purificación de esta proteína, así como su posición actual en el mercado.

Palabras clave: estreptoquinasa, uroquinasa, activador tisular del plasminógeno, agentes trombolíticos

Introduction

The appearance of blood clots can cause the blocking of arteries or veins, with serious consequences that can induce death. The hemostasis of a healthy individual induces clots in order to prevent the body's blood loss, but the failures of the haemostatic equilibrium can induce events such as stroke pulmonary embolism, deep venous thrombosis and the acute myocardial infarction. The disorders that induce those failures and the development of clots require the administration of thrombolytic agents [1-3]. This kind of drug has also been used for the treatment of clots formed in the catheters by administering drugs for long periods of time in patients with treatment of hemodialyse. Streptokinase (Sk) is one of those agents. Other thrombolytic or fibrinolytic agents that can be found now in the market include urokinase (Uk) and their derivative products of the tissue type plasminogen activator (tPA).

Some clinical studies have compared the effectiveness of the recombinant tPA and Sk, but do not show a clear preference by one of them. Sk is as effective as the recombinant tPA in the treatment of acute myocardial infarction [4] and has a better cost-effectiveness relationship [5]; however, its use is not risk-safe. The Sk research continues in order to make the product better as a thrombolytic agent. This product is the most frequently prescribed for treating the acute myocardial infarction and is

present in the market of the health systems of the poorest countries.

Thrombolysis

The physiology of the formation of clots of fibrin is described in several studies [6, 7]. A thrombus or clot is formed when blood cells are contained in a matrix of the protein fibrin, an enzyme that can act on the dissolution of clots. This process is known as thrombolysis or fibrinolysis. In the blood circulation of mammals, the enzyme responsible for this process is plasmin, a seric protease similar to trypsin [8].

Plasmin is the fibrinolytically active form produced from the inactive zymogene, called plasminogen that is present in blood circulation. The conversion of plasminogen into plasmin is mediated by a proteolytic cut of the Arg 561-Val 562 bond that is mediated by several plasminogen activators [9]. The plasminogen activators present in blood are tPA and uPA. The fibrinolytic activity in circulation is modulated by the inhibitors of the plasminogen PAI-1) and plasmin activators (α 1-antiplasmin, α 2-macroglobulin) (Figure 1) [10].

Plasmin acts on the fibrin network and changes it into degradation products (PDF).

The recombinant types of human plasminogen activators (tPA and uPA) appear during the clinical

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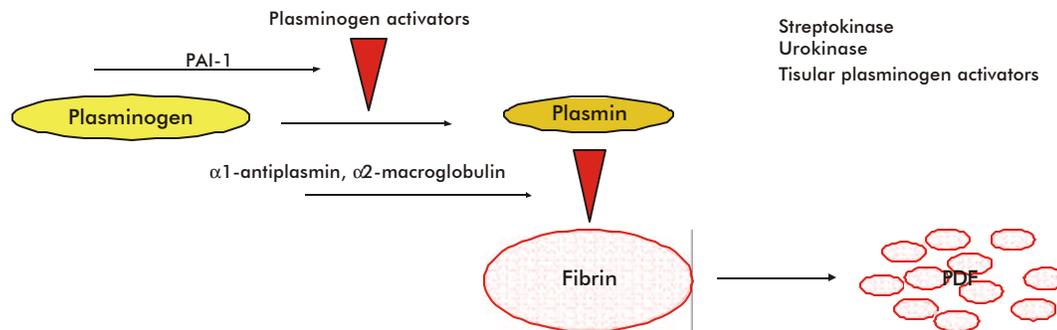


Figure 1. Chain of reactions involved in fibrin clots dissolution.

treatment. Also, Sk, a protein isolated from the streptococcus bacterium, not found in natural condition in human circulation appears during the clinical treatment. Sk, tPA and uPA do not have a direct fibrinolytic activity, their therapeutic action is performed through the activation of the plasminogen present in blood and its conversion into plasmin.

Different from tPA and uPA that are proteases, Sk does not have any enzymatic activity [8]. This protein acquires its activating capability through the formation of a complex with the plasminogen or the plasmin present in blood circulation. That is a stoichiometric (1:1) highly similar that plays the function of a very specific protease, when activating other molecules of plasminogen in its conversion into plasmin [8, 11].

Plasminogen activators

The main plasminogen activators, incorporated into fibrinolytic therapy are shown in table 1.

The non-fibrin specific drugs, such as Sk and Uk convert into plasmin both the circulating plasminogen and the clot-bonded plasminogen, and it induces not only the lysis of the fibrin in the clot, but also an important systemic fibrinogenolysis, a fibrinogenemia and an increase of the circulating products from the fibrin degradation (PDF).

Because of the relative selectiveness of the plasminogen-fibrin binary complex, the fibrin-specific activators mainly give place to fibrin lysis on the clot surface without theoretically affect the circulating fibrinogen [15].

Structurally modified molecules of the tisular plasminogen activator and of Sk that are derived from the proteins identified as activators of plasminogen

Tabla 1. Main plasminogen activators incorporated to fibrinolytic therapy.

tPA	uPA	Sk
Seric protease	Seric protease	It is not an enzyme
Fibrin specific activator	Non-fibrin specific activator	Non-fibrin specific activator
Molecular weight: 70 kDa	Molecular weight: 55 kDa	Molecular weight: 47 kDa
Produced by the vesicle endothelium cells [12]	Produced in the kidney and secreted in urine [13]	Produced in the culture of β -hemolytic streptococci of the C group [14]

are currently in the market for thrombolytic therapy. The thrombolytic drugs have also been classified as first-second and third generation drugs, according to the moment when they have been incorporated to the clinical use for thromboembolic diseases. Their classification is shown in table 2.

Urokinase

Uk was first used for treating acute myocardial infarction the 60s and it is now the most used thrombolytic agent in Japan. Its thrombolytic activity is similar to that of Sk, but its clinical use is limited because of its high cost and of the non-existence of random studies that demonstrate its efficacy.

Uk is a seric protein similar to tripsin, composed of two polypeptidic chains (A and B) with 20 000 and 34 000 Daltons, respectively, that are linked by a disulphur bridge that is its native structure. The appearance of Uk is preceded by that of an inactive proenzyme: pro-urokinase (pro-Uk), with a unique chain and 54 000 Daltons molecular weight. Its activation occurs by the plasmin action that hydrolyzes the peptidic bond lis158-159 iso1 and gives birth to the double-chain [16].

Uk activates the Glu-plasminogen by a cut of the peptidic bond Arg 561-Val 562 to Glu-plasmin. Just like Sk, Uk is a non fibrin-specific activator, so it induces a systemic lytic condition. During the treatment with Uk, besides fibrinolysis, changes in coagulation can occur when the plasma levels of fibrinogen and the V and VII factors of coagulation are reduced.

The plasmatic kinetics of Uk is bi-exponential, with a first stage of 4 minutes and a final average life that can be from 10 to 20 minutes [17].

There is a linear correlation between the plasma thrombolytic activity and the Uk dose that can be given by its mechanism of action and the absence of neu-

Table 2. Generational classification of the thrombolytic drugs.

First generation	Second Generation	Third generation
Urokinase	Tisular plasminogen activator	Reteplase (r-PA) TNK
Streptokinase	Sk-plasminogen activating complex (APSAC)	
	Pro-urokinase (scum-PA)	

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tralyzing agents. Its main secondary effect is hemorrhage, and it does not have antigenic properties in humans.

Tissue type plasminogen activator

In 1983, the definitive proof that tPA is synthesized in the endothelial cells was obtained [18].

In 1980, tPA was identified in melanoma cells and a year later, the enzyme was purified. The gene coding for the protein was isolated from the 8 chromosome and was introduced into a vector that was used for transforming ovary cells of hamsters (CHO) for its expression, what made easier its production on a large scale.

tPA is a glycoprotein of 68 000 Daltons, composed of 530 amino acids. At first, it is synthesized and excreted as a single-chain molecule (sct-PA, Alteplase), that is later divided into a double-chain molecule (tct-PA, Duteplase), joined by only one disulphur bridge, because of the cut of the Arg278-Ile279 link, by the action of a series of endogenous proteins, such as plasmin, tissue kallikrein and activated X factor.

Structurally, the amino-terminal area is composed of several domains that have homology with other proteins:

- The 1-43 residues are homologues to those of fibronectin, called *finger domain*.

- The residues contained into the 44 and 91 amino acids are homologue to the *kringle* areas of the plasminogen.

The *kringle 2* area, and the *finger* domain are essential for the binding with fibrin. The carboxyl-terminal (279-530) is homologue to that of other seric proteases and contains the region responsible for the catalytic activity, composed of the following amino acids: His322, Asp371 and Ser478 [19].

In the absence of fibrin, the tPA poorly activates the plasminogen because it is a fibrin specific agent. However, when fibrin is formed, tPA and plasminogen are joined to the clot and, orderly and sequentially, the activation of plasminogen are produced.

The tPA specificity for fibrin enhances the increase of its enzymatic activity due to the conformational changes of tPA and the plasminogen formed after the union with fibrin. The tPA and the plasminogen are joined to the fibrin by the presence of the *kringle* domains, the first of them has a high affinity for fibrin.

The relative specificity of tPA on the fibrin seems to increase the speed of this agent for obtaining the coronary recanalization with regards to other non fibrin-specific agents, besides increasing the ability for lysing relatively old clots [20]. On the contrary, this early rate of re-canalization appears with a higher incidence of re-occlusions: 13% compared to 8% regarding non fibrin-specific agents [21].

The starting average life of recombinant tPA in healthy individuals is 5-6 minutes, and the end average life is approximately, 64 minutes [22].

The most usual adverse effect, just as occurs with other thrombolytic agents, is the risk of hemorrhage, practically similar to that for Sk or Uk, in spite of the affinity of tPA to fibrin. The incidence of hemorrhage is lower when it is used for the treatment of the acute myocardial infarction, compared to other diseases, such as the deep venous thrombosis, since the lapse of treatment is longer for the last case. No serious

immunological or allergic reactions have been described, although some reactions of low hyper-sensitivity, such as stitch and Urticaria have been observed.

Third-generation thrombolytics agents

The group of the third-generation thrombolytic agents is composed of the thrombolytic agents that fight better the characteristics of the first and second generation agents. Few products from this group have been used for humans, after hard *in vitro* research and with animals. The most advanced product is Reteplase (r-PA) and its design was based on the natural tissue type plasminogen activator and is manufactured by recombination genetic techniques in *E. coli* [23]. This product consists of a single-chain molecule containing 355 amino acids corresponding to the encoding sequences from 1 to 3 and from 176 to 527 amino acids from the native tPA. Its expression in *E. coli* produces a non glycosylated protein that accumulates into the cells as inactive inclusion bodies and that are purified to restore the native active structure.

This product keeps the *kringle 2* and seric protein domains in its structure, but does not have the *finger*, EGF and *kringle 1* domains in the native tPA [24].

Reteplase is a non glycosylated plasminogen activator and its structural differences with Alteplase give them a longer average life (18 minutes; while in tPA is from 3 to 6 minutes) with two derived consequences: to administer the first one, a lower dose of the drug is needed for keeping high therapeutic levels, and the second, can be administered as intravenous bolus, what starts thrombolysis more rapidly and therefore, an early reperfusion is obtained.

Compared to tPA, Reteplase has a lower affinity for fibrin, because it does not have the finger domain.

This product, like tPA is inhibited by the PAI-I and it is less effective than Alteplase for platelet-rich plasmatic clots lysis and old thrombi [24].

The adverse effects and the safety of r-PA have been widely assessed in different clinical assays INJECT [25], RAPID-1 [26], RAPID-2 [26], and GUSTO III [28]. The internal hemorrhage can be intracranial, retroperitoneal, gastrointestinal, genitourinary or respiratory.

The differences between Reteplase and Alteplase observed in the RAPID-1 and RAPID-2 studies were not significant for any of the cardiovascular effects. The incidence of the allergic complications in the INJECT study was lower for the r-PA group (1.1%) than for the Sk (2%).

The Tecnetepase product (TNKase) is the most recent thrombolytic drug approved by FDA for its application as a *bolus* for the treatment of the acute myocardial infarction. It is a glycoprotein with 517 amino acids obtained by modifications of complementary DNA of the human natural tPA.

That molecule has substitutions of the 103 threonin by asparagine, substitution of the 117 asparagine by glutamine within the *kringle 1* domain and four substitutions of alanine in the 296-299 positions in protease domain.

The modifications performed overcome the pharmacological properties of the recombinant tisular plasminogen activator (Alteplase), with an increase

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of the average life from 5 to 7 times, the specificity is increased 14 times *in vitro* reduces the systemic depletion of fibrinogen and plasminogen and increases about 80 times its resistance for PAI-1 [29-31]. The efficacy of TNKase was compared to the application of a single dose of Alteplase in the ASSENT study [32].

At least three new modifications of tPA could be found in the group of third-generation thrombolytic drugs studied for their marketing: Monteplase [33], Parniteplase [34] and Lanoteplase [35].

Streptokinase

Streptokinase is an extra cellular non-enzymatic protein, formed by a polypeptidic chain composed of 414 amino acids without disulphur bridges [36]. This protein has its maximal activity at pH 7.5 and its isoelectric point is 4.7 [37-39]. In its structure, it does not include cysteine, cistin, phosphorus, conjugated carbohydrates or lipids. The streptokinases produced by diverse groups of streptococci have different structures [40, 41].

The activation of plasminogen by Sk is species-specific [42]. It is formed by several structural domains [α , β and γ] with different functional properties. The calorimetric scanning analysis suggests that the protein is formed by two structural domains [43]. They are: amino-terminal domain, including the residues from 1 to 59 and with a low affinity for plasminogen and the residues from the protein (60-414) related to the formation of the complex with plasminogen [44]. The carboxy-terminal domain of Sk is involved in the recognition of the substrate: plasminogen and in its activation [46, 47] and specifically the Asp 41-His 48 region of the molecule participates in the joining with plasminogen [48], while the function of the adjacent region of the 48-59 residues has been very discussed [49].

The coiled region of the streptokinase γ -domain is considered as essential for the activation of plasminogen [50] and similarly, the β domain is involved in the formation of the Sk-plasminogen complex that is responsible for activating plasminogen [51].

Sk is joined to plasminogen through the sites of linkage with lysine, to get the appropriate conformational structure for activating plasminogen [52, 53].

The first 59 residues of the molecule amino acids have more than one function in the process of formation of the complex and the activation of plasminogen [54, 55]. Without those amino-terminal residues, the Sk has an unstable secondary structure and significantly reduces the structure of the remaining fragment of Sk (60-414) [55]. Several studies have been performed with fragments of Sk that have provided some information about their fibrinolytic activity when they combine and form the active complex with plasminogen [45, 55-60].

Sk, itself, has no proteolytic activity. Its union with plasminogen in a 1:1 ratio forms the activator complex that hydrolyses the Arg 561-Val 562 of the rest of the circulating plasminogen and changes it into plasmin [61].

As we mentioned, Sk is a non-specific fibrinolytic because it not only activates the plasminogen with lysine, but also the plasmatic plasminogen, what can induce hyperplasminemia, depletion of the circulating

fibrinogen (up to 20%) and of the V and VIII coagulation factors, with the concomitant increase of the products from the degradation of plasminogen into plasma. In spite of the systemic lytic condition that can induce a dose of 1 500 000 IU, almost an equal incidence of hemorrhagic complications to those occurred with other thrombolytic agents showing a higher affinity for fibrin [62] has been observed.

On the other part, plasmin stipulates the conversion of kalikreinogen into kalikreine, so, the Sk infusion produces a release of quinines. It is partly responsible for the hypotensive effect seen in most of the patients receiving Sk.

The kinetics of this drug is not well known, its concentration in plasma and its average life depends on its affinity for substrate and on the plasmatic concentrations of anti-Sk antibodies. Its difference from the fibrin-specific activators is that its fibrinolytic effect is not directly proportional to the dose administered, what can change from one to another patient due to the reasons above mentioned.

Sk is removed from the blood circulation in a biphasic way: the most rapid phase is the inactivation of Sk by the specific antibodies (4 minutes, approximately); in the second phase and once the complex is formed, Sk is removed with an average life of 30 minutes [63].

The titers of the anti-Sk antibodies increase 5 or 6 days after administration and reach maximal concentrations several weeks later. They are normal 4-6 months later, so their administration is very controversial in this period [62].

As other thrombolytic agents, the main complication of the treatment by Sk is hemorrhage that is related to the dose and the duration of the intravenous infusion.

Because of its bacterial origin, Sk is antigenic, and therefore, it can induce allergic reactions. Four percent of the patients of the Second International Study of Infarct Survival-ISIS-2 [64] that received Sk had allergic reactions, fever, shivers, urticaria or rash. The anaphylactic shock is fortunately very rare (0.1-0.5%), however, arterial hypotension needed resuscitation through fluid therapy in 7-10% patients.

Modifications of the Sk molecule

The therapeutic possibilities of Sk are limited by its immunogenicity and short average life in blood. It is degraded by plasmin in blood circulation.

The works of structure modification of the molecule intend to extend its average life and/or reduce its immunogenicity by improving the activation of plasminogen. The first of those modifications and the only one present in the thrombolytic products' market was the non-covalent equivocal activating complex formed by Sk and human lys-plasminogen, whose catalytic center has been reversibly acylated by a p-anisole derivative (APSAC) that increases the molecule's average life regarding Sk [65]. Some papers have stated that hypotension is less frequent than when the patient is medicated with Sk [66].

Several studies have been carried out in searching new modifications of the molecule, based on the structure-fusion relationship and its domains [46-48, 60, 67-75].

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The structural variants of Sk have been obtained through genetic mutation, recombinant DNA techniques and chemical or enzymatic modifications. Mutants that improve its stability [76], variants modifying the highest proteolytic action sites Lis 59 and Lis 38 and that have made possible to obtain plasmin-resistant mutants [63] have been obtained. Those forms resistant to plasmin action are as active as the native molecule. The recombinant Sk produced in *Pichia pastoris* yeast is glycosylated and so, it has some resistance to proteolysis [77]. Complexes of Sk with polymers, such as polyethyleneglycol (PEG) have been prepared in order to increase its average life [78, 79].

The immunogenicity of the Sk domains is different [80] and some research reports the results in identifying the most antigenic regions [81-83]. Recombinant Sk mutants with reduced immunogenicity have been obtained [84]. A mutant without the 42 amino acids region of the terminal carboxy region is less immunogenic than the native molecule [72].

Production of Streptokinase

The production of Sk through streptococci was discovered in 1874 by Billroth in exudates from infected wounds. Later, similar bacteria were detected in the blood of patients with scarlet fever, and in 1919, streptococci sp. were classified in α , β and γ variants according to their hemolytic reactions on agar blood plates.

In 1933, Lancefield obtained a differentiation of β hemolytic strains in A to O groups from serologic analysis [85]. Sk was isolated from the A, C and G groups and is preferentially produced by group C, which lacks some toxins excreted by the two other groups.

The H46A strain (ATCC 12449) of *Streptococcus equisimilis* group C, isolated from a human source in 1945, has been frequently used for manufacturing. It was isolated among hundreds of fibrinolytic strains because the Sk obtained was more active.

The H46A strain grows in a semi-synthetic medium and excretes great amounts of Sk [86-67]. Also, it has been frequently used in the isolation of the Sk gene for its expression in other microorganisms [88-90].

Recombinant Streptokinase

There is sufficient information on the gene, its transcriptional control and its promoter that have made possible the cloning and the secure expression of the recombinant streptokinase in bacteria that are not pathogenic for humans. Malke sequenced the Sk gene [88]. The Sk gene transcriptional control has been studied enough [91], as well as the functional analysis of its promoter [92].

The isolation of the gene and the studies about it suggest that it is a polymorph gene [41]. The gene isolated from the H46A strain has been cloned in several strains of Gram negative bacteria, such as *Bacillus subtilis* [63,90] and *Escherichia coli* [36, 93-98]. The expression of Sk in *Pichia pastoris* yeast has been obtained, too.

The insertion of a genetic construction with the Sk gene and erythromycin as a selection marker was introduced in the *S. equisimilis* H46A strain in order to select super producing clones of protein [99].

The isolation of *S. equisimilis* from the nucleotide sequence of the gene codifying Sk and its expression in *Escherichia coli* and *Pichia pastoris* was patented in 1992 and it was demonstrated that the protein obtained could be used for the treatment of different types of thrombosis [100].

Fermentation

The group A streptococci frequently needs rich and complex media, supplemented with nutritional factors for their growth [101].

A medium usually used for producing Sk is brain-heart infusion [36], where a satisfactory growth of *S. equisimilis* H46A has been obtained. The maximal concentration was detected in the growth exponential stage. This is a chemically defined medium for the satisfactory growth of group A streptococci [42]. The growth velocity in that medium was comparable to the velocity obtained in more complex media.

The influence of temperature in the culture of *S. equisimilis* H46A plays a significant function in the growth and the production of Sk, the optimal value is 28 °C and the amount of Sk produced per unit of biomass depends on it [102].

The optimal conditions of fermentation for mutants of the wild streptococcus strain and for other genetically manipulated organisms are discussed in many studies. From the Sk gene of *S. equisimilis* expressed in *E. coli*, Sk concentrations higher than those obtained in group C streptococcus culture have been obtained through a medium composed of casein hydrolyate, yeast extract and salts [93]. Recombinant Sk has also been obtained in *E. coli* with a LB medium [69].

Biological activity

The assays used for determining the Sk activity are based on the activation of plasminogen to plasmin and its activity is measured by the proteolytic action of plasmin on a substrate indicator, such as fibrin clots, casein, other proteins and several synthetic esters (lysine methyl ester, lysine ethyl ester, p-sulphonyl-toluene-L-arginine-methyl ester).

In 1949, Christensen created the first quantitative method to measure the activity of this protein; it determined the amount of Sk necessary to induce the lyses of a standard clot of fibrin in 10 minutes at 37 °C and pH 7.4. Some modifications of this procedure have been used for Sk quantification. [103-107].

The casein digestion for Sk quantification was established in 1947 and the method of radial caseinolyse in agarose gel with the example of casein and human plasminogen is usually used [108].

The method of fibrin plate, used at first to measure the proteolytic activity in blood [109] has been widely used to measure fibrinolysis. Several modifications have been made to that method [110].

In the studies of lysis of fibrin clots by Sk, pH, salt concentration in the buffer and the plasminogen concentration have been identified as the variables that influence the most on the determination. The concentration of fibrinogen also affects the assay [111].

For the last years, the amino acid synthetic esters have been used as sensitive substrates in the proteolytic activity assay [112] adapted for the determination of plasmin [113-116].

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An assay with a chromogenic substrate in plates [117], the plasmin generated hydrolyzes a specific tripeptide, H-D-valyl-leucyl-lysine-p-nitroanilide. Some modifications of that assay have been established to determine Sk fibrinolytic activity, with a study of the effect of salt and fibrinogen concentration in the generation of the plasmin proteolytic activity [118].

Sk quantification as a pharmaceutical product of clinical use is described in British Pharmacopeia. The method is based on the *in vitro* formation of a clot and that clot lysis by the quantified Sk according to a pattern curve. It is an industrious expensive method depending on the permanent observation of the analyst. For the last years, the National Institute of Biological Standard and Control (NIBSC) of England, has performed studies to approve a quantitative method for determination of Sk, based on the use of a chromogenic substrate for plasmin. The results indicate that the approval of the method could be ready for the next year, when the collaborative study will be complete; several laboratories from different countries take part in that study. The study includes the assessment of the method and the use of streptokinases from diverse origins and manufacturers.

Purification

Several schemes for Sk purification have been described, including the commercial preparations obtained from the culture of several strains of streptococci [37, 119-121].

One of the first purification systems described was the ionic exchange combination on a DEAE- cellulose gel and column electrophoresis on a sucrose gradient to obtain a 5-6 increase in purity [37]. To obtain higher levels of purity, it was necessary to repeat the same procedure several times.

Better results are obtained by combining ionic exchange on DEAE-Sephadex A-50 with gel filtration on Sephadex G-100.

In 1968, Tomar [122] purified Sk from a Varidasa preparation by using a different procedure by dividing it into different procedure by dividing it into fractions through chromatography on hydroxyapatite or precipitation with ammonium sulphate. Precipitation in 40-50% ammonium sulphate increases two or three times the specific activity of the preparation. The precipitate was recovered by centrifugation and was dialyzed with sodium chloride. The product was applied on DEAE-cellulose and was eluted through a gradient.

The affinity chromatography has been used very much for Sk purification. One of the first procedures used plasmin treated by disopropylfluorophosphate (DIP) immobilized on sepharose [123]. The obtainment of the ligand was carried out by treating plasminogen with Uk to obtain plasmin and then its proteolytic activity was inhibited by DIP. This process induced a 30% loss of activity by the incomplete inhibition of immobilized plasmin.

The use of immunoaffinity for Sk purification has also been described in the literature [124].

Other purification scheme was the use of human plasminogen or acylated plasmin immobilized on a sepharose support [125]. The acylation of plasminogen or plasmin was performed with p-nitrophenyl-p-guanidinobenzoate (NPGB). It allowed the use of that

ligand in affinity and did not require a previous activation of plasminogen to plasmin and this considerably reduced Sk proteolysis by immobilized plasmin. The combination of plasminogen sepharose with an affinity system and the use of a monoclonal antibody as ligand for the purification of Sk, and a product with 93% purity were obtained [59].

NPGB as acylating agent of plasminogen for Sk purification was used with a buffer urea as eluent [126], with a 9 times purification factor and a recovery higher than 90%. The specific activity of the purified material was 74 000 IU/mg.

Sk has also been purified from the filtrate of the streptococci culture by using hydrophobic supports, such as phenyl sepharose and octyl sepharose, with an elution through an ammonium sulphate gradient and its combination with ionic exchange and filtration gel steps.

The purification of recombinant Sk, intracellularly expressed and insoluble in *E. coli* was performed from the mechanical cell rupture, solubilization with urea and after the renaturalization of protein in gel filtration a step of hydrophobicity (TSK-butyl) and a ionic exchange were performed to obtain a product with a purity higher than 99%, a specific activity over 70 000 IU/mg and 49% recovery of process [121]. A similar yield (45%) and 97% purity were obtained when recombinant Sk produced in *E. coli* inclusion bodies [97].

Streptokinase: Current position in the Market

Natural Sk, as product for clinical use was launched in the market several years ago. Hoechst Marion Roussel and Kabivitrum (Pharmacia and Upjohn) were the leaders for that product. They registered the product as Streptase[®] and Kabikinase[®] that now cover a significant place in the market. Other pharmaceutical manufacturers have obtained licenses for producing those drugs. The generic product can be found as Propinase[®] (Emcure Pharmaceuticals Ltd), Ekinase[®] (Dabur Pharmaceuticals Ltd.), Stapase[®] (Cadila Pharmaceuticals Ltd.), Icinase[®] (ICI India Limited), Thrombosolv[®] (VHB Life Sciences), Fibrokinase[®] (Otsira Genetica), Solustrep[®] (DONG KOOK PHARM Co. Ltd), Zikinase[®] (Indon).

The recombinant Sk preparations are added to the natural Sk preparations: Heberkinasa[®], commercialized by HEBER BIOTEC. That was the first Sk in the market. It was clinically tested by the TERIMA studies [127] that included 224 patients and compared a natural preparation to the recombinant one. The studies demonstrated that there are no difference between both products and that the benefits obtained with the natural preparation can be also found for recombinant Sk and TERIMA-2 [128] was the extension of the use of Heberkinasa[®] in 52 hospitals and 2 923 patients. Thus, the use of the product was extended and its potentiality for reducing mortality (28 % reduction of intra-hospital mortality) in patients with acute myocardial infarction was demonstrated. The level of antibodies and the neutralizing activity in patients treated with Heberkinasa[®] and Streptase[®] were tested in the TERIMA study and there it was demonstrated that for both preparations, the level of

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antibodies was low a year after the infusion of the product, and that can make possible to re-administer the drug [129].

Heberkinasa[®] has also been used in patients suffering from thrombosis of cardiac valves and the deep venous thrombosis. For the first case, a clinical assay was performed with 15 patients suffering from thrombosis in cardiac valves prostheses. In 30 to 50% of the cases, obstruction appears independently from the anticlotting to which those patients are kept. The administration plan was performed with a starting dose of 250 000 IU for 30 minutes and a maintenance dose of 100 000 IU an hour for 72 hours or less. The results are monitored by the imaging assessment of thrombi lysis. The thrombolytic therapy in those patients is a safe alternative for the thrombosis of prosthesis. Even though it is incompletely effective, it can offer some additional time for preparing the patient for surgery and protecting him/her from distal embolisms during surgery.

In deep venous thrombosis, Heberkinasa[®] was used in a group of patients that received a starting dose of 250 000 IU, intravenously, in a time lapse of 30 minutes, followed by a keeping dose of 100 000 IU every hour in continuous infusion for 24-72 hours, depending on the moment of the thrombus dissolution. For the 100 % of the patients, there was dissolution of the thrombus. This response was classified as total or partial. The incidence of suspicion of adverse reactions in the studied population was high, but they were controllable and did not cause any risk for the patients' life.

Those results increase the scientific support for the use of this product because of the low degree of severity of the adverse reactions and of the drug-tolerance.

The treatment of the complicated parapneumonic loculated pleural effusion (DBTPC) and pleural empyema (EP) were also treated by this product in a pilot assay in 4 patients (3 cases of DBTPC and 1 case of EP). The doses, as in former cases, are high and sometimes they need the administration of the product for 2-4 days. A complete resolution of the clinical frame was obtained with a re-expansion of the affected lung in 3 patients. The improvement was evident after the radiological test. Those results suggest that the treatment of those diseases by Heberkinasa can be safe and efficacious.

Recently, Shankinase[®] by Shantha Biotechnics, of recombinant origin was launched in the market.

In the market of thrombolytic drugs, Sk has an important place, especially in the less developed countries. The cost of a dose of a second- or third-generation thrombolytic drug can be over \$ 2 196.00* for Alteplase or Reteplase and the \$ 2 750.00* in the case of Tecnetplase, the cost of Sk can be 10 times lower.

* Cost/dose, From the American Heart Association.

An analysis of the early thrombolysis in the acute myocardial infarction and its economic assessment was performed by Boland *et al.* (2003). This study covered the analyses performed since 1980 to 2001 and the main criterion for its inclusion was the comparison among drugs (Alteplase, Reteplase, Sk and Tecnetplase) for the early treatment of the acute myocardial infarction.

Finally, the results of 20 studies reported in 50 papers were considered; 14 corresponded to comparative studies and 142 907 patients received some thrombolytic treatment. The results intended to obtain conclusions to answer the following question: Which is the most appropriated thrombolytic for treatment in the acute myocardial infarction?

The conclusions of this study were based on the efficacy of the thrombolytic treatment (mortality in 30-35 days). Sk is as effective as the Alteplase infusion. Tecnetplase is as effective as the application of a bolus of Alteplase and Reteplase is as effective as Sk.

Those results could generate new questions: Is Tecnetplase better than Sk or not?

Can Reteplase be as effective as Tecnetplase?

The performance of new clinical studies can be necessary to answer those questions; the statistical analysis, itself, does not offer the appropriate answer.

From the analysis performed it is clear that mortality is low after the thrombolytic treatment with any of the studied drugs.

The complications by cerebrovascular diseases were significant after the use of those drugs, with a lower velocity for Sk that, however, caused the higher per cent of allergic reactions.

The economic assessment of the thrombolytic therapy and the similarity of the clinical results make evident that the cost-benefit relationship can be determined by the drugs acquisition cost, what, no doubt, widely displace the cost-effectiveness balance to Sk.

Conclusions

Sk is the most ancient thrombolytic drug. No doubt, it is one of the best clinically assessed and characterized. It is the most prescribed yet for the treatment of the acute myocardial infarction, with an attractive manufacturing cost if compared to some homologous. Its adverse reactions are very well defined and can be clinically treated.

The understanding of its structure-function could allow, in a near future, the obtainment of modifications of the molecule useful to reduce the allergic reactions they induce to make possible to keep it in a market, that now satisfactorily share with the new generations of thrombolytic drugs.

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