RESEARCH

Formulation development of a recombinant Streptokinase suppository for hemorrhoids treatment

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ABSTRACT

Recombinant Streptokinase (rSK) is a protein of bacterial origin currently used in the treatment of acute myocardial infarction. Its thrombolytic and anti-inflammatory action makes it attractive for the treatment of hemorrhoids. In this work the influence of absorption enhancer, preservative and emulsifiers on rSK stability was accessed by determining its biological activity under stress conditions. According to the results obtained, were selected as excipients for the formulation: Sodium salicylate as enhancer and anti-inflammatory agent, thimerosal as preservative, Span 60 as emulsifier and Witepsol W25 as suppository base and 100 000 IU per gram of suppository for stability study. Afterwards, three lots at pilot scale were manufactured, packed in aluminum blister shell and stored at 5 ± 3 °C. Immediately after preparation, the samples were evaluated at months 0, 3, 6, 9, 12 and 18 having into account the physical, chemical and biological properties. The stability study demonstrated that the formulation containing rSK as active pharmaceutical ingredient was stable during 18 months under refrigerated conditions.

Keywords: streptokinase, suppository, hemorrhoids, formulations development, rectal administration, thrombolytic, long term stability

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RESUMEN

Desarrollo de una formulación en supositorio con estreptoquinasa recombinante para el tratamiento de las hemorroides. La estreptoquinasa recombinante (SKr) es una proteína de origen bacteriano que se utiliza para el tratamiento del infarto agudo de miocardio. Su acción trombolítica y antinflamatoria la hace atractiva para el tratamiento de las hemorroides. Se evaluó la influencia de promotores de la absorción, preservantes y tensoactivos sobre la estabilidad de la SKr, mediante la determinación de su actividad biológica bajo condiciones de estrés. De acuerdo con los resultados, se seleccionaron el salicilato de sodio como promotor de la absorción y agente antinflamatorio, el tiomersal como preservante, el Span 60 como tensoactivo y el Witepsol W25 como base del supositorio, y 100 000 UI de SKr por gramo de supositorio para el estudio de la estabilidad. Posteriormente se prepararon tres lotes a escala piloto y se almacenaron en blíster de aluminio a 5 ± 3 °C. Inmediatamente después de la preparación, se evaluaron las muestras a los 0; 3; 6; 9; 12 y 18 meses, teniendo en cuenta las propiedades físicas, químicas y biológicas. El estudio de estabilidad demostró que la formulación en forma de supositorio es estable durante 18 meses bajo condiciones de refrigeración.

Palabras clave: estreptoquinasa, supositorio, hemorroide, desarrollo de formulaciones, administración rectal, trombolítico, estabilidad en tiempo real

*I*ntroduction

The delivery of therapeutics and proteins remains a priority for many pharmaceutical companies, being a huge challenge the need to find non-parenteral routes for their administration [1, 2]. The rectal route is one of them. The rectum has been an accepted site for drug delivery, and used for systemic and local action with potential for the delivery of peptides. Many studies have been focused in the bioavailability of a variety of peptides such as calcitonin [3], insulin [4, 5], Beta interferon [6] and also Streptokinase [9] among others.

Streptokinase is a high molecular weight protein which nowadays is used for the treatment of acute myocardial infarction, deep vein thrombosis, permanent vascular access thrombosis and other diseases with thrombotic origin. In this sense, it is presented in different pharmaceutical forms like lyophilized for parenteral use [6, 7]. It has also been used for mucosal administration, in orally administered pills VARIDASE®, for edema relief of inflammatory process [8]. It has also been reported its rectal administration with the main objective of systemic action for the treatment of hematoma re-absorption or just for comparing its concentration in the blood after rectal and oral administration [9]. However, its local administration at the rectum remained to be studied.

Rectal Streptokinase administration using a suppository formulation is possible in order to achieve the elimination of the thrombus in hemorroidal disease, with the consequent decrease of the inflammation and pain in the affected zone, without systemic thrombolytic effects. Hemorroids treatments are often divided between non-operative management, office

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procedures and surgical management. Topical agents using astringents, analgesics, and steroids help on providing relief in an acute setting, but there is no evidence showing their benefit for prevention or long-term treatment of hemorrhoidal disease [10]. The use of rSK in hemorrhoidal disease could allow solving the pathology locally, in a neither painful nor invasive ways. Additionally, it would not require specialized personnel or equipment for its application and it makes possible the treatment of the acute hemorrhoidal disease in a non-surgical way, avoiding the inconvenient inherent to this kind of treatment. Thus, the purpose of our study was to develop a formulation and to determine the biological, physical-chemical stability of streptokinase in a suppository formulation during 18 months in order to verify de viability of the product.

Materials and methods

Materials

Recombinant Streptokinase was obtained from the Center of Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). Ethylendiamine tetraacetic acid salt dehydrate (EDTA) was purchased from Merck (Darmstadt, Germany). Benzalkonium chloride, thimerosal, Tween-20, Tween-80, Span 20, Span 60 were obtained from Merck-Schuchardt, Hohenbrunn, Germany. Suppository base (Witepsol W25) was provided by Sasol (Witten, Germany). Methyl paraben, propyl paraben, sodium salicylate and sodium deoxycholate were obtained from Sigma-Aldrich (USA).

Excipient selection for a rSK suppository formulation

Selection of the absorption enhancer

rSK was diluted at 100 000 IU/mL and mixed with three different enhancers: Sodium salicylate, sodium deoxycholate and EDTA, each at 0.5 %. The biological activity was tested 0, 3, 7 and 15 days later. During the study the solutions were stored at 37 ± 2 ºC.

Selection of the preservative

rSK was diluted at 100 000 IU/mL and the solution was mixed with different preservatives: 0.01 % Benzalkonium chloride, 0.18 % methyl paraben, 0.02 % propyl paraben or 0.001 % thimerosal) and stored at 28 ± 3 ºC. Biological activity and organoleptic characteristics were tested 0, 3, 7 and 30 days later.

Selection of the emulsifiers

The following non ionic tensoactives were used to elaborate suppository formulations: (Tween-20, Tween-80, Span 20 and Span 60 at 1, 2, 4 %). Then, the physical characteristics were evaluated.

Suppository preparation

Three consecutive, independent batches of suppository formulations were produced to investigate their long term stability. Suppositories were prepared, by melting the suppository base in a water bath at 54 ºC. Thimerosal at 0.001 % and sodium salicylate at 0.5 % were added continuously at constant stirring until a homogenous dispersion was reached. The dispersion was cooled down up to 36 ºC and, subsequently, rSK (100 000 IU/g of suppository) was incorporated with constant agitation. The mixture was then spilled in cold suppository molds of 2 g. Suppositories were left to solidify and the excess was removed until final ejection. Then they were refrigerated at 5 ± 3 ºC and periodically analyzed.

Drug extraction

The content of Streptokinase in a suppository was extracted after one unit was melted in 4 mL of water at 37 ºC. The dilution was mixed for 1 minute in a Vortex-type mixer and then centrifuged at 8000 rcf for 2 minutes. The solutions were stored at 5 ± 3 ºC until its analysis.

Determination of Recombinant Streptokinase by substrate chromogenic

This test is based on the formation of an activating complex mol/mol between the Streptokinase and the plasminogen, that is capable of activating the free plasminogen in the plasma or plasmin. It is measured quantitatively for the liberation of a colored molecule that is directly proportional to the units of Streptokinase used [11].

SDS-PAGE analysis

The samples were analyzed by SDS/PAGE as described by Laemmli [12]. The gels were scanned and densitometrically analyzed after Coomassie blue staining. A reference standard of rSK provided by the Quality Control Division of CIGB was used as the positive control.

Other determinations to suppository stability

Softening time, weight, organoleptic characteristics, microbiological tests were all determined in compliance with acceptance levels criteria (USP) [13].

Long term stability studies

Suppositories with the best excipient formulation were assessed for rSK stability after storage at 5 ± 3 °C. Suppositories were tested for weight, organoleptic properties, softening time, microbiology, and preservative effectiveness (Table 1), and also for biological activity and purity.

The long-term stability assay was designed in agreement with the principles of the International Conference on Harmonisation guideline "Q1A Stability Testing of New Drug Substances and Products" to propose a retest period or shelf life in a registration application (Table 1) [14]. The results were compared using a two-tailed ANOVA with replication, after a comparison of the homogeneity of variance (Bartlett's test) [15].

Results and discussion

The preformulation strategy plays a vital role to obtain a proper and stable formulation dosage form. It involves the application of biopharmaceutical principles to the physicochemical parameters of the drug. This approach helps the formulator to reduce preparing unnecessary formulations, leading to reduced costs and time effectiveness. In order to obtain a safe and efficacious suppository formulation, different excipients were evaluated.

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Table 1. Frequency of the assay in long term stability studies

Assay	Months	Acceptance criteria
Substrate chromogenic	0, 3, 6, 9, 12, 18	140 000 and 260 000 IU/suppository
Purity by SDS-PAGE	0, 3, 6, 9, 12, 18	≥ 90 %
Weight of suppository	0, 3, 6, 9, 12, 18	1.80-2.2 g
Organoleptic Test	0, 3, 6, 9, 12, 18	Suppository of white cream with torpedo form, flat surface and without hollows
Softening time	0, 6, 9, 18	< 30 min
Microbiological test	0, 6, 12, 18	Not more than 10^3 aerobic bacteria and not more than 10^2 fungi per g Absence of *Escherichia coli* Absence of *Pseudomonas aeruginosa* Absence of *Candida albicans*
Preservative effectiveness	0, 18	Effective against microorganism tested

SDS-PAGE: sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Influence of the absorption enhancers over the biological activity of rSK

The passage of drug molecules through the skin could be an important and rather troublesome stage in percutaneous drug delivery. Most molecules penetrate through skin via intercellular microroute and, therefore, require disrupting or bypassing its molecular structure [16]. Chemical substances temporarily diminishing the barrier of the skin and known as accelerants or absorption promoters can enhance drug flux. Many classes of chemical permeation enhancers have been used including sulfoxides, fatty acids, surfactants, chelating agents and others [17].

In this study, the effects of different promoter enhancers (sodium deoxycholate, sodium salicylate and EDTA) on rSK biological activity were evaluated. According to the values obtained in the biological activity of the rSK diluted with the absorption enhancers tested, the protein was stable for 15 days at 37 ºC.

No statistical differences were obtained during that time or when the biological activity was compared to different absorption promoters ($p > 0.05$) (Figure 1).

Some studies have reported that bile salts and its derivates as sodium deoxycholate affect the intestinal glycocalyx structure and decrease gastric and intestinal mucous. A transcellular absorption enhancing effect is suggested by the phospholipid disordering action of unconjugated and conjugated bile salts. Furthermore, the paracellular absorption-promoting effect is suggested to be intermediated by binding of Ca^{2+} [18]. However, the use of sodium deoxycholate as enhancer was eliminated from the study, because it is reported certain damage caused by bile salts on the mucosa and the possible carcinogenic effects of its rectal administration [19].

Sodium salicylate and EDTA were selected in this study, because they are chelating agents that form complexes with calcium and magnesium ions present between intestinal epithelial cells and ultimately lead to the opening of tight junctions, and thereby increase permeability for exogenous substances [18].

Due to the good stability of rSK formulated with sodium salicylate and EDTA as enhancers, they were used in an animal model of hemorrhoidal thrombosis. One suppository containing 100 000 IU/g rSK was administered to rabbits, the best results obtained with the rSK formulation with sodium salicylate, and the inflammatory process was reversed in the anal region [20]. Another advantage found in a previous study of sodium salicylate is that it induces urokinase-type plasminogen activator (uPA) production by activating its promoter [21]. According to these results, sodium salicylate was selected as enhancer to develop the suppository formulation.

Influence of the antimicrobial preservative over biological activity of rSK and evaluation of organoleptic characteristics of the suppository

The effects of methyl and propyl parabens, benzalkonium chloride and thimerosal on rSK and suppository formulation were evaluated. Methyl and propyl parabens are stable and non-volatile compounds used as antimicrobial preservatives in foods, drugs and

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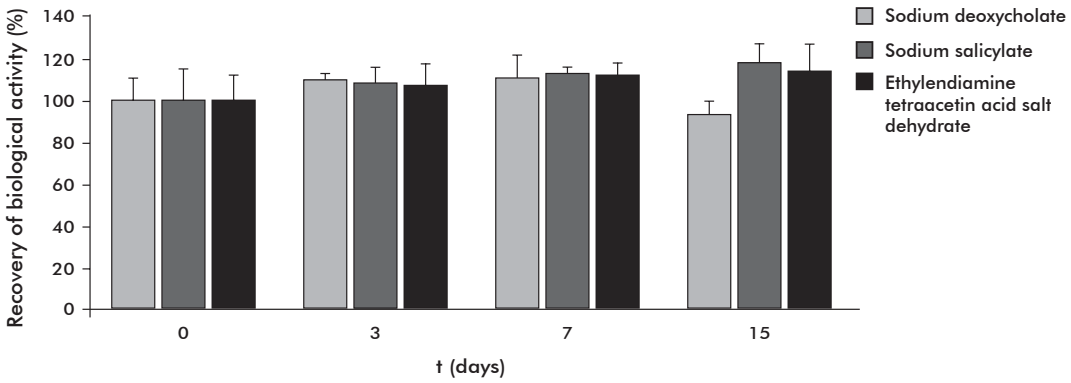

Figure 1. Effect of different absorption enhancers on recovery percent of biological activity of the Recombinant Streptokinase, after 15 days of storage at 37 °C. Biological activity was determined by the S-2251 chromogenic-substrate method.

cosmetics for over 50 years. Acute toxicity studies in animals indicated that they are relatively non-toxic. Their mechanism of action may be linked to mitochondrial failure depending on the induction of membrane permeability transition, accompanied by the mitochondrial depolarization and depletion of cellular ATP through uncoupling of oxidative phosphorylation [22]. According to their characteristics, they were evaluated to formulate a suppository with rSK.

Methyl paraben and propyl paraben have low solubility in water at room temperature. This implies that temperature must be increased above 90 ºC to dissolve them [23]. At that temperature, the biological activity of proteins decreases because of its degradation or denaturation. Hence, the protein was added after dissolving the parabens. Suppository formulated by this procedure resulted in undesired organoleptic characteristics for the formulation due to phase separation.

Another antimicrobial preservative used was benzalkonium chloride. For different formulations, it is the preservative of choice and the American College of Toxicology had concluded that benzalkonium chloride can be safely used as an antimicrobial agent at concentrations up to 0.1 % [24]. A white, precipitate was detected when it was added to the aqueous phase containing rSK. According to literature this precipitation could be due to incompatibilities with the protein [25].

Then benzalkonium chloride, methylparaben and propylparaben were rejected from the study because of incompatibilities with rSK or suppositories.

The other preservative, thimerosal, is an organomercurial preservative broadly used in the microbial preservation. At concentrations of 0.001 to 0.01 % it has shown great effectiveness against a wide spectrum of microorganisms in immunoglobulin preparations, skin antigens test, nasal and ophthalmic preparations and hepatitis B vaccines [26]. To elaborate the suppository, thimerosal was dissolved without difficulty in the rSK solution, showing appropriate organoleptic characteristics. Also, the rSK biological activity was evaluated in solution and in the suppository, with no statistically significant differences ($p > 0.05$) for 15 days (Figure 2).

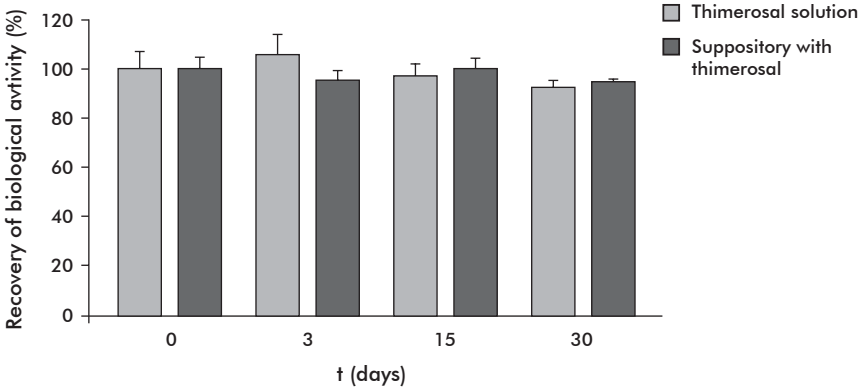

Figure 2. Effect of thimerosal on the recovery percent of biological activity of Recombinant Streptokinase, after 30 days of storage at 28 °C. Biological activity was determined by the S-2251 chromogenic-substrate method.

Influence of emulsifiers on the physical properties of the suppository

Emulsifiers derived from natural sources like non-ionic surfactants are expected to be safer than the synthetic ones. They are less toxic compared to ionic surface-active agents. The high and low hydrophilic and lipophilic balance (HLB), and subsequent hydrophilicity or hydrophobicity of surfactants are required for the immediate formation of oil-in-water (o/w) or water-in-oil (w/o) droplets, respectively, and/or rapid spreading of the formulation in the aqueous or oil environments, providing a good dispersing/self-emulsifying performance [27].

The emulsifiers tested to formulate the rSK suppository were: Tween-20, Tween-80, Span 20 and Span 60 at 1, 2 and 4 % all of them. All of these surfactants, being non-ionic, are much less damaging to the skin than other classes and could allow better permeation of rSK [28, 29].

When preparing the suppository formulation by adding Tween-20 or Tween-80, the preparation showed a flocky aspect and suppositories didn't get the torpedo shape. The high HLB is necessary for the formation of o/w droplets of the formulation but the oleaginous phase in the suppository is bigger than the aqueous phase and with emulsifiers with low HLB better results were obtained.

After formulated, suppositories using Span 20 and Span 60, best results were obtained with Span 60 at 1 %, favoring the emulsion corresponding with a propriate physical characteristics. Neither fluky aspect nor cavities were observed (Figure 3). The final formulation selected for the stability study was 100 000 IU/g of rSK, 0.5 % sodium salicylate, 0.001 % thimerosal, 1 % Span 60 and Witepsol W25 up to 100 %.

Stability studies for long term of rSK suppository

The aforementioned results obtained during the preformulation screening of the candidate stabilizing excipients allowed us to expect a long shelf life for this formulation. It has been recognized that these types of preformulation studies can only generally provide the basis for starting long term stability studies, but they may fail to reflect or predict the desired behavior of proteins. Three independent and consecutive batches were manufactured using rSK at 100 000 IU/g by suppository and real time stability studies at the purpose storage condition (5 ± 3 ºC) were undertaken (Table 2).

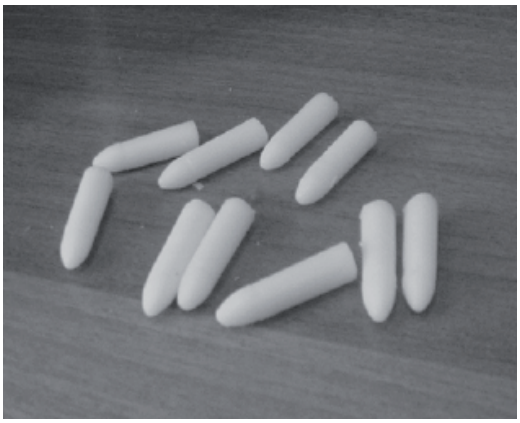

Figure 3. Suppository formulated with 1 % Span 60.

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Table 2. Long-term stability of recombinant streptokinase suppository formulation

Months	Biological activity* (IU/suppository)			Purity by SDS-PAGE (%)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
0	205737.78	201225.19	197250.13	98	98	97
3	209551.11	254506.67	192334.09	95	96	97
6	248348.89	206652.22	185964.08	95	96	99
9	207904.00	201852.22	192542.22	95	95	98
12	193354.44	183378.89	166502.22	90	93	96
18	153580.08	153451.13	153451.13	98	97	97

* Determination of the biological activity of the recombinant streptokinase by the S-2251 chromogenic-substrate method.
SDS-PAGE: sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Prepared suppositories were randomly selected, visually inspected, cut longitudinally and their surfaces were examined with naked eye. The initial examination of the formulation indicated no physical change, such as color, odor, and the surface was flat and without cavities. Also determination of weight uniformity was measured and no suppositories were deviated from average weight by more than 10 % of established weight.

The release of the active ingredient from the vehicle is related to the melting point of the vehicle and the solubility of the drug in the vehicle. Some studies have indicated that the release of drug is higher from bases with low melting range than from those of comparatively higher melting range [30]. In rSK suppository the time taken to melt them was measured when immersed in a water bath and maintained at a constant temperature of 37 ± 0.5 ºC and it was no longer than 30 minutes in all suppositories tested, which complied with the limits established [31].

The current formulation was stable for 18 months at 5 ± 3 ºC (Table 2). During this storage the biological activity of the active ingredient varied from 209 551 to 153 451 IU/suppository remaining between 70 and 130 % of its nominal value. The acceptance limit was high due to the variation of the method, no statistical difference was obtained between lots ($p > 0.05$).

The purity of rSK as determined by SDS/PAGE remained above 90 %. Other studies have described the good stability of rSKr conserved at 5 ± 3 ºC in freeze-dried formulation [32] but there were no report on the stability of rSK in suppository form. In the period evaluated, no degradation was observed by this method.

The manufacture and packaging ensured microbial quality of the formulation during the time (no more than 10^3 aerobic bacteria and no more than 10^2 fungi per gram, and *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were absent), and the effectiveness of the chosen preservative was demonstrated at 0 and 18 months.

All the evaluated parameters were found within the limits established. rSK is one of the most characterized and evaluated thrombolytic agents in clinical trials, and a common prescription in heart attack [33]. A clinical trial in acute hemorrhoidal disease was carried out with the rSK suppository formulation obtained in this work. The result showed that it was safe and tolerable, and complete recovery was achieved in most of the patients (90 %) [34].

Conclusions

A suppository with rSK as active pharmaceutical ingredient was developed. The excipients were selected according to the compatibility with rSK and the best quality properties of the suppository. Excipients included sodium salicylate as enhancer and anti-inflammatory agent, thimerosal as preservative, Span 60 as emulsifier and Witepsol W25 as base. This formulation was biologically, chemically, physically and microbiologically stable when stored for 18 months between 2 and 8 °C.

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