Analysis of five streptokinase formulations using the euglobulin lysis test and the plasminogen activation assay

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Abstract

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Streptokinase, a 47-kDa protein isolated and secreted by most group A, C and G ß-hemolytic streptococci, interacts with and activates human protein plasminogen to form an active complex capable of converting other plasminogen molecules to plasmin. Our objective was to compare five streptokinase formulations commercially available in Brazil in terms of their activity in the *in vitro* tests of euglobulin clot formation and of the hydrolysis of the plasmin-specific substrate S-2251™. Euglobulin lysis time was determined using a 96-well microtiter plate. Initially, human thrombin (10 IU/ml) and streptokinase were placed in individual wells, clot formation was initiated by the addition of plasma euglobulin, and turbidity was measured at 340 nm every 30 s. In the second assay, plasminogen activation was measured using the plasmin-specific substrate S-2251™. Streptase™ was used as the reference formulation because it presented the strongest fibrinolytic activity in the euglobulin lysis test. The Unitinase™ and Solustrep™ formulations were the weakest, showing about 50% activity compared to the reference formulation. All streptokinases tested activated plasminogen but significant differences were observed. In terms of total S-2251™ activity per vial, Streptase™ (75.7 ± 5.0 units) and Streptonase™ (94.7 ± 4.6 units) had the highest activity, while Unitinase™ (31.0 ± 2.4 units) and Strek™ (32.9 ± 3.3 units) had the weakest activity. Solustrep™ (53.3 ± 2.7 units) presented intermediate activity. The variations among the different formulations for both euglobulin lysis test and chromogenic substrate hydrolysis correlated with the SDS-PAGE densitometric results for the amount of 47-kDa protein. These data show that the commercially available clinical streptokinase formulations vary significantly in their *in vitro* activity. Whether these differences have clinical implications needs to be investigated.

Introduction

In 1933, Tillet and Garner (1) first described the exogenous plasminogen activator isolated and secreted by most group A, C and G ß-hemolytic streptococci. This activator was named "streptokinase" by Christensen and MacLeod (2). Streptokinase, a 47-kDa protein, interacts with the protein plasminogen to form a streptokinase-plasminogen complex capable of converting other plasminogen molecules to plasmin. Streptokinase does not require fibrin for efficient plasminogen activation in blood (3). It is a catalytically inert bacterial protein that has no structural homologues (4,5). Streptokinase can form an activator complex with plasmin, which contains a functional active site, or with plasminogen (lys-plasminogen) in which it induces non-proteolytically the formation of a functional active site (5,6).

The success of the complex formed by streptokinase as a therapeutic agent has been attributed to its unique mechanism of fibrin-dependent plasminogen activation. When administered to humans, the streptokinase activator complex rapidly generates plasmin in the circulating blood at sites distant from fibrin clots. This mechanism depletes the plasminogen substrate and exhausts the clot-dissolving (or fibrinolytic) potential of plasminogen (7-9).

Streptokinase is now a leading agent for the treatment of acute myocardial infarction. Approximately 400,000-500,000 patients receive thrombolytic therapy per year worldwide. Many investigators (10-15), based on several randomized multicenter clinical trials, recommend 1,500,000 IU as the appropriate dose for the treatment of an acute myocardial infarction. From a clinical perspective, the possible effect of significant deviations from the recommended dose is unknown. Note that the assays reported here were not the same as used for the recommendation of 1,500,000 IU.

Five streptokinase preparations commercialized in Brazil for clinical use by different manufacturers were analyzed to determine their activity, content and homogeneity. Streptase™ was used as a reference material. The activity of the samples was determined on the basis of euglobulin lysis time and of a chromogenic assay using S-2251™. The homogeneity of the protein content of the samples was determined by SDS-PAGE.

The study revealed significant differences among commercially available preparations in Brazil in terms of total streptokinase activity per vial.

Material and Methods

Streptokinase

Five streptokinase preparations from different manufacturers, containing 1,500,000 IU per vial as a powder, were tested (Table 1). Five milliliters of cold Milli-Q water was added to each flask to dissolve the solid. Samples nominally containing 300,000 IU/ml were frozen at -70ºC until use. During analysis all solutions were immersed in ice.

Preparation of human plasma and euglobulin fraction

Blood was obtained by venipuncture from the antecubital vein of 2 healthy volunteers at rest, with minimum stasis. Samples were collected into a 0.1 volume of 3.8% sodium citrate and plasma was prepared by centrifu-

Table 1. Streptokinase formulations for clinical use evaluated in the present study.

Name	Streptokinase supplier	Lot No.
Streptase	Hoechst Marion Roussel S/A	2482601F
Unitinase	Meizler Comércio Internacional S/A	717102
Solustrep	Laboratório Químico Farmacêutico Bergamo Ltda.	DK0456
Streptonase	Blausiegel Ind. & Com. Ltda.	KH07K150
Strek	Libra Produtos Laboratoriais Ltda.	717102

These streptokinase preparations are commercially available in Brazil and are identified by the commercial name, manufacturer and specific lot number. All formulations nominally contained 1,500,000 IU of fibrinolytic activity per vial.

gation (2,000 *g*) for 15 min at 4ºC. Plasma samples were pooled and kept at -70ºC until use.

The plasma euglobulin fraction was prepared by 10-fold dilution of citrated plasma and acidification at pH 5.0 by the addition of 1% acetic acid. After standing for 1 h at 4ºC the diluted plasma was centrifuged (2,000 *g*) for 15 min at 4ºC. The precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 7.4.

Euglobulin clot lysis assay

We used a 96-well microtiter plate assay to determine the euglobulin lysis time (16-18). Control samples were prepared by adding 20 µl human thrombin (10 IU/ml) and 30 µl 0.1 M Tris-HCl buffer, pH 7.4, to individual wells. Test samples were prepared by adding 10 µl thrombin, 10 µl buffer and 20 µl streptokinase solution. Clot formation was initiated by the addition of 150 µl of the plasma euglobulin fraction. The turbidity in the wells was measured as absorbance at 340 nm every 30 s for 20 min using a SPECTRAmax Microplate Spectrophotometer and the software SOFTmax PRO (Molecular Devices Corporation, Sunnyvale, CA, USA). Each streptokinase dilution was assayed in triplicate. To evaluate the effect of the different streptokinases on euglobulin lysis time, we used the software GraphPad Prisma 3.0 to plot the log of streptokinase IU against log of the area under the curve. Linear regression was applied and the efficiency of each streptokinase formulation was compared to the reference Streptase™.

To evaluate the streptokinase activity after storing the stock solution, the fibrinolytic activity was also evaluated after 24 h at 4ºC.

Chromogenic assay

Plasminogen activation by streptokinase was assayed using the synthetic substrate S-2251™ (Chromogenix-Instrumentation Laboratory, Milan, Italy). S-2251™ is a chromo-genic substrate for both plasmin and strep-tokinase-activated plasminogen. The method for the determination of activity is based on the difference in absorbance between the pNA formed and the original substrate. The rate of pNA formation is measured at 405 nm and this photometric signal is directly proportional to the plasmin activity, which reflects the amount of functional plasminogen originally present in the sample.

The reaction was performed using 96-well plates. Control samples were prepared by adding 20 µl 0.1 M Tris-HCl buffer, pH 7.4, 30 µl substrate S-2251 (0.6 mM) and 100 µl plasminogen solution (0.1 CU/ml). In the test samples the reaction was performed after addition of 20 µl streptokinase solution tested at different concentrations (150 to 600 IU/ml), 30 µl S-2251 and 100 µl plasminogen solution. The plate was immediately placed in the plate reader previously heated at 37ºC. The absorbance of the wells was measured at 405 nm every 30 s for 30 min. Plates were shaken for 3 s before reading the absorbance using a SPECTRAmax Microplate Spectrophotometer and the software SOFTmax PRO. Each streptokinase dilution was assayed a minimum of three times.

One unit was defined as the amount of enzyme activity that converts 1 µmol of substrate per minute per liter. Since the enzymatic activity is normally calculated using a cuvette with 1 cm of optical path, we adapted the formula to the 96-well plate taking into account a total reaction volume of 150 µl and an optical path of 0.4 cm. Under these conditions, the extinction coefficient of pNA is 3840 mol l^{-1} cm^{-1}.

Polyacrylamide gel electrophoresis

Samples were prepared for SDS-PAGE analysis by diluting 50 µl of each streptokinase stock solution in 950 µl Milli-Q water. Finally, 100 µl of the diluted streptokinase solution was mixed with 100 µl of 2X Laemmli buffer (19). Samples were heated at 95ºC

for 4 min to complete the reaction with the SDS and ß-mercaptoethanol and 20 µl of each sample was loaded onto the stacking gel. PAGE was performed using a discontinuous system (19). Samples were initially loaded and stacked in 4% polyacrylamide gel (stacking gel). Proteins were resolved in 10% polyacrylamide gel (resolving gel). SDS was used in combination with ß-mercaptoethanol to reduce disulfide bonds, denature the proteins and make the proteins negatively charged. Electrophoresis was conducted at 20 mA per gel (100-200 V) until the bromophenol blue marker reached the bottom of the gel. Proteins were stained with Coomassie brilliant blue solution (0.025%

Coomassie blue in 10% acetic acid) for at least 1 h, then destained with 10% acetic acid for 2 h. Mobility was plotted against the molecular weight of standard proteins (SigmaMarker Wide Range, Sigma, St. Louis, MO, USA) using a semi-log scale and the molecular weight of the proteins was estimated by interpolation.

Densitometric analysis of the protein bands was performed using the software Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

Protein quantification

Total protein concentration was determined with the QuantiPro™ BCA assay kit (Sigma) using bovine serum albumin as standard (20).

Results and Discussion

Euglobulin clot lysis

Clot formation was observed immediately after starting the reaction and turbidity increased until clot lysis was initiated by activated plasmin. The resulting graph was a hyperbole and the area under the curve increased with decreasing streptokinase concentration. Since the lines obtained were not perfectly parallel, we performed the comparison using 100 points covering the total line length. The relative streptokinase activity is represented as the mean ± SD of these 100 points.

Streptase™ showed the strongest fibrinolytic activity in the euglobulin lysis test. Unitinase™ and Solustrep™ were the weakest proteins, showing about 50% of the activity of the Streptase™ formulation (Table 2). The data show a more than 50% difference in activity between the most active (Streptase™) and least active (Unitinase™) streptokinase preparations as measured by euglobulin clot lysis. Streptokinase activity decreased dramatically after 24 h at 4ºC (Table 2).

Table 2. Comparison of streptokinase activity measured by euglobulin lysis in five clinical formulations.

Streptokinase	Streptokinase activity (%) relative to Streptase	Loss of streptokinase activity after 24 h at 4ºC (%)
Streptase™	100	62.0 ± 4.7
Streptonase™	83.3 ± 15.8	63.3 ± 9.5
Strek™	71.6 ± 15.6	70.2 ± 3.0
Solustrep™	52.2 ± 10.6	49.9 ± 6.7
Unitinase™	46.8 ± 2.4	59.0 ± 3.3

Activity was measured by the euglobulin lysis test and is reported as percent of the activity present in the Streptase™ formulation. Data are reported as the mean ± SEM for three independent experiments performed in duplicate. The data for the stability study at 4ºC for 24 h represent the total loss (%) of the fibrinolytic activity compared to the values reported in the first column.

Table 3. Comparison of streptokinase activity measured by the hydrolysis of the chromogenic peptide S-2251™.

Streptokinase	Total units	Ratio to the reference Streptase	N
Streptonase™	94.7 ± 4.6	1.25	16
Streptase™	75.7 ± 5.0	1.00	14
Solustrep™	53.3 ± 2.7	0.70	15
Strek™	32.9 ± 3.3	0.43	14
Unitinase™	31.0 ± 2.4	0.41	12

Plasminogen activation induced by streptokinase was measured spectrophotometrically with S-2251™, a substrate for both plasmin and streptokinase-activated plasminogen. One unit is defined as the amount of enzyme activity that converts 1 µmol of substrate per minute per liter. Each of the streptokinase formulations tested was compared to the reference Streptase™. Results are reported as the mean ± SEM for the number of determinations shown in the right column.

Hydrolysis of S-2251™

All streptokinase preparations tested hydrolyzed the plasminogen-like synthetic peptide substrate. However, the activity varied amongst the streptokinase formulations, as indicated in Table 3. Streptonase™ (94.7 ± 4.6 units/vial) and Streptase™ (75.7 ± 5.0 units/vial) were the most active formulations, while Strek™ and Unitinase™ were the weakest (32.9 ± 3.3 and 31.0 ± 2.4 units/vial, respectively). Solustrep™ (53.3 ± 2.7 units/vial) presented intermediate activity (Table 3).

SDS-PAGE

Electrophoretic analysis showed two major bands. The upper band corresponded to human albumin (~67 kDa) and was the most prominent protein (Figure 1A). The lower band was identified as the streptokinase protein on the basis of its apparent molecular mass of 47 kDa (data not shown), Streptase™, Solustrep™ and Streptonase™ showed contaminating proteins of a wide molecular mass range below 67 kDa. The bands corresponding to Unitinase™ and Strek™ were resolved into two components.

Densitometric analysis of protein bands confirmed a significant variation in the amount of both streptokinase and human albumin protein in each formulation (Figure 1B). The total amount of albumin is only indicated on the label of the following formulations: Unitinase™ (195 mg), Solustrep™ (200 mg) and Streptonase™ (200 mg). After protein quantitation by three independent determinations the variability observed for total protein amount observed in each flask agreed with the densitometric results: Unitinase™ (87.5 ± 8.5 mg), Streptase™ (207.1 ± 9.8 mg), Solustrep™ (374.0 ± 32.1 mg), Streptonase™ (433.3 ± 47.2 mg), and Strek™ (107.8 ± 5.1 mg).

The amount of protein corresponding to streptokinase is not indicated on the product labels. The variations among the different formulations for both euglobulin lysis test and chromogenic substrate hydrolysis presented a close correlation with the densitometric data for the streptokinase main band (Figure 1B).

The euglobulin lysis test and the chromogenic substrate assay presented similar results in ranking the streptokinase activity of five commercial clinical preparations. This suggests that either assay can be used to determine streptokinase activity. The chromogenic substrate assay has the advantage of being fast and highly reproducible. In the
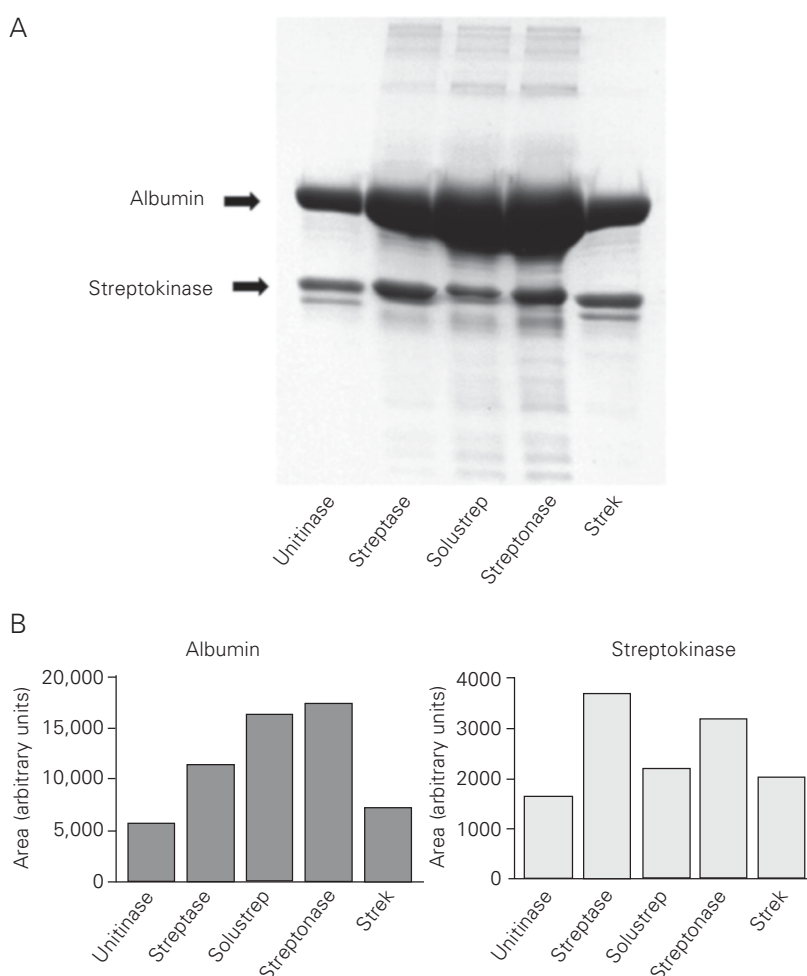

Figure 1. SDS-PAGE analysis of the protein content of five streptokinase formulations. *A*, PAGE was performed using a 10% polyacrylamide gel in a discontinuous system at 20 mA per gel (100-200 V). Proteins were stained with Coomassie brilliant blue. This result is representative of four independent experiments. *B*, Densitometric analysis of the albumin and streptokinase bands. The areas of the bands are reported in arbitrary units. Note that the scale for albumin is five times that of streptokinase.

euglobulin test, the preparation of the euglobulin fraction is laborious and this material has also the disadvantage of being unstable.

The present study revealed significant differences among the commercially available streptokinase preparations. Only Streptonase™ presented similar fibrinolytic activity when compared to Streptase™. All other streptokinases presented significantly less fibrinolytic activity. There are also significant densitometric differences regarding protein content associated with the SDS-PAGE band.

Whether these differences have clinical consequences deserve further evaluation by both medical professionals and regulatory authorities.

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