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Validation of the Chromogenic Bioassay for the Potency Assessment of Streptokinase in Biopharmaceutical Formulations

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Abstract

Streptokinase (STK) is a thrombolytic agent clinically used to treat patients with acute myocardial infarction and venous and arterial thrombosis. An *in vitro* chromogenic substrate end point bioassay was validated for the potency evaluation of biopharmaceutical formulations. The dose-response curve was linear over the concentration range of 2.50-40 IU/mL (r^2=0.999), with a quantitation limit of 2.50 IU/mL and a detection limit of 1.10 IU/mL, respectively. Specificity was established in studies with spiked samples. The accuracy was 100.34% with bias lower than 0.53%, and method validation demonstrated also acceptable results for precision and robustness. The validated method was applied to the potency assessment giving potencies between 92.20% and 108.97%. In addition, the activity of streptodornase and streptolysin were also evaluated giving values lower than 9.79 IU per 100 000 IU STK, and 1.47 for the absorbance ratio, respectively. The validated bioassay was applied in combination with the purity evaluation, contributing to assure the batch-to-batch consistency and quality of the bulk and finished biotechnology-derived medicine.

Keywords: Streptokinase; Chromogenic bioassay; Streptodornase; Streptolysin; Validation; Biotechnology-derived medicine

Introduction

Streptokinase (STK) is clinically used world-wide as a thrombolytic agent to treat patients with acute myocardial infarction, deep vein thrombosis, arterial thrombosis and embolism [1-3]. The structure of STK consists of a 414 amino acids polypeptide chain with a molecular mass of 47 kDa. The protein exhibits its maximum activity at a pH of approximately 7.5 and its pI is 4.7. Most of the native Streptokinases (STKs) are obtained from pathogenic β-hemolytic streptococci A, C and G, being the group C preferred as they lack erythrogenic toxins. Recombinant STKs have been produced with reduced immunogenicity, and the gene from *Streptococcus equisimilis* H46A was first cloned and expressed in *E. coli* releasing substantial amounts of STK into the culture medium [4-6]. Besides, the STK isolated hitherto may contain the enzymes streptodornase and streptolysin O, which are active even in small quantities [7].

The biological activity has been assessed by the *in vitro* fibrin clot lysis assay and the chromogenic plasminogen activation substrate assay, that were also used in an international collaborative study organized to establish the 3rd international Standard for streptokinase. It was demonstrated that the chromogenic substrate assay (SCSA), due to its ability to activate the fibrinolytic system, converting plasminogen to plasmin, is a suitable procedure for the potency determination of the streptokinase preparations [8,9].

Sixteen preparations of STK available world-wide for clinical use were compared by a SCSA and SDS-PAGE electrophoresis showing wide variations of the activities, purity and composition [10]. Potencies of different preparations of STK were also evaluated by the euglobulin lysis test and the SCSA, showing significant variations between the products available for clinical use [11]. The gene from *Streptococcus equisimilis* was cloned in a vector of *E. coli* to overexpress the profibrinolytic protein, and almost all the recombinant STK was exported to the periplasmic space and the bioactivity was evaluated by the chromogenic assay [12]. STK expressed as inclusion body in *E. coli* was refolded into active forms proteins, purified and characterized by chromatographic methods, MALDI-TOF, and the bioactivity was evaluated by the chromogenic assay [5].

Streptokinase produced from species of *Streptococcus pyogenes* was quantified by the method of Lowry, its electrophoretic mobility and molecular weight determined by SDS-PAGE, and the biological activity evaluated by the radial caseinolysis assay [13]. Functional characteristics such as substrate specificity and the effects of pH and temperature on the activity of Streptodornase in marketed product, against the native double stranded DNA were evaluated showing a possible existence of semi-denatured "meta-stable" conformations with reduced levels of DNase activity [7]. But, the validation of the method recommended for biopharmaceutical products, is essential to show that the procedure is suitable for its intended purpose [14].

The aim of this article was to validate a specific, sensitive and stability-indicating chromogenic substrate assay to assess the potency of streptokinase; carry out *in vitro* bioassays to evaluate streptodornase and streptolysin present in the product; thus contribute to improve the quality control and to assure the therapeutic efficacy of the biotechnology-derived product.

Experimental

Chemicals and reagents

The 3rd international standard of streptokinase (IS-STK WHO

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00/464), containing 1030 IU/vial, the 2nd international standard of streptodornase (IS-STD WHO 08/230) and human anti-streptolysin O were obtained from the National Institute for Biological Standards and Control-NIBSC (Hertz, UK). A total of twelve batches of streptokinase, containing 1 500 000 IU/vial and 750 000 IU/vial of streptokinase were obtained, respectively, from Bergamo and Blausiegel (São Paulo, Brazil). The samples were acquired from commercial sources and used within their shelf life period. Plasminogen of bovine plasma, sodium deoxyribonucleate, human serum albumin (HSA), imidazole, and glutamate were acquired from Sigma-Aldrich® (St. Louis, USA). Chromogenic substrate S2251 was purchased from Chromogenix® (Milan, Italy). Tris (hydroxymethyl) aminomethane, sodium thioglycolate, sodium phosphate dibasic anhydrous, calcium sulphate, magnesium sulphate, sodium hydroxide, hydrochloric acid, perchloric acid, and acetic acid were obtained from Merck® (Darmstadt, Germany). All chemicals used were of pharmaceutical or special analytical grade. Ultrapure water was obtained using an Elix 3 coupled to a Mili-Q Gradient A10 system Millipore (Bedford, USA).

Apparatus

The absorbances of the assays were measured on a Multiskan FC microplate reader Thermo Scientific® (Vantaa, Finland), and on a UV-1601 PC-UV-VISIBLE Spectrophotometer Shimadzu® (Kyoto, Japan).

Procedure

Samples and standard solutions: Working standard and sample solutions of STK were prepared daily for the chromogenic assay, by diluting the IS-STK and the samples in 25 mM tris (hydroxymethyl) aminomethane with HSA solution at pH 7.7, to final concentrations between 2.5 and 40 IU/mL. Solutions for the streptodornase assay were prepared by diluting the IS-STD and the samples in imidazole buffer solution (IBS) pH 6.5 to obtain, respectively, solutions containing 20 IU/mL and 150 000 IU/mL of STK. For the streptolysin assay, solution of the sample was diluted in phosphate buffer solution pH 7.2, to obtain a final concentration of 1 000 000 IU/mL of STK.

Streptokinase chromogenic substrate assay (SCSA): The bioassay was performed as described elsewhere for the concentrated solution [9], modified accordingly. Volumes of 25 µL of the IS-STK and the sample solutions with concentrations between 2.5 and 40 IU/mL, were added to the 96-well plate, respectively in triplicate, and allowed to equilibrate at 37°C in water-bath for 1 min. Then, 100 µL of the chromogenic substrate (diluted 1:1 in water) were added to each well, and the plate was incubated for exactly 2 min, followed by the addition of 50 µL of the 1 mg/mL plasminogen. The reaction was stopped 10 min after, by adding 90 µL of 20% acetic acid. The absorbance was measured at 405 nm in the microplate reader and the biological potencies were calculated against the IS-STK by the parallel line statistical method using the CombiStats software (European Directorate for the Quality of Medicines and HealthCare, EDQM Council of Europe).

Streptodornase assay: The bioassay was performed as described elsewhere [9], adapted. Briefly, volumes of 0.5 mL of 1 mg/mL sodium deoxyribonucleate solution in IBS pH 6.5, were added in duplicate to six centrifuge tubes, followed by the addition of 0.25 mL, 0.125 and 0 mL of IBS, respectively. Then, was added 0.25 mL of the sample solution in all the tubes, followed by the addition in sequence, of 0 mL, 0.125 mL and 0.25 mL of the 20 IU/mL solution of IS-STD. The solutions were mixed up and heated at 37°C for 15 min. Two additional tubes were prepared by adding 0.25 mL of IBS and 0.25 mL of the sample solution, maintained without incubation. Then, 3.0 mL of 2.5% perchloric acid were added to all of the tubes, mixed, centrifuged at about 3000 g for 5 min, and the absorbances of the supernatant measured at 260 nm. The mean of the absorbances measured for the 0.25 mL sample concentration spiked in duplicate each one with 0.125 and 0.25 mL of the IS-STD, respectively, was subtracted from the sum of the two absorbances obtained only with the sample. This result was compared and should be higher than the value obtained as a difference between the sample with and without incubation, which means that the sample comply with the requirement that specify a maximum of 10 IU of streptodornase per 100 000 IU of STK.

Streptolysin assay: The bioassay was performed as described elsewhere [9], adjusted. Briefly, a volume of 0.5 mL, equivalent to 500 000 IU of STK of the sample prepared with the diluent composed by 1 volume of phosphate buffer solution pH 7.2 and 9 volumes of a 0.9% sodium chloride, was transferred to a polystyrene tube. A reference solution was prepared in parallel using only the diluent. Then, 0.4 mL of a 2.3% solution of sodium thioglycolate was added, and heated in a water-bath at 37°C for 10 min. A volume of 0.1 mL of a solution of human antistreptolysin O containing 5 IU/mL was pipette, and heated at 37 °C for 5 min. Then, 1 mL of rabbit erythrocyte suspension was added, heated at 37°C for 30 min, and centrifuged at about 1000 g for 10 min. The absorbance of the supernatant was measured at 550 nm, and should be not more than 1.5 times higher than that of the reference solution.

Validation of the Streptokinase chromogenic assay

The assay was validated using samples of a biopharmaceutical formulations of streptokinase with a label claim of 1 500 000 IU/vial and 750 000 IU/vial by determinations of the following parameters: linearity, range, precision, accuracy, detection limit (DL), quantitation limit (QL), robustness and stability, following the guidelines adapted for the *in vitro* bioassay [14,15].

Linearity: Linearity was determined for the assay by constructing three analytical curves, each one with eight concentrations of the IS-STK over the 2.50-40 IU/mL range. The absorbance's were plotted against the respective concentrations of streptokinase to obtain the analytical curve. The results were subjected to regression analysis by the least squares method to calculate the calibration equation and determination coefficient.

Precision: Assay precision was determined by means of repeatability (intra-days) and intermediate precision (interday). Repeatability was examined by six evaluations of the same streptokinase concentration, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by analysis of two samples on three different days (interday) and also by submitting the samples to analysis by other analysts in the same laboratory (between-analysts).

Accuracy: The accuracy was evaluated by applying the proposed assay to the analysis of pharmaceutical solutions with concentrations at 1 200 000, 1 500 000 and 1 800 000 IU/mL equivalent to 80, 100 and 120% of the nominal analytical concentrations, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation; it was expressed as the percentage relative error (bias %) between the measured mean concentrations and the added concentrations.

Limits of detection and quantitation: The detection limit (DL) and the quantitation limit (QL) were calculated by using the mean values of the three independent analytical curves, determined by a linear regression model, where the factors 3.3 and 10 for the detection

and quantitation limits, were multiplied by the ratio of the standard deviation of the intercept and the slope, respectively. The QL was also evaluated in an experimental assay.

Robustness: The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for the routine analysis. The robustness of the SCSA was determined by analyzing the same samples containing 1 500 000 IU/mL and 750 000 IU/mL, respectively, under a variety of conditions of the assay parameters, such as: reaction time with plasminogen (5, 10 and 15 minutes), reaction time with substrate chromogenic (1, 2 and 3 minutes), pH of buffer solution (pH 7.4, 7.7 and pH 8.0), assay temperature (34, 37, 40°C) and stability of the analytical solution at 2-8°C.

Results and Discussion

Method validation

The procedure was performed to demonstrate that the performance characteristics of the SCSA meet the requirements for the potency assessment of streptokinase in biopharmaceutical formulations. The dose-response curve was constructed plotting the experimental values of absorbances versus the logarithms of the concentrations in triplicate. The analytical curves were found to be linear over the concentration range of 2.50-40 IU/mL. The determination coefficient calculated from $y=(55817 \pm 218.57)x-(103001 \pm 8245.10)$, where, x is the concentration and y is the absorbance, was $r^2=0.999$, indicating the linearity of the analytical curve for the assay.

Specificity of the assay for the biomolecule was assessed by determination of the potency of the samples spiked with higher concentrations of the excipients, HSA, glutamate and sodium phosphate. In addition, the samples were also spiked with volumes equivalent to 2 IU/mL of heparin, 2 IU/mL of enoxaparin, 1 EU/mL of bacterial endotoxins, 0.87 nKat/mL of factor Xa, and 1.25 IU/mL of factor IIa, showing non-significant differences ($p>0.05$).

The precision of the SCSA was studied by calculating the relative standard deviation (RSD %), for six analyses at a concentration of 1 500 000 IU/mL, performed on the same day and under the same experimental conditions. The obtained RSD was 1.62%. The intermediate precision was assessed by analysis of two samples of the biopharmaceutical formulation on three different days (interday), giving RSD values of 0.57 and 0.48%, respectively (Table 1). Between-analysts precision was determined by calculating the mean values and the RSD after analysis of two samples of the same biopharmaceutical formulation by three analysts; the values were found to be 0.34 and 0.66%, respectively, as given in Table 1.

The accuracy of the SCSA was assessed from three replicate determinations of three solutions at concentrations of 1 200 000, 1 500 000 and 1 800 000 IU/mL, respectively. The absolute means obtained with a mean value of 100.34, with bias lower than 0.53% (Table 2), confirmed that the method is accurate within the desired range.

The DL and QL of the SCSA were calculated from the slope and the standard deviation of the intercept determined by a linear-regression model, by using the mean values of the three independent calibration curves. The obtained values were 1.10 and 2.61 IU/mL, respectively. The experimental value determined for the QL was found to be 2.50 IU/mL.

The results of the bioassay and the experimental range of the selected variables evaluated in the robustness test are given in Table 3, together with the optimized values. There were no significant changes in the potency results when modifications were introduced into the experimental conditions, thus showing the assay to be robust. The stability of the STK sample solutions was assessed and the data obtained showed non-significant changes, relative to freshly prepared samples, when maintained at 2-8°C for 24 h.

Method application

The validated SCSA was applied to the potency assessment of streptokinase in biopharmaceutical products, giving values within 92.20 and 108.97% of the stated potency, as shown in Table 4, meeting the specifications which claim 90-110% of the stated potency [8]. In addition, as the production of STK can be accompanied by the formation of streptodornase, the activity was assessed by the *in vitro* assay, giving results lower than 9.79 IU per 100 000 IU of STK, as demonstrated in Table 5. Due to the interference of the impurities, the assay was performed with the samples spiked with the IS-STD, calculating the content based on the difference between the absorbances. The streptolysin activity was also evaluated showing absorbances up to 1.47 times higher than the reference solution, in accordance with the specifications, showing the quality of the products.

Conclusion

The results of the validation studies show that the chromogenic substrate assay is specific, sensitive, with a QL of 2.50 IU/mL, and possesses excellent linearity and precision characteristics, and were successfully applied for the potency assessment of STK in biological products. In addition, the results obtained with the bioassays performed to evaluate also the presence of streptodornase and streptolysin, contribute to ensure batch-to-batch consistency and the quality of the biotechnology-derived medicine.

Sample	Inter-days			Between-analysts		
	Day	Recovery[a] %	RSD[b] %	Analysts	Recovery[a] %	RSD[b] %
1	1	99.83	0.57	A	100.97	0.34
	2	99.12		B	99.42	
	3	101.57		C	100.15	
2	1	100.75	0.48	A	98.45	0.66
	2	101.07		B	99.57	
	3	100.88		C	99.12	

[a]Mean of three replicates
[b]RSD=relative standard deviation

Table 1: Inter-days and between-analysts precision data of chromogenic substrate assay for streptokinase in biopharmaceutical formulations.

Nominal concentration IU/mL	Mean concentration measured[a] IU/mL	RSD[b] %	Accuracy %	Bias[c] %
1 200 000	1 204 560	2.16	100.38	0.38
1 500 000	1 501 500	1.69	100.10	0.10
1 800 000	1 809 540	1.87	100.53	0.53

[a]Mean of three replicates
[b]RSD=relative standard deviation
[c]Bias=[(measured concentration-nominal concentration)/nominal concentration] × 100

Table 2: Accuracy of chromogenic substrate assay for streptokinase in the biopharmaceutical formulations.

Variable	Range investigated	STK[a] %	Confidence interval (P=0.95)	RSD[b] %	Optimized value
Plasminogen reaction time	5 minutes	100.72	96.41-109.11	1.44	10 minutes
	10 minutes	100.12	97.23-113.65	0.82	
	15 minutes	101.17	94.42-111.59	1.17	
Chromogenic substrate incubation	1 minute	100.51	98.62-102.14	1.04	2 minutes
	2 minutes	100.31	92.51-109.17	0.41	
	3 minutes	99.00	89.91-109.03	0.88	
Buffer pH	pH=7.4	98.48	87.88-110.24	0.95	pH 7.7
	pH=7.7	99.80	98.40-101.20	0.36	
	pH=8.0	101.12	91.25-112.03	1.14	
Assay temperature	34°C	102.01	92.11-112.97	1.12	37°C
	37°C	99.92	90.13-110.82	0.69	
	40°C	98.33	89.94-107.75	1.27	
Solution stability	Initial	102.90	98.01-120.83	0.75	-
	24 hours (2-8°C)	100.91	96.57-120.88	0.91	

[a]Mean of three replicates
[b]RSD=relative standard deviation

Table 3: Conditions and range investigated during robustness testing with the one-variable-at-a-time (OVAT) procedure for the streptokinase (STK) assay.

Sample	Potency Stated IU/vial	Potency Found[a]		Confidence Intervals (P=0.95)
		IU/vial	%	
1	1 500 000	1 515 150	101.01	96.40-105.80
2	1 500 000	1 582 200	105.48	100.10-111.10
3	1 500 000	1 383 000	92.20	85.90-98.90
4	1 500 000	1 576 950	105.13	97.20-113.60
5	1 500 000	1 589 100	105.94	102.90-109.10
6	1 500 000	1 600 350	106.69	102.50-110.90
7	1 500 000	1 499 550	99.97	93.21-111.65
8	1 500 000	1 476 300	98.42	95.00-101.90
9	1 500 000	1 569 750	104.65	100.30-109.00
10	750 000	806 475	107.53	103.50-111.60
11	750 000	798 000	106.40	97.50-116.10
12	750 000	817 275	108.97	98.10-115.10
Mean	-	-	103.53	-
SD[b]	-	-	4.77	-

[a]Mean of three replicates
[b]SD=Standard deviation

Table 4: Potency, confidence intervals (P=0.95) of streptokinase in biopharmaceutical products by the chromogenic substrate assay.

Sample	Streptodornase		Streptolysin
	Absorbances	Activity	Absorbances
	Sample<Standard+Sample	IU/100 000 IU STK	Sample/Reference Solution
1	0.595<0.815	7.29	1.17
2	0.624<0.792	7.88	1.30
3	0.606<0.803	7.55	1.27
4	0.560<0.933	6.00	1.27
5	0.720<0.875	8.23	1.29
6	0.550<0.840	6.55	1.13
7	0.637<0.859	7.41	1.18
8	0.690<0.705	9.79	1.47
9	0.730<0.825	8.85	1.16
10	0.520<0.945	5.51	1.17
11	0.670<0.980	6.84	1.17
12	0.750<0.940	7.97	1.28

Table 5: Activity evaluation of streptodornase and streptolysin in biopharmaceutical products by *in vitro* bioassays.

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