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Validation of an SDS-Page Method Used to Determine Purity of Recombinant Streptokinase Extracted From Suppositories Applied in Hemorrhoidal Disease Treatment

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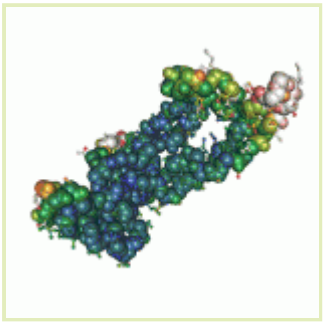

ABSTRACT

Validation of analytical procedures used to determine purity of proteins used as active pharmaceutical ingredients contained in suppositories has not been reported yet. In consequence, this study validated a sensitive and accurate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure applied to determine purity of recombinant Streptokinase (rSK) extracted from suppositories (Proctokinasa®) applied for treating hemorrhoidal disease. Results showed a high specificity, since the smallest protein amount consistently visualized (detection limit) was equal to 100 ng, the precision analysis evidenced a coefficient of variation ?0.1% (repeatability), 0.4% (intermediate precision) and 3% (reproducibility). The study performed using two rSK samples, two acrylamide reagents and different running buffer pH-values corroborated procedure robustness, since it fulfilled all approved criteria for each parameter. Moreover, electrophoretic profile and molecular size of rSK extracted from suppositories and rSK reference material were similar, and no interference with placebo was evidenced. The rSK purity was not affected by suppository manufacturing process and storage conditions (in suppository aluminum foil from 2 to 8 °C). As conclusion, the SDS-PAGE procedure validated in this study is sensitive, precise, consistent and robust and thus can be used for determining purity of rSK extracted from suppositories applied in hemorrhoidal disease treatment.

INTRODUCTION

Streptokinase (SK) is a non-enzymatic extracellular protein secreted by different strains of beta hemolytic streptococci formed by a polypeptide chain composed of 414 amino acids. It is capable of converting plasminogen into plasmin, which degrades fibrin, allowing restoring blood flow to clogged arteries or veins (Suresh et al., 2012; Thelwell, 2014). Because of this, SK is used as a thrombolytic agent in the treatment of different disorders such as acute myocardial infarction, pulmonary thrombolism, surgical complications and permanent vascular access thrombosis (Sikri and Bardia, 2007). On the other hand, anti-inflammatory effects of SK have been also reported (Stringer et al., 1997).

Unlike typical activators such as Urokinase and Tissue Type Plasminogen Activator, SK has no proteolytic activity. Activation

occurs due to formation of an equimolar complex between SK and plasminogen. This complex converts plasminogen into plasmin, by breaking linkage Arg561 - Val562, either from the rest of free plasminogen, such as that which is adsorbed to the clot. In that way, the enzyme smooths thrombus, giving rise to appearance of fibrin degradation products, which possess anticoagulant activity (Koji et al., 2003). That is why; this molecule could be considered as a proper candidate for treating the hemorrhoidal disease.

Hemorrhoids are the most frequent proctologic illness, being a worldwide health problem where thrombosis and/or inflammation with micro-thrombi may be present (Hardy and Cohen, 2014; Zagriadski? et al., 2018). Hemorrhoids are defined as a dilation of hemorrhoidal plexus vein varicose nodules. When they become inflamed, they bleed and ache. About five percent of world population has symptoms related to hemorrhoidal disease, reaching a prevalence close 50% after 50 years of age.

The initial treatment of this disease is based on hygienic-dietetic and symptomatic measures, in addition to the use of local application formulations that focus its main action on inflammation and pain reduction (Altomare and Giannini, 2013). However, benefits are not enough, so a large patient number require invasive procedures such as surgeries (Hackford et al., 2013).

To treat this pathology, a suppository formulation containing recombinant (rSK) as active pharmaceutical ingredient (API) was developed in about a decade ago (Aguilera et al., 2013; Aguilera et al., 2014). The rSK was produced cloning the gene coding SK, isolated from Streptococcus equisimilis from group C of Lancelfield in Escherichia coli (Estrada et al., 1992). The protein expression was evidenced in Escherichia coli under the control of the tryptophan promoter in a multi-copy plasmid (López et al., 2002). Furtherly, the established biotechnological production process allowed producing rSK consistently, with a purity degree ? 90%, high specific activity and non-pyrogen contamination (Suresh et al., 2012; Hernández et al., 2015).

Suppositories are solid dosage forms (Boylan et al., 1986), made to be inserted into mucosal cavities such as rectum, vagina, or urethra, and are intended to exert a local or systemic action. In that sense, suppositories are used when other routes of administration are not practicable. In this case, the disease is treated locally without intervention of specialized personnel or equipments; drug does not come into contact with digestive enzymes that can degrade the API. In some drugs, rectal route is as effective as parenteral route, due to the high irrigation of area by hemorrhoidal veins and lymphatic circulation (van Hoogdalem et al., 1991; Prasanna et al., 2012). Nonetheless, the procedure used for measuring rSK purity formulated into the suppositories was not validated (ICH Q2A, 2005).

Regarding this, all analytical methods and procedures have to be applied taking into consideration API properties. For instance, API chemical instabilities are produced by reactions that occur in the amino acids included in the primary structure (Gupta and Sharma, 2009). In consequence, API suffering changes in physical-chemical characteristics and thus significant losses of biological activity. Thus, multiples considerations must be analyzed in the stability study programs in concordance with principles of physical, chemical, biochemical and immunochemical methods and procedures used for evaluating biological activity, target molecule purity and degradation product percentage of (ICH Q1E, 2001).

Considering this, this paper describes validation of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure used to corroborate the high purity of rSK formulated in Proctokinasa® suppositories for treating the hemorrhoidal disease.

MATERIALS AND METHODS

Materials

All chemicals (analytical grade) used were supplied by Merck, Darmstadt, Germany.

Recombinant Streptokinase

The rSK reference material (RM) and rSK (API) were produced by the Center for Genetic Engineering and Biotechnology, Havana, Cuba, following the procedure described by (López et al., 2002).

Suppository Preparation and Extraction of Recombinant Streptokinase from Suppositories

Suppository formulation was done by the fusion method (Boylan et al., 1986), the mixture was dropped into 2 g cold

suppository molds to be solidified and stored from 2 - 8 °C until rSK purity analysis. For protein extraction, 4 mL of phosphate buffered saline (PBS) (potassium chloride 0.0026 mol/L, sodium chloride 0.136 mol /L, potassium dihydrogen phosphate 0.0019 mol/L, disodium hydrogen phosphate 0.0076 mol/L, pH 7.3) were centrifuged in a 15 mL centrifuge tube and placed in a thermostat bath at 37 °C (Julabo, Seelbach, Germany). As soon as the tube reached 37 °C, suppository was then introduced into a tube for 15 minutes and strongly agitated during five seconds. The tube was placed again at 37 °C for another 15 minutes and next sample contained in the tube was centrifuged at 178 g to collect aqueous phase (Aguilera et al., 2014).

Determination of Protein Concentration

Protein concentration was determined by the modified Bradford method (Bradford, 1976). About 10 ?L of sample was taken at an appropriate dilution and 200 ?L of prepared Bradford reagent as follow (10% orthophosphoric acid (v/v), 5% absolute ethanol (v/v), 0.01% Coomassie G-250 (m/v). Sample concentration was measured on a calibrated spectrophotometer (Varioskan® Flash, Vantaa, Finland) at a wavelength of 580 nm. A bovine serum albumin (BSA) preparation at a concentration of 2 mg/mL was used as reference material (RM). Sample protein concentration was calculated by interpolating absorbance values.

Determination of Recombinant Streptokinase Purity

The rSK purity determination was carried out according to procedure described by Laemmli (1970). Run was performed at 30 mA/cm and staining with bright blue Coomassie G-250, for 2 h. Distribution was carried out with a mixture of 10% methanol and 10% acetic acid, in distilled water. The rSK-RM, BSA and formulated product (FP) were used as samples. The mean, confidence interval, standard deviation and percentage of error were calculated using Microsoft Office Excel software. Acceptance criteria for FP purity were ? 90% and an electrophoretic profile similar to those observed in RM sample.

VALIDATION OF SDS-PAGE PERFORMED TO MEASURE PURITY OF RECOMBINANT STREPTOKINASE FORMULATED FROM SUPPOSITORIES

Specificity

About 20 ?g of total proteins were applied. In the first part, it was stained with Coomassie blue G-250 (Bradford, 1976). Samples placed were RM, FP, Placebo (P) and molecular weight marker (MWM). In the second part, an anti-rSK Western-Blot was performed placing RM, FP, and P as a negative control. BSA was also applied. Next, proteins were transferred to a nitrocellulose membrane for rSK immunoidentification. Subsequently, membrane was incubated in 5% milk dissolved in PBS, for 1 h at 37 °C. The membrane was washed (2 times x 2 min) with PBS and incubated with a rSK specific antibody preparation conjugated to horseradish peroxidase (Merck, Darmstadt, Germany) at 37 °C for 1 hour. Subsequently, membrane was washed (six times for five min) with PBS and incubated at room temperature with substrate solution (11 mL of PBS, 5 mg of diaminobenzidine, 5.5 ?L of H_2O_2). Once color was developed on membrane, substrate solution was removed and immersed in distilled water (Taylor and Posch, 2014).

Detection Limit Determination

Different amounts of rSK RM (0.1, 0.2, 0.5, 1, 2, 5, 10, 15, 20 and 30 ?g) were applied into SDS-PAGE. The detection limit corresponded with the smallest amount of protein consistently visualized.

Precision Determination

Six suppositories were extracted in each trial described above and 20 ?g of total proteins from each sample were applied into the SDS-PAGE.

Repeatability Determination

The coefficient of variation (CV) of purity values among determinations performed by the same analyst in the same day was determined for each test using as acceptance criterion ? 2% (Intermediate precision). The procedure for extraction of rSK contained in six suppositories was done as it is described above. About 20 ?g of total proteins of each sample were applied in two different SDS-PAGE (two analysts). The CV of purity values was calculated to verify variability by and among analysts. The acceptance criterion was ? 3%. Subsequently, an ANOVA test was performed for two groups of purity values assuming

equal variances and means were compared by Student's t-test for determining whether or not statistical differences existed. Acceptance criteria: CV ? 3%, if t calculated ? t tabulated, there were no significant differences for a value of ? equal to 0.05.

Robustness Analysis

Two samples of different batches of acrylamide reagent were evaluated in different SDS-PAGE. In each electrophoresis RM, FP, P and MWM samples were applied. On the other hand, run buffer used were: 0.1% sodium dodecylsulfate; glycine 1.44%; Tris- (hydroxymethyl) aminomethane 0.3% (running buffer), at pH 8.3 (variant 1) and at pH 8.1 (variant 2). As acceptance criteria, the test was robust if electrophoretic profile of FP was similar to the electrophoretic profile of RM used in the technique. The molecular size of samples was determined by the software Quantity One 4.6.5.

RESULTS AND DISCUSSION

Multiples tests have been established in Cuba to evaluate medicine quality for the sanitary registration, which are in harmony with guidelines of the International Conference on Harmonization (ICH Q1E, 2003). In that sense, quality control of suppositories is carried out in three moments: raw material control, control during process through organoleptic, physical and mechanical examinations, for example: weight, liquefaction or fusion time and hardness; and FP control to see if it complies with its physical, chemical and microbiological properties (Chul et al., 2005).

In general, therapeutic proteins require preservation of their native status for the lifetime of API or any changes in their folding during processing has to be reversible (Wang, 2015). Related to this, physical degradation is related to alterations in secondary, tertiary or quaternary structures of proteins among which, denaturation, aggregation, precipitation, and adsorption may have a large impact on the protein purity and biological activity (Savale, 2016). The main contributing causes to this phenomenon are shear forces, creation of interfaces, presence of surfactant, pH, ionic strength, buffer composition and temperature, which could be also the main responsible for rSK degradation during suppository preparation (Zapadka et al., 2017). For instance, purity decrease provoked by temperature is a phenomenon observed in protein formulations because it decreases free energy associated with native structure modifications (Kamerzell et al., 2011). In the case of rSK, it has been demonstrated the beta domain is the most heat-labile or susceptible to the temperature effect (Beldarraín et al., 2001). Therefore, estimation of rSK purity during suppository formulation and store conditions with a validated method or procedure is mandatory for product approval and application.

Results of the validation experiments performed in this study showed firstly, a high specificity of SDS-PAGE procedure. It was corroborated with the help of electrophoretic separation and estimation of rSK molecular size compared with rSK-RM and with the use of the Western-blot anti-rSK identification procedure. In detail, Figure 1 illustrates a 12.5%-SDS-PAGE representation done for separating rSK extracted from suppositories. Both rSK RM and FP are in the same height of MWS corresponding with reported molecular weight for rSK (43 kDa) (Beldarraín et al., 2001). Moreover, no band was detected when extraction method was applied to P-sample, corroborating the method is specific and interferences of suppository excipient were not produced. The Western blotting technique, using an antibody specific for rSK allowed detecting rSK over the same time period in RM, FP and MWS samples. As results no signals were observed for negative control and P-formulation samples. Besides, values of purity and molecular weight estimated for rSK contained in the FP and RM samples were 94± 1% and 43 kDa, respectively. The molecular weight estimation was made by interpolating in the graph the Rf value (relative electrophoretic mobility) and the Logarithm of the MW (molecular weight of each known protein) (Figure 2). These results fulfilled the test acceptance criteria of method, corroborating that electrophoretic profile of rSK extracted from suppositories was similar to the rSK RM electrophoretic profile.

FIGURE 1

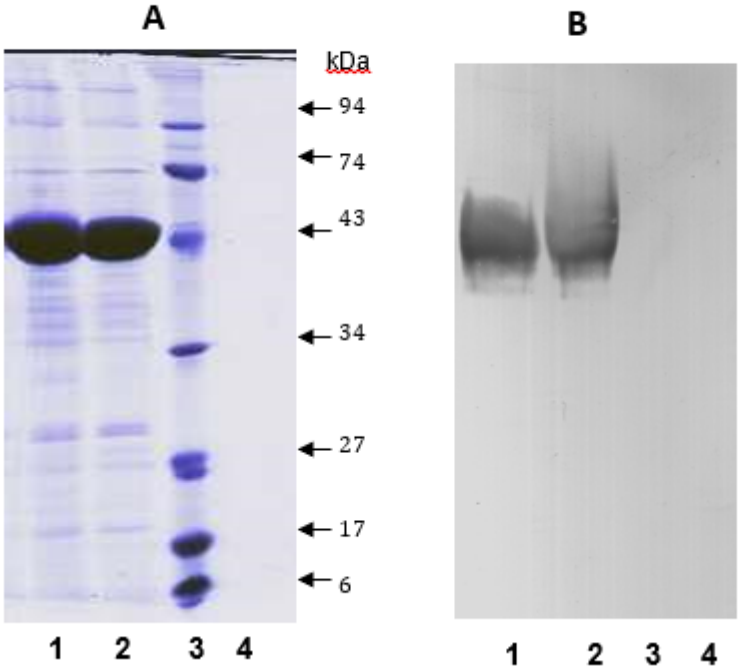

Figure 1. *Results of specificity determination. (A) 12.5%-SDS-PAGE for separating rSK extracted from suppositories. (A) Lane 1, RM rSK. Lane 2, WM (SERVA). Lane 3, FP. Lane 4, Placebo suppository. (B) Western-blot anti-rSK: Lane 1, RM rSK. Lane 2, WM (SERVA).Lane 3, FP batch. Lane 4, placebo suppository.*

In regard to SDS-PAGE detection limit determination, different rSK RM amounts: 0.1; 0.2; 0.5; 1; 2; 5; 10; 15; 20; and 30 µg[LP2] of rSK were applied to SDS-PAGE. The FP and P-samples evidenced that the smallest visualized amount of rSK visualized was 0.1 µg (Figure 3). As it can be noted, in FP sample, no band showed higher intensity than intensity of band corresponding to 10% of RM (Lane 4, 1 ?g). In that sense, many dyes have been described for staining proteins, but Coomassie Blue is the most popular. Because of its high extinction coefficient and high affinity for proteins, detection limit reported is close to 1 µg (Dorri and Kurien, 2018), which is coincident with results obtained in this study. In addition, none of excipients (Placebo) used in the formulation had interferences in SDS-PAGE assay, corroborating again the specificity of SDS-PAGE method for rSK.

The measurement of the precision of a method is carried out on the basis of a sufficient number of determinations of a homogeneous mixture of the product, in the same conditions, on the same sample, by the same analyst, in the same laboratory and with the same equipment and reagents; generally in a short interval of time, which is why it evaluates the intrinsic variability of the process, also named within-assay precision.

FIGURE 2

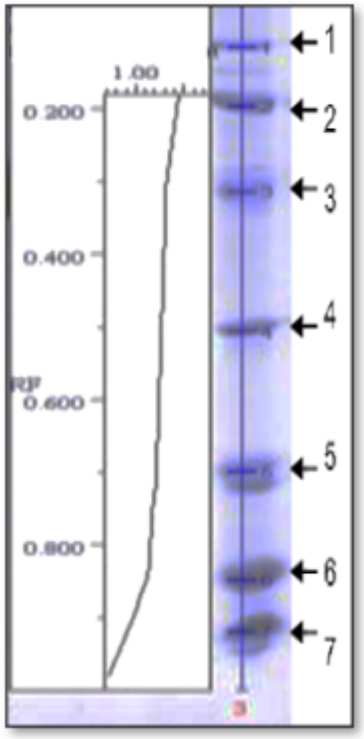

Figure 2. *Results of specificity Assay. Molecular weight estimation was made by interpolating the relative electrophoretic mobility value (Rf)) and the Logarithm of the MW (molecular weight of each known protein) in the graphic. Lanes 1-7, MWS.*

Results revealed no significant differences between the purity values obtained by each analyst for the six extractions performed in the repeatability test (94 ± 0.1; 94 ± 0.3, 94 ± 0.2; 94 ± 0.4). The coefficient of variation (CV) obtained for each test was less than 2% (Table 1). The statistical analysis demonstrated a tabulated t for 2 tails (2.23) value greater than calculated t (0.14).

FIGURE 3

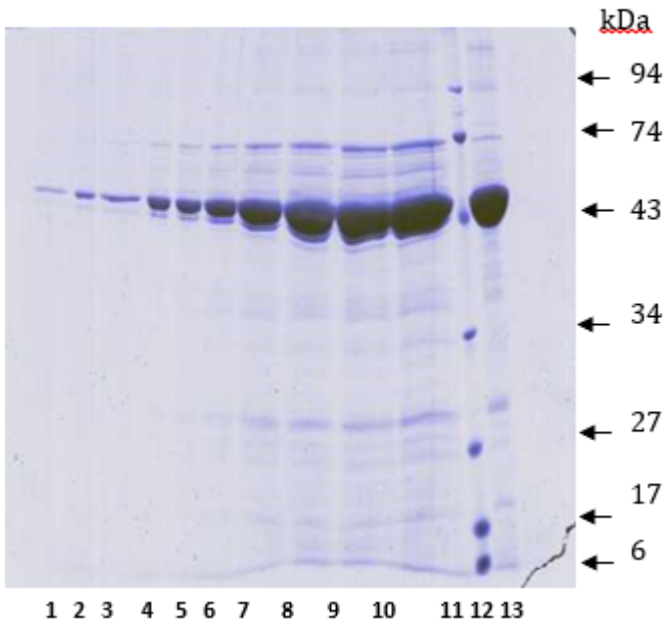

Figure 3. *Illustration of the detection limit determination. SDS-PAGE made by analyst 1. Lane 1, 0.1 µg. Lane 2, 0.2 µg. Lane 3, 0.5 µg. Lane 4, 1 µg. Lane 5, 2 µg. Lane 6, 5 µg. Lane 7, 10 µg. Lane 8, 15 µg. Lane 9, 20 µg. Lane 10, 30 µg SKr RM. Lane 11, MWS. Lane 12, FP. Lane 13, P sample.*

TABLE I

Samples	Parameter	Analyst 1		Analyst 2	
	Average Value (%)	94	94	94	94
A	SD	0.1	0.3	0.2	0.4
	CV (%)	0.1	0.4	0.2	0.4
	Average Value (%)	94	94	94	94
B	SD	0.1	0.3	0.2	0.4
	CV (%)	0.1	0.4	0.2	0.4

Table 1. *(A) Purity values of samples of six independent extractions from suppository. (B) Purity values of samples of 10 replicas of six extraction mixture (Intermediate precision)*

On the other hand, Figure 4 (Analyst 1) shows an electrophoretic profile of FP, which was similar to electrophoretic profile of rSK RM. Therefore, the validated SDS-PAGE method fulfilled acceptance criteria for the repeatability test satisfactorily. In addition, it was also observed that CV for each test performed by the same analyst in two different gels was ?0.4%, and thus the criterion established to evaluate the intermediate precision was also fulfilled (? 3%). There were no significant differences between purity results obtained by each analyst after mixing of the six extractions analyzed in each gel (two gels per analyst and 10 replicates for each gel). It was corroborated by the Student's t-test where the tabulated t (2.10) was greater than the calculated t (0.36 for analyst 1 and 0.19 for analyst 2), Figure 5. In addition, the CV between tests was calculated by analyst being 0.26% for analyst 1 and 0.31% for analyst 2; lower than the established criteria (Table 1). The CV between analysts was 0.29%, fulfilling the acceptance criterion for the intermediate precision analysis (variability among analysts) in the control of FP samples (?3%). In the Student t test, the tabulated t for 2 tails (2.02) was greater than calculated t (0.79), so it also corroborated no significant statistical differences. The CV values between molecular sizes of replicates were considered satisfactory, considering that they were less than 3%. Then, this method was accurate because there were no significant differences between analysts and days.

FIGURE 4

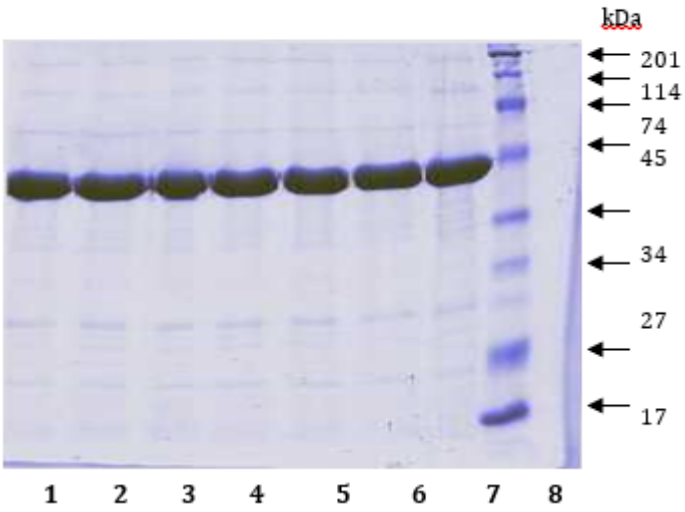

Figure 4. *Results of repeatability assay performed by analyst 1. Lanes 1 to 6, 20 µg FP. Lane 7, RM rSK. Lane 8, MWS.*

FIGURE 5

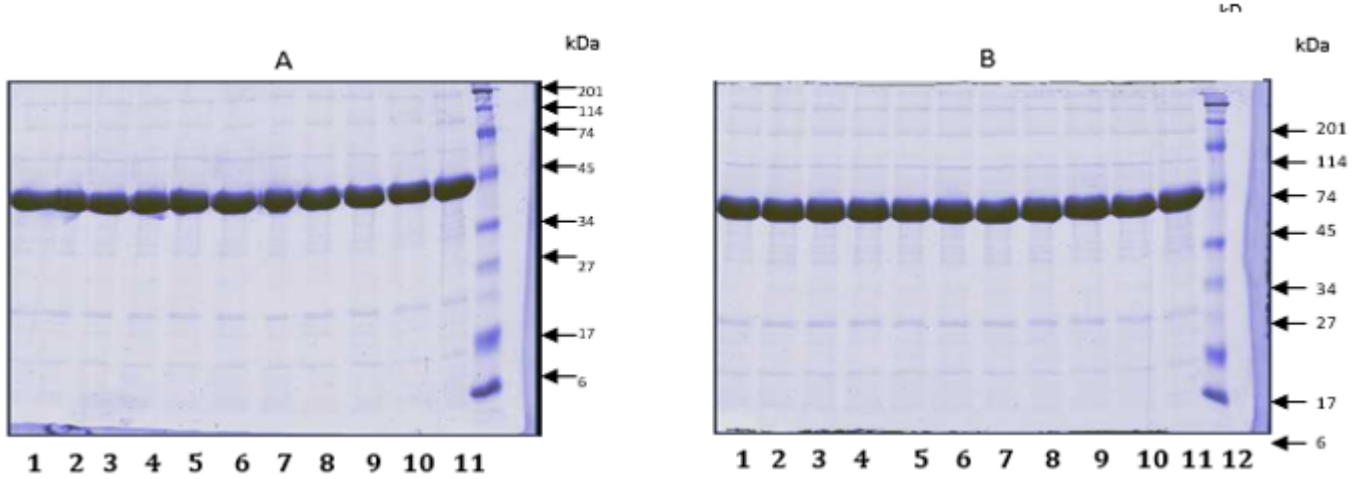

Figure 5. *Results of intermediate precision assay. (A). Analyst 1; Lanes 1-10, 20 ?g of FP. Lane 11, rSK RM. Lane 12, MWS. (B). Analyst 2. Lanes 1-10, 20 ?g FP lot. Lane 11, rSK RM. Lane 12, MWS*

Finally, a robustness study was also carried out to conclude validation of SDS-PAGE procedure standardized to estimate purity of rSK extracted from suppositories. In regard to this study, it is important to know first that the acrylamide is a chemically inert support frequently used in gel electrophoresis, with uniform properties, and easy and reproducible preparation. It also forms transparent gels with high mechanical stability, insoluble in water, relatively non-ionic and allows good visualization of bands for a relative long time. Furthermore, it has the advantage that by varying the concentration of polymers, the pore size could be modified in a controlled manner, so variations in this reagent modify the gel resolution and thus the protein separation. Concerning to this, the use of high quality reagents and deionized water are prerequisites that have to be considered to guarantee reproducible and high resolution gels, in particular, this is very important for acrylamide, since it is the most abundant constituent of the gel. As its main impurity is residual acrylic acid, due to, among other causes, absorption of laboratory environment water, which could delay the electrophoretic mobility of proteins (Maizel, 2000).

In this study, two different batches of acrylamide, from the same supplier and prepared under the same laboratory conditions were used to study behavior of rSK electrophoretic profiles. A shown in Figure 6: Acrylamide batch 1; Lane 1: RM of rSK; Lane 2 FP. Lane 3 MWM and Lane 4 P and B: Acrylamide batch 2; Lane 1: RM of rSK; Lane 2 P, Lane 3 FP and Lane 4 MWM, the electrophoretic profiles fulfilled the test acceptance criteria, corroborating that the electrophoretic profile of rSK in suppository FP was similar to the profile rSK RM and thus differences in these two batches of acrylamide did not affect the electrophoretic

profile and molecular size of FP samples corresponded to the expected molecular size (43 kDa). In addition, no interferences of excipients were detected in the rSK purity analysis.

Therefore, these results allowed concluding assessed samples met product quality specification (? 90%) in the acrylamide batch independent manner.

Secondly, most biomolecules (proteins, nucleic acids and polysaccharides) have certain electrical charge with anionic and cationic groups capable of dissociating. The net charge of particles is given by pH of the medium and can be modified by interaction with small molecules of ions or other macromolecules, so pH may also has influences on migration speed of target molecule (Chandra, 2006). In the case of SDS electrophoresis, the molecule is unfolded and negatively charged, but it has been reported that changes in pH of running buffer could also affect electrophoretic mobility (Zahid et al., 2014). In this sense, it is known that pH value of Tris-glycine buffer changes with temperature (Chandra, 2006), so the theory support the existing of independent two ionic species Cl- (conductive ions) and glycinate (lagging ions), which migrate in the same direction of molecules. At the beginning of electrophoresis, the Cl- ions tend to move rapidly, separating from the rest of ionic species and leaving behind an area of low conductivity (Jovin, 1973). The specific conductivity is inversely proportional to the strength of electric field; a voltage gradient is formed in this region accelerating the movement of the lagging ions, which causes them to migrate behind the conductive ions and at the same speed. As consequence, changing in pH values could shift the equilibrium to glycine hydrochloride, which could affect the area of low conductivity and prevent an adequate concentration of proteins in the gel. Considering this, another study was conducted to know influence of pH values. Figure 7 illustrates results using running buffer with different pH values. In A, run buffer was used at pH 8.3 (variant 1, pH 8.3 and variant 2, pH 8.1) after separation of rSK from suppositories. Ass results, electrophoretic profile of FP was similar to electrophoretic profile of rSK RM. Table 2 shows values of purity and the estimated molecular weight for analyzed sample, where the molecular size of FP also corresponded with expected value of rSK RM. Besides, all values were in the same order in both buffers with different pH values. Summarizing, this SDS-PAGE procedure is robust, since it was not affected by small variations of the acrylamide batches and pH of the functioning buffer. As supplementary information a stability study to the current formulation was made demonstrating stability of the rSK in FP for 18 months. The rSK purity remained above 90% in 18 months and no degradation was neither detected (Aguilera et al., 2013).

FIGURE 6

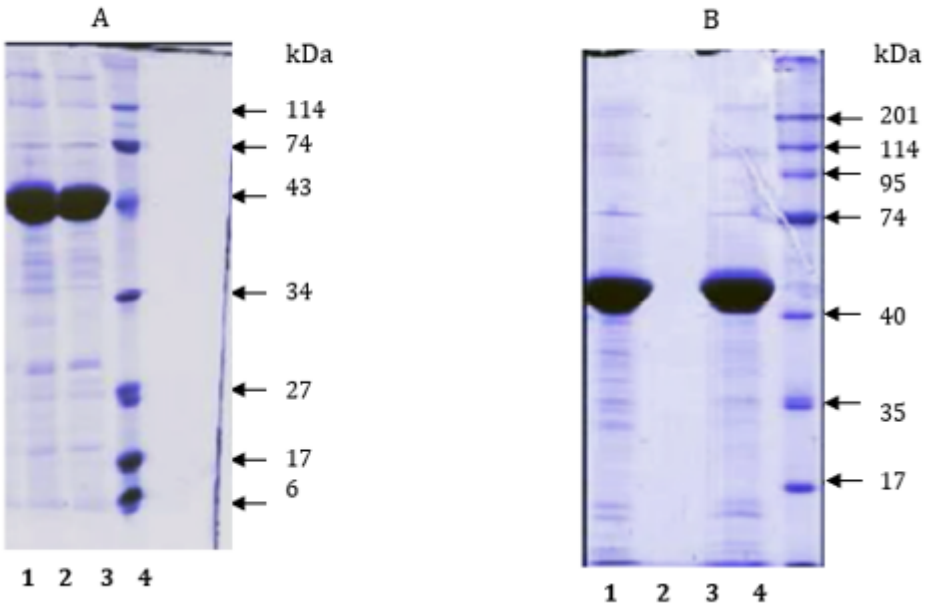

Figure 6. *Results of electrophoresis profile of two different batches of acrylamide, from the same supplier, prepared under the same laboratory conditions. A: Acrylamide batch 1; Lane 1, RM of rSK. Lane 2, FP. Lane 3, MWS. Lane 4, Placebo. B: Acrylamide batch 2; Lane 1, RM of rSK. Lane 2, Placebo. Lane 3, FP. Lane 4, MWS.*

.Figure 7

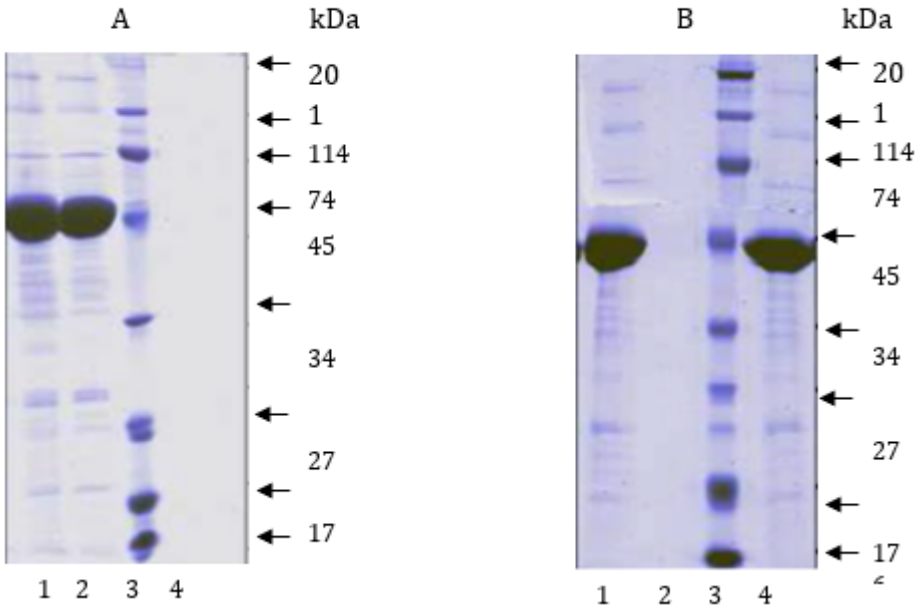

Figure 7: *Results electrophoresis profiles in SDS-PAGE using running buffer with different pH values. A: Running buffer pH 8.3; Lane 1, rSK. Lane 2, FP. Lane 3-MWS. Lane 4, Placebo B: Running buffer pH 8.1; Lane 1, rSK RM; Lane 2, P sample. Lane 3, MWS, Lane 4, FP*

TABLE II

Samples	Purity (%)		Molecular Weight (kDa)*	
	Variant 1	Variant 2	Variant 1	Variant 2
RM (SKe-06-0210)	94	93	43	43
FP	94	93	43	43

Table 2. *Results of the purity and molecular weight determination of samples assessed in the robustness study*
**Molecular weight was estimated by interpolating in the graph of the Rf value (relative electrophoretic mobility) and the logarithm of the MW of each known protein).*

CONCLUSIONS

The SDS-PAGE method validated in this study is sensitive, precise, consistent and robust and therefore can be used for determining the purity of rSK extracted from suppositories applied in hemorrhoidal disease treatment. Purity of rSK is not affected by suppository manufacturing process and neither by store[LP3] conditions since purity and molecular profile of the extracted rSK is similar to the non-formulated rSK reference material.

CONFLICT OF INTEREST

The work described here has not been published previously and none of authors have any conflicts of interest to declare that limit publication of this paper in this journal.

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