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Method Development for Identification of Streptokinase in Pharmaceutical Formulation by SDS-PAGE/Western Blot as an Alternative to Pharmacopoeia Method

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ABSTRACT Streptokinase is currently used for the treatment of thromboembolic blockages such as stroke. Due to its high price, illegal streptokinase has been found in pharmaceutical market. According to Drug Act of B.E. 2510 (1967), the punishment can be done if the object belonging to the culprit is proved as real medicine. Thus, the identification test of the drug is especially important for the investigation process. The identification method for streptokinase in pharmacopoeia requires the use of animal plasma; however, the reduction of animal usage in drug test by using alternative method is widely accepted in every corner of the world. Therefore, our study aimed to develop the identification test for streptokinase using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as an alternative for pharmacopoeia method. Various factors including concentrations of primary and secondary antibodies, incubation period, and limit of detection (LOD) for streptokinase standard and streptokinase pharmaceutical formulation were validated. The optimal concentrations of primary and secondary antibodies were at 7.5 × 10-3 and 2 × 10-3 mg/ml, respectively. The incubation periods for both primary and secondary antibodies at 1 h provided the strongest signal. LOD values of streptokinase standard and streptokinase pharmaceutical formulation were at the concentrations of 0.5 and 20 IU/ml, respectively. The human albumin, which is used as stabilizer in the streptokinase pharmaceutical formulation, was not interfered with the test. The results of the study confirmed that the method is reliable and applicable for the identification of streptokinase in pharmaceutical formulation.

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Introduction

Streptokinase (SK) consists of 415 amino acids in a polypeptide chain[1]. SK is an extracellular protein and originally extracted from certain strains of beta hemolytic streptococcus[1, 2]. SK is a potent activator of plasminogen, an inactive precursor of plasmin, which degrades fibrin clot[3-6] and currently used for the treatment of thromboembolic blockages including coronary thrombosis such as stroke and myocardial infarction[7, 8]. More than ten brands of SK products have been launched in the global pharmaceutical market[9], while information from the Thai FDA website informs that there are two trademarks of SK products available in Thailand[10]. Due to its high price, illegal SK drug product has been reported in the pharmaceutical market and leads to prolong illness or possibly death. According to Drug Act of B.E. 2510 (1967), the punishment can be done if the illegally-imported object belonging to the culprit is proved as real medicine. Therefore, the identification test is a key milestone to prove the fictitious SK. However, the identification method for SK drug product by studying clot-lysis method in pharmacopoeia requires the animal plasma. The reduction of animal usage in drug test by using alternative method is widely accepted in every corner of the world. There were studies reporting the SK identification test using SDS-PAGE and gel clot methods[11, 12]. However, the SDS-PAGE is not specific for only SK as it detects all proteins in sample. The gel clot method needs the human plasma and thrombin and therefore the study requires the ethics approval, which usually takes time for consideration. Thus, our study aimed to develop an alternative identification test for SK, which reduces the use of animal in the experiment and also specific to SK in pharmaceutical formulation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB) has benefits as it requires small quantity of sample and does not need animal plasma. In addition, the use of specific antibody for reacting with SK by WB brings advantage for the identification test in term of specificity.

Materials and methods

1. Materials

Ultrapure water (Type I) was used throughout the study. Methanol, hydrochloric acid, orthophosphoric acid and acetic acid were from Carlo Erba Reagents (Rodano, Italy). Normal saline solution was from Thai Nakorn Patana Co., Ltd. (Nonthaburi, Thailand). Sodium chloride and human serum albumin was from Sigma (Buchs, Switzerland). Potassium chloride and glycerol were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), bromophenol blue, glycine, 30% acrylamide/bis solution (37.5:1), ammonium persulfate, Tetramethylenediamine (TEMED), Tris (hydroxymethyl)-aminomethane (Tris), Tween-20 and nonfat dry milk (blotting-grade blocker) and Horseradish peroxidase (HRP) substrate kit were obtained from BioRad Laboratories (California, USA.). The silver staining kit was obtained from GE HealthcareBio Sciences AB (Uppsala, Sweden). Trichloroacetic acid was from Fluka (Steinheim, Germany). Sheep antiSKpolyclonal antibody (primary antibody) and rabbit anti sheep IgG-H&L-HRP

antibody (secondary antibody) was from Abcam (Cambridge, UK). Protein marker (Molecular weight (MW) ranging from 12 to 225 kDa) was from GE Healthcare (Buckinghamshire, UK). The SK pharmaceutical formulation (Streptase®) was a generous gift from Sanofi-aventis (Thailand) Co., Ltd. SK standard was purchased from NIBSC (Heartfordshire, UK).

2. SDS-PAGE/Western blot

SDS-PAGE/WB is composed of two techniques, which base on the separation of proteins according to their sizes by SDS-PAGE and the use of antibody to react with a specific protein in sample by WB. SDS-PAGE/WB for SK was performed as follows:

2.1 SDS-PAGE

Resolving and stacking gels were prepared at the concentrations of 12.5 and 4% (w/v), respectively. The resolving gel mixture (1 gel) consisted of 4.125 ml of 30% acrylamide/bis solution (37.5:1), 2.5 ml of 2M Tris buffer at pH8.8, 3.375 ml of water, 50 µl of 10% (w/v) ammonium persulfate and 5 µl of TEMED. The stacking gel (1 gel) was composed of 1.32 ml of 30% acrylamide/bis solution (37.5:1), 2.5 ml of 2 M Tris buffer at pH 6.8, 6.1 ml of water, 50 µl of 10% (w/v) ammonium persulfate and 10 µl of TEMED. Loading buffer (10 ml) was composed of 3.1ml of Tris-HCl pH 6.8, 5 ml of glycerol, 0.5 ml of 1% (w/v) bromophenol blue and 1.4 ml of water.Running buffer was composed of 14.40 g of glycine and 3 g of Tris dissolving to the volume of water at 1000 ml. SK standard, SK pharmaceutical formulation and human albumin were dissolved and diluted with normal saline solution to obtain the required concentrations. The sample and loading buffer were then mixed at ratio of 4:1 before loading into gel. The gel was run in a chamber containing running buffer at 200 V until the dye front was about 5 mm from the bottom and the machine was stopped.

2.2 Western blot

The protein on the polyacrylamide gel was then transferred onto nitrocellulose membrane by using the Mini Trans-Blot® Electrophoretic Transfer Cell at 100 V for 1 h. The blotted nitrocellulose membrane containing SK was firstly incubated with primary and then secondary antibody. The immunoreactivity of antibody and specific protein was detected using the HRP substrate colorimetric detection kit.

3. Optimization of SDS-PAGE/Western Blot

The study of optimum concentration of antibody was performed using a cross-table method.The concentrations of primary antibody were investigated at 2.5×10^{-3}, 7.5×10^{-3} and 12.5×10^{-3} mg/ml, while concentrations of secondary antibody were evaluated at 5×10^{-4}, 2×10^{-3} and 3.5×10^{-3} mg/ml. Both primary and secondary antibodies were diluted with blocking buffer containing 5% (w/v) nonfat dry milk in Tris buffer saline to obtain the required concentrations. The incubation periods for primary and secondary antibodies were also examined at 30, 60 and 90 min.

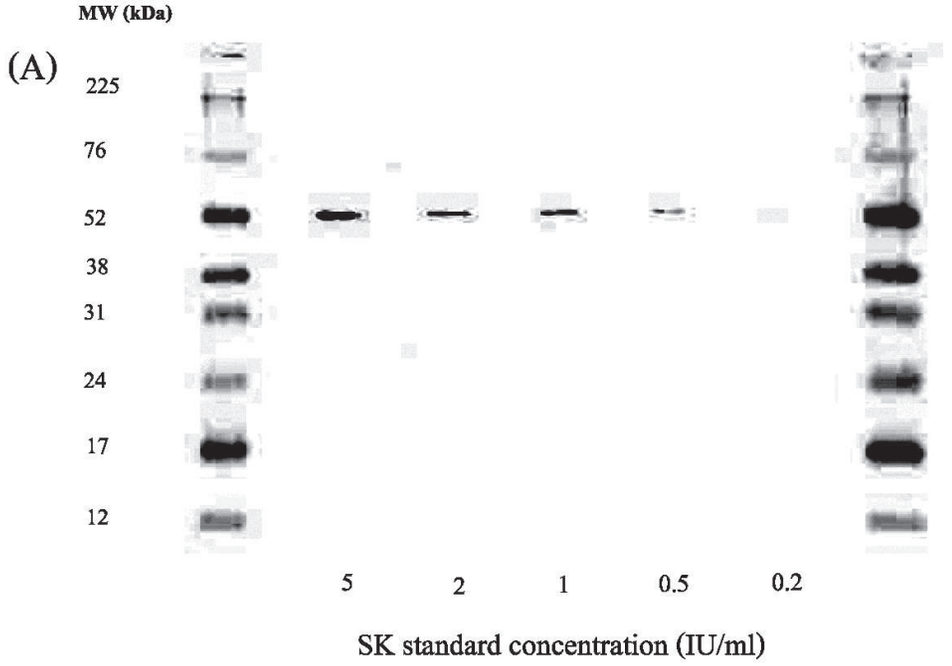
4. Validation of SDS-PAGE/Western Blot

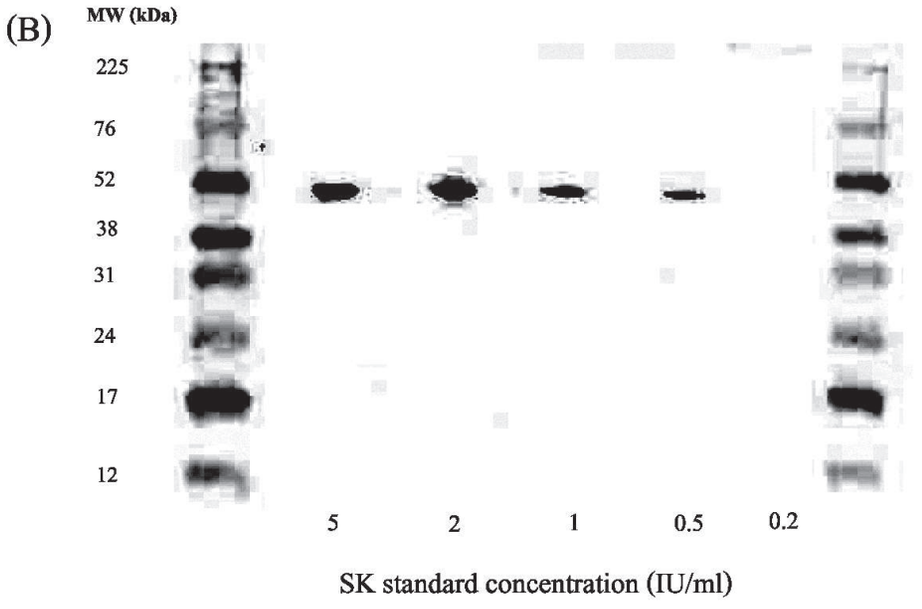
The limit of detection (LOD) of SK standard and SK pharmaceutical formulation (Streptase®) was carried out at concentrations of 0.2-50 and 1-100 IU/ml, respectively. The specificity of the method was also performed by running SDS-PAGE/WB of SK standard (2 IU/ml), SK pharmaceutical formulation (100 IU/ml) and human albumin (negative control, 5 mg/ml) at the same time.

The precision of the SDS-PAGE/WB for the identification test of SK was investigated for three days consecutively. The concentrations of primary and secondary antibodies were used in the study at 7.5×10^{-3} and 2×10^{-3} mg/ml, respectively. The incubation periods of both primary and secondary antibodies were at 1 h. The concentrations of SK standard, SK pharmaceutical formulation and human albumin were performed at concentrations of 2 IU/ml, 100 IU/ml and 5 mg/ml, respectively. The study was detected by both WB and silver staining.

Results

The conditions for the identification of SK in pharmaceutical formulation using SDS-PAGE/WB were developed. The important factors affecting the method including the concentrations and incubation period of primary and secondary antibodies, and the interference of albumin in SK formulation were validated. The influences of primary and secondary antibody concentrations were shown in figure 1 and 2.





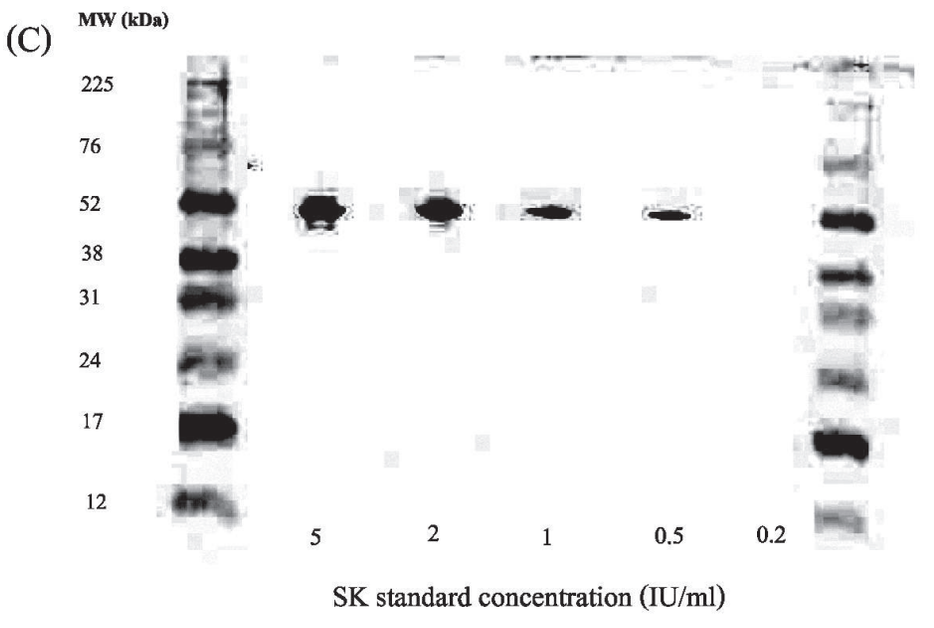


Figure 1 Determination of optimum concentration of primary antibody: (A) 2.5×10^{-3} mg/ml (B) 7.5×10^{-3} mg/ml and (C) 12.5×10^{-3} mg/ml. The incubation periods for both primary and secondary antibodies were 1 h. The concentration of secondary antibody was fixed at 2×10^{-3} mg/ml.

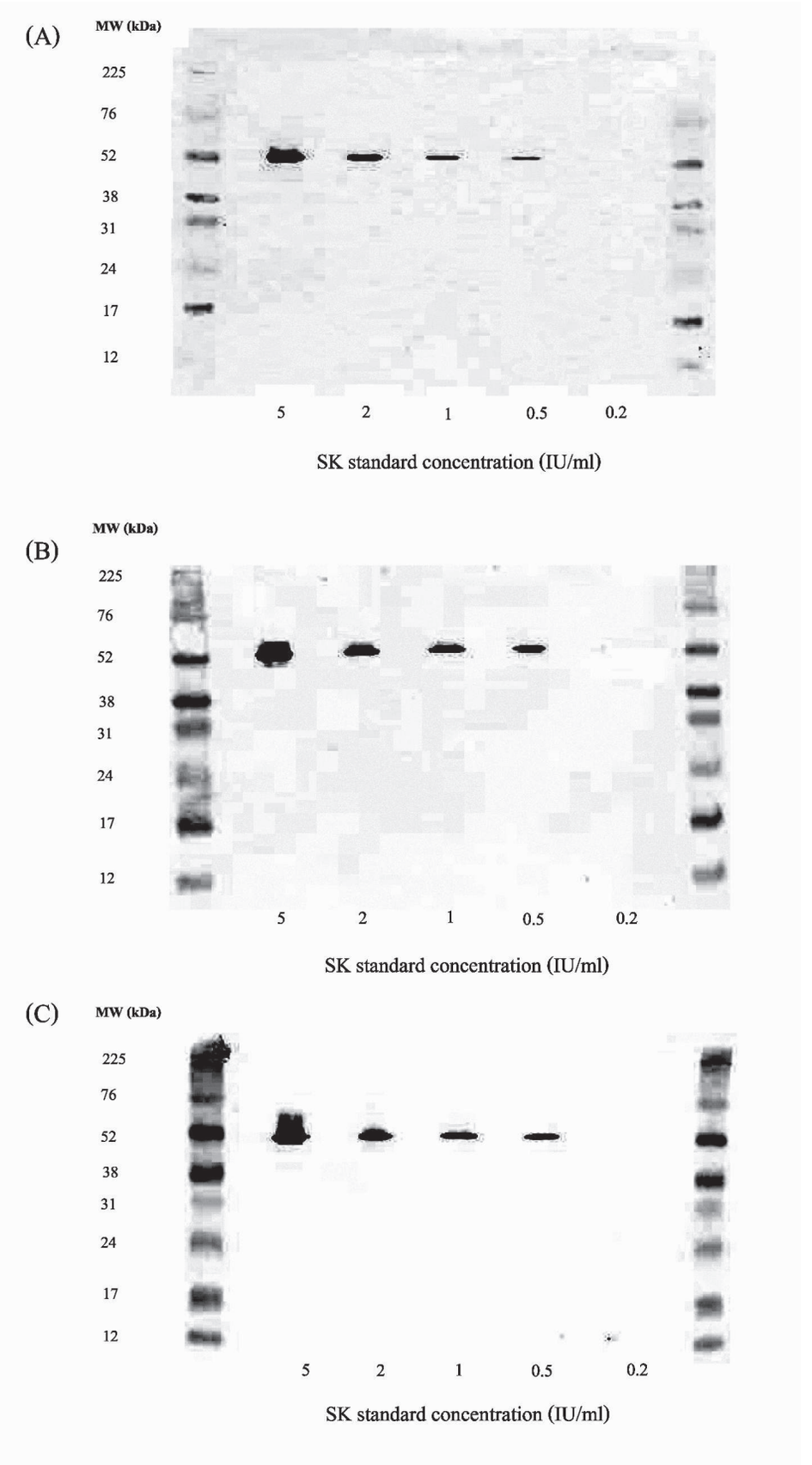


Figure 2 Determination of optimum concentration of secondary antibody: (A) 5×10^{-4} mg/ml (B) 2×10^{-3} mg/ml and (C) 3.5×10^{-3} mg/ml. The incubation periods for both primary and secondary antibodies were 1 h. The concentration of primary antibody was fixed at 7.5×10^{-3}mg/ml.

The effect of incubation periods for primary and secondary antibodies was demonstrated in figure 3 and 4, respectively.

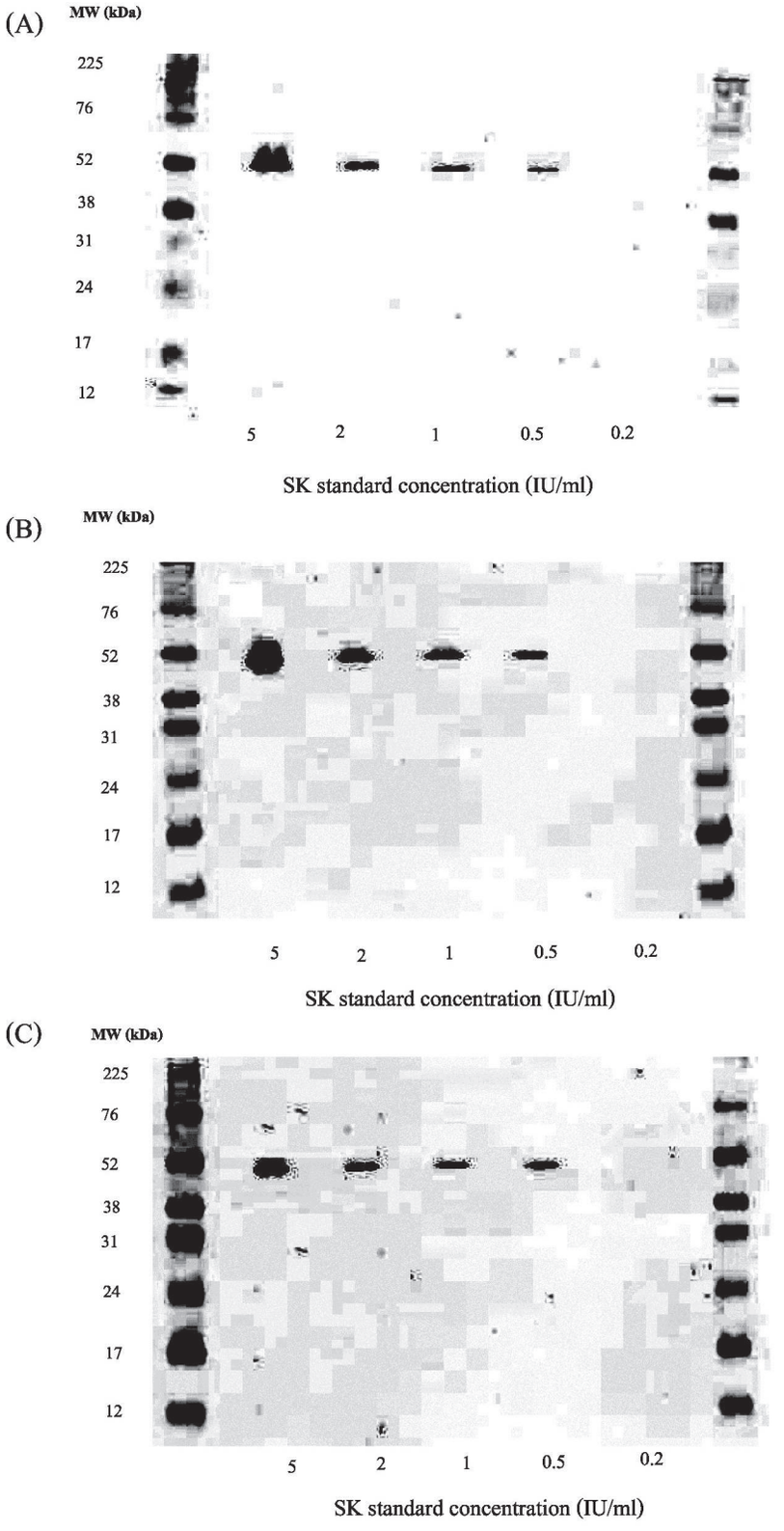


Figure 3 Determination of incubation period for primary antibody (A) 30 min, (B) 60 min and (C) 90 min. The concentrations of primary and secondary antibody were at 7.5×10^{-3} and 2×10^{-3} mg/ml, respectively.

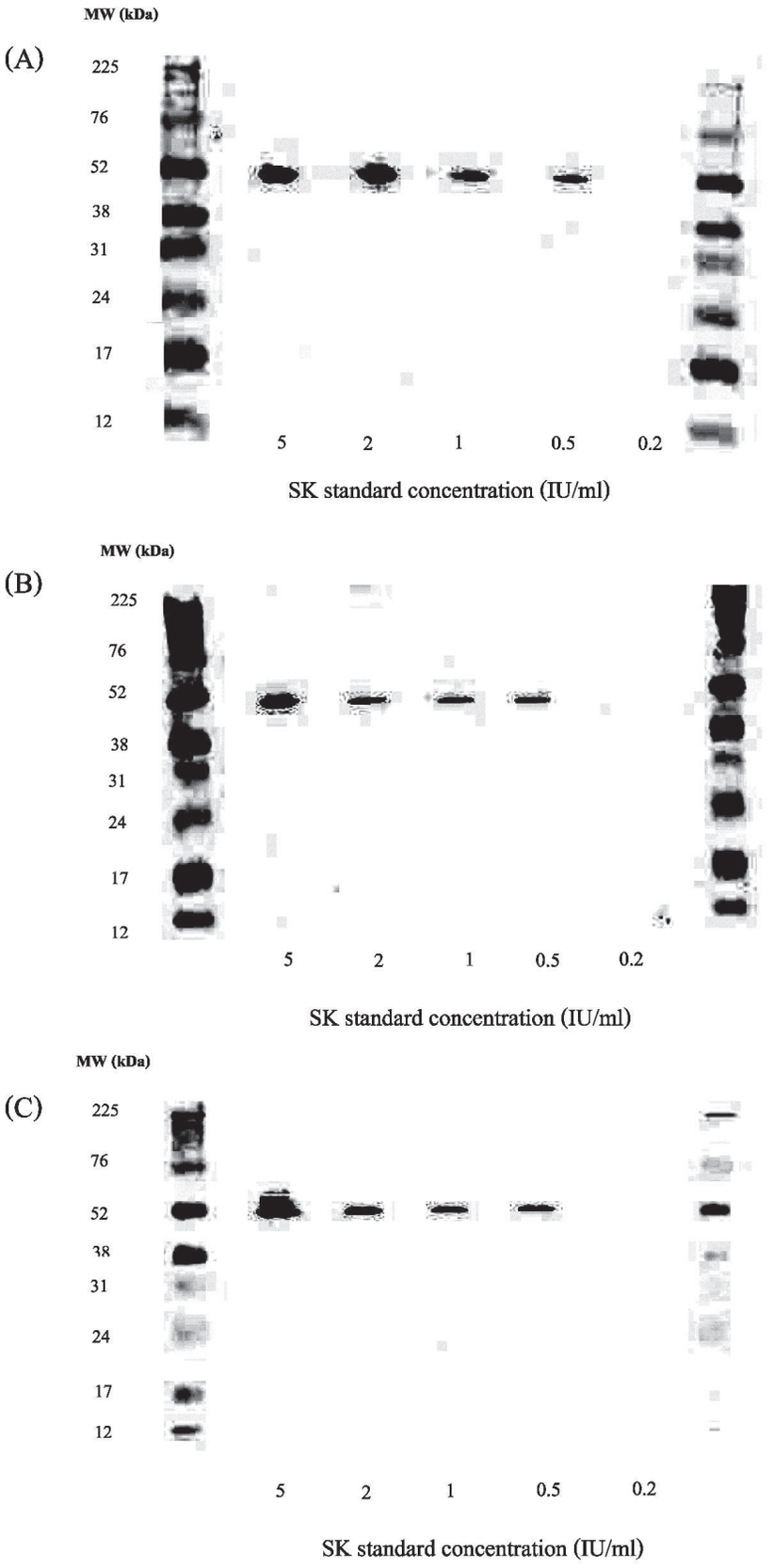


Figure 4 Determination of incubation period for secondary antibody:(A) 30 min, (B) 60 min and (C) 90 min. The concentrations of primary and secondary antibody were at 7.5×10^{-3} and 2×10^{-3} mg/ml, respectively.

The determination of LOD for SK standard (SD) and SK pharmaceutical formulation (Streptase®, SP) was performed and the result was indicated in figure 5.

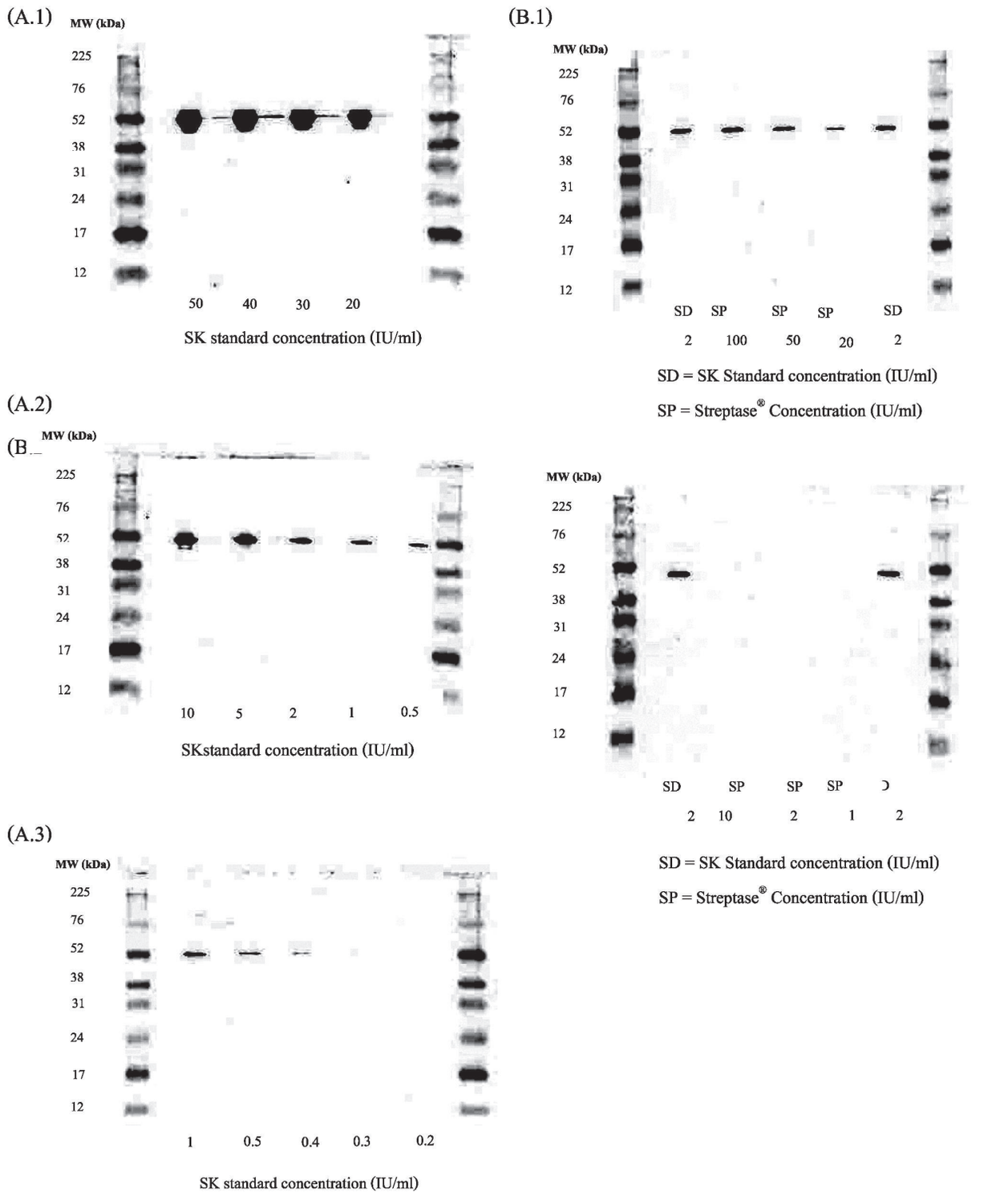
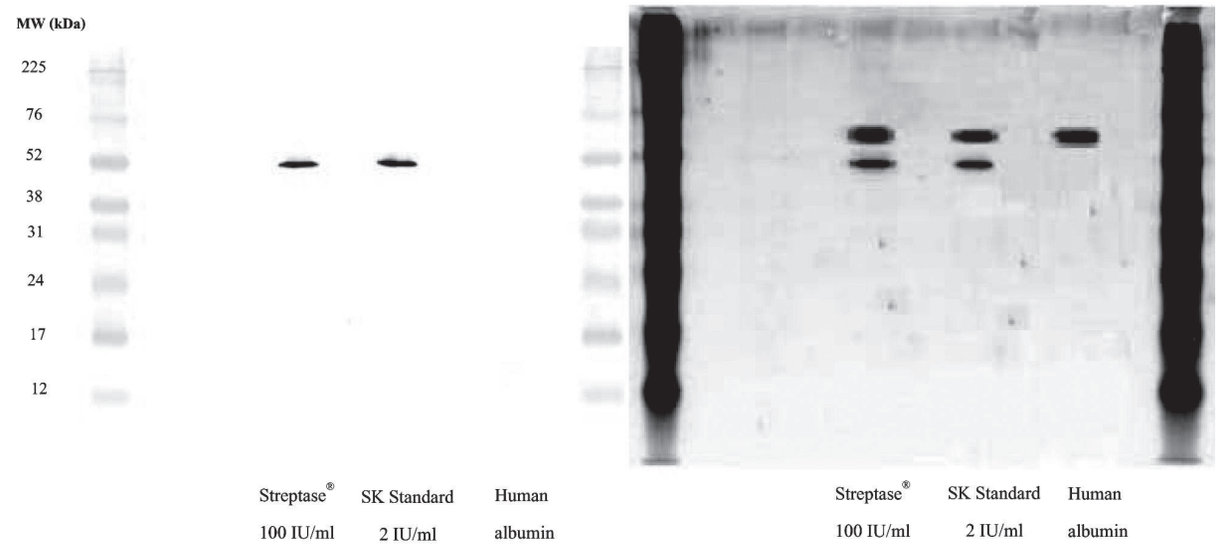


Figure 5 The study of LOD for SK standards (SD) and SK pharmaceutical formulation (Streptase®, SP) was performed at concentrations of 0.2-50 IU/ml (A.1-A.3) and 1-100 IU/ml (B.1-B.2), respectively. The concentrations of primary and secondary antibodies were at 7.5×10^{-3} and 2×10^{-3} mg/ml, repectively. The 1 h incubation of both primary and secondary antibodies was used.

The SK standard, Streptase® and human albumin were run using SDS-PAGE/WB with the validated conditions and the result was determined in figure 6.

Figure 6



The precision study of all three consecutive days showed the same result as that in figure 6. It was found that there was no band of albumin detected by the WB, while the silver staining detected both human albumin and SK.

Discussion

The SK band was found at 47 kDa,which was in line with the study of Dubey and co-workers[13]. The study for the optimum concentration of primary antibody showed that the SK band was not clearly observed at concentration of 2.5×10^{-3} mg/ml, (Figure 1). On the other hand, the SK at the concentrations from 0.5 to 5 IU/ml was detected at primary antibody concentrations of 7.5×10^{-3} and 12.5×10^{-3} mg/ml; however, at these concentrations, SK standard at concentration of 0.2 IU/ml was not detected (Figure 1). The similar results were recognized using secondary antibody at concentrations of 5×10^{-4}, 2×10^{-3} and 3.5×10^{-3} mg/ml.The SK bands at concentrations from 0.5 to 5 IU/ml were obviously seen at the concentrations of 2×10^{-3} and 3.5×10^{-3} mg/ml but the concentration of SK at 0.2 IU/ml was not determined (Figure 2). Therefore, the optimal concentrations of primary and secondary antibodies for the identification of SK were at 7.5×10^{-3} and 2×10^{-3} mg/ml, respectively. It has been notified that the incubation period may affect the presence of protein on membrane[14]. This study proved that there was not much different in the appearance of SK bands using the incubation periods at 30, 60 and 90 min. However, the SK bands at the concentration of 0.5 IU/ml were clearer monitored using

incubation at 60 and 90 min for both primary and secondary antibodies (Figure 3 and 4). The incubation times of both primary and secondary antibodies for this study were chosen at 60 min. The study also demonstrated that the concentrations at 0.5 and 20 IU/ml were the LOD values of SK standard and SK in pharmaceutical formulation (Streptase®, SP), respectively (Figure 5). The requirement of higher concentration of SP for the test was not surprised as the pharmaceutical formulation usually contains more ingredients than the standard and this might lead to lower sensitivity for the detection of SP than that of SK standard. It has been notified that two brands of SK products are available in Thailand. It was regret that we could obtain only one brand of SK product (Streptase®). As this drug is strictly used, it has to be prescribed only by physician and we cannot directly buy from the drug company. However, the searchable information found that the major protein components in both two brands are SK and human albumin. Therefore, it is important to ensure that human albumin used as stabilizer in the SK pharmaceutical formulation had no effect on the method. We performed the test using human albumin as negative control and the outcome showed that there was no band of albumin detected by the WB (Figure 6). Nevertheless, the albumin was observed by silverstaining (Figure 6). The study indicated that WB was more specific than silver staining, which detected every protein. The precision test showed the consistent results of all three consecutive days. This proved that the method is appropriate for the identification test of SK.

Conclusion

The SDS-PAGE/WB for identification of streptokinase has an advantage as it can reduce the use of animal plasma. In addition, the validated result proved that albumin in SK pharmaceutical formulation was not interfered with the test. The study concluded that the SDS-PAGE/WB for the identification test of SK in pharmaceutical formulation can be used as an alternative method for pharmacopeia, since the technique is specific and reliable.

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การพัฒนาวิธีการตรวจเอกลักษณ์สำหรับเภสัชภัณฑ์ Streptokinase โดยวิธี SDS-PAGE/Western Blot เพื่อทดแทนวิธีวิเคราะห์ตามเภสัชตำรับ

บุณฑริกา บุญญาภิวัฒน์ และญานิกา รัตนสุวรรณ
สำนักยาและวัตถุเสพติด กรมวิทยาศาสตร์การแพทย์ ถนนติวานนท์ นนทบุรี 11000

บทคัดย่อ Streptokinase ใช้ในการรักษาโรคหลอดเลือดอุดตัน เช่น โรคหลอดเลือดสมองตีบตัน (Stroke) เนื่องจากในปัจจุบันยา streptokinase มีราคาสูง จึงมีการลักลอบนำเข้ามาใช้ในประเทศแบบผิดกฎหมายตามพระราชบัญญัติยา พ.ศ. 2510 บทลงโทษผู้กระทำผิดจะทำได้เมื่อมีการพิสูจน์ได้ว่าของกลางคือยา ดังนั้นการตรวจพิสูจน์เอกลักษณ์ยา streptokinase จึงมีความสำคัญในกระบวนการพิสูจน์หลักฐานวิธีการตรวจเอกลักษณ์ยา streptokinase ในเภสัชตำรับสากลจำเป็นต้องใช้พลาสม่าจากสัตว์ทดลอง ซึ่งขัดแย้งกับการพยายามลดการใช้สัตว์ทดลองในการตรวจวิเคราะห์ยาซึ่งปัจจุบันเป็นที่ยอมรับและถือปฏิบัติทั่วโลก การศึกษานี้จึงมีเป้าหมายในการพัฒนาวิธีการตรวจเอกลักษณ์ยา streptokinase โดยวิธี sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ร่วมกับวิธี Western Blot เพื่อทดแทนวิธีตามเภสัชตำรับสากลการศึกษาความถูกต้องของวิธีวิเคราะห์มีการศึกษาปัจจัยที่เกี่ยวข้องที่มีผลต่อวิธีวิเคราะห์ เช่น ความเข้มข้นของแอนติบอดีทั้งชนิดปฐมภูมิและทุติยภูมิระยะเวลาการบ่ม และปริมาณต่ำสุดของ streptokinase ที่สามารถตรวจพบในสารมาตรฐานและในเภสัชภัณฑ์ จากการศึกษาพบว่าความเข้มข้นที่เหมาะสมสำหรับแอนติบอดีชนิดปฐมภูมิและทุติยภูมิคือ 7.5×10^{-3} และ 2×10^{-3} mg/ml ตามลำดับ โดยมีระยะเวลาที่เหมาะสม ในการบ่มสำหรับแอนติบอดีทั้งสองชนิดที่ 1 ชั่วโมง ปริมาณ streptokinase ต่ำสุดที่ตรวจพบในสารมาตรฐานและในเภสัชภัณฑ์คือที่ความเข้มข้น 0.5 and 20 IU/ml ตามลำดับ นอกจากนี้ในการศึกษาพบว่าอัลบูมินที่ใส่ในเภสัชภัณฑ์ยา streptokinase เพื่อเป็นสารทำให้ตำรับคงตัวไม่รบกวนวิธีวิเคราะห์ดังกล่าว ผลจากการศึกษานี้ชี้ให้เห็นว่าวิธีวิเคราะห์ที่พัฒนาขึ้นเป็นวิธีที่เชื่อถือได้สามารถนำไปใช้ในการตรวจเอกลักษณ์ยา streptokinase ในเภสัชภัณฑ์ได้