Original article

Development of human thrombin production at National Blood Centre, Thai Red Cross Society

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Abstract:

Introduction: *Human thrombin (HT) has been widely used in hemostatic products and used as an active ingredient to produce fibrin sealant or fibrin glue. Fibrin sealant was produced by National Blood Centre, Thai Red Cross Society (NBC, TRCS) since 1997 using imported HT. It was clinically applied in many surgeries such as dental and heart procedures. However, since 2010, the manufacturer has denied to supply the product. As a result, fibrin sealant production was terminated. The development of HT from local plasma was initiated in phase I during 2016-2020 in order to utilize proteins in plasma to maximize their benefits in patient treatment.* ***Objective:*** *This study aims to develop a process for producing HT from plasma at the NBC, TRCS instead of importing from abroad which is expensive.* ***Materials and Methods:*** *The production of thrombin started with prothrombin complex (PTC) separation, where pooled cryo-removed plasma (CRP) was processed with DEAE Sephadex resin and eluted to obtain crude PTC before converting PTC to thrombin by glycine, calcium chloride, and pH control. Solvent detergent (SD) treatment was performed to ensure viral safety before thrombin purification using SP Sephadex column chromatography and nanofiltration. The prothrombin and purified thrombin were analyzed for activity and purity using coagulation assays, spectrophotometry, gas chromatography, and SDS-PAGE.* ***Results:*** *HT production from three 20 L of CRP batches yielded titers of 12,665, 11,143 and 12,727 IU/mL, with specific activities of 1,550, 1,450, and 1,509 IU/mg, respectively. SD residues were within acceptable limits in 2 batches, and SDS-PAGE showed no other protein bands post-purification. Scaling up to 40 L of CRP for two lots produced results consistent with the 20 L batches in terms of thrombin titer, specific activity, and SD residues.* ***Conclusion:*** *NBC, TRCS developed scalable HT from plasma production meeting the criteria for medical and research applications.*

Keywords : ● *Human thrombin* ● *Production* ● *Purification* ● *Human plasma*

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นิพนธ์ต้นฉบับ

การพัฒนาการผลิตทรอมบินของมนุษย์ของศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทย

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บทคัดย่อ

บทนำ *ทรอมบินของมนุษย์ ถูกนำมาใช้อย่างแพร่หลายในผลิตภัณฑ์ห้ามเลือด และถือเป็นส่วนประกอบในตัวยาออกฤทธิ์ที่สำคัญของกาวไฟบริน ซึ่งถูกนำมาใช้ในทางคลินิกสำหรับการผ่าตัด เช่น ศัลยกรรมช่องปากและหัวใจ โดยศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทยได้เริ่มผลิตกาวไฟบรินตั้งแต่ปี พ.ศ. 2540 โดยใช้ทรอมบินที่นำเข้าจากต่างประเทศซึ่งภายหลังผู้ผลิตจากต่างประเทศได้เลิกจำหน่าย จึงทำให้ต้องหยุดผลิตกาวไฟบรินในปี พ.ศ. 2553 ศูนย์บริการโลหิตแห่งชาติจึงมีแนวคิดในการพัฒนาการผลิตทรอมบินจากมนุษย์ขึ้นใช้เองในประเทศ โดยเริ่มจากการสกัดทรอมบินจากพลาสมาในระยะที่ 1 ช่วงปี พ.ศ. 2559-2563 เพื่อให้เกิดประโยชน์สูงสุดในการนำโปรตีนที่มีอยู่ในพลาสมามาใช้ในการรักษาผู้ป่วย* ***วัตถุประสงค์*** *การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนากระบวนการผลิตทรอมบินจากพลาสมา ณ ศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทย แทนการสั่งซื้อจากต่างประเทศซึ่งมีราคาแพง* ***วัสดุและวิธีการ*** *การผลิตทรอมบินเริ่มต้นด้วยการแยกโปรทรอมบินคอมเพล็กซ์ (PTC) โดยพลาสมาที่ผ่านการแยก cryoprecipitate ออก ถูกนำไปผ่านเรซิน DEAE Sephadex และทำการล้างเพื่อให้ได้ PTC ขั้นต้น ก่อนเปลี่ยน PTC เป็นทรอมบินโดยใช้ไกลซีน แคลเซียมคลอไรด์ และควบคุมค่า pH การนำไปผ่านสารละลายและสารชะล้าง (SD) ถูกดำเนินการเพื่อความปลอดภัยจากไวรัส ก่อนทำการแยกทรอมบินบริสุทธิ์ด้วยคอลัมน์โครมาโทกราฟี SP Sephadex และการกรองระดับนาโน ทรอมบินและโปรทรอมบินที่บริสุทธิ์ถูกวิเคราะห์หาค่าความแรงและความบริสุทธิ์โดยใช้การทดสอบการแข็งตัวของเลือด สเปกโตรโฟโตเมตรี โครมาโทกราฟีแบบแก๊ส และ SDS-PAGE* ***ผลการศึกษา*** *การผลิตทรอมบินจากพลาสมาขนาด 20 ลิตร 3 ชุด ให้ค่าความแรงทรอมบินที่ 12,665, 11,143 และ 12,727 IU/mL โดยมีค่าความแรงจำเพาะที่ 1,550, 1,450 และ 1,509 IU/mg ตามลำดับ ปริมาณสาร SD ที่เหลือตกค้าง พบว่าสองรุ่นการผลิต มีค่าอยู่ในเกณฑ์ที่ยอมรับได้ และ SDS-PAGE ไม่พบแถบโปรตีนอื่น ๆ หลังการทำให้บริสุทธิ์ การเพิ่มขนาดการผลิตเป็น 40 ลิตรสำหรับ 2 รุ่นการผลิต ให้ผลลัพธ์ที่สอดคล้องกับขนาดการผลิต 20 ลิตรในแง่ของค่าความแรงทรอมบิน ค่าความแรงจำเพาะ และปริมาณสาร SD ที่เหลือตกค้าง* ***สรุป*** *ศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทยได้พัฒนาการผลิตทรอมบินจากพลาสมาที่สามารถขยายขนาดการผลิตได้ โดยสอดคล้องตามเกณฑ์คุณภาพมาตรฐานที่จะนำไปประยุกต์ใช้ทางการแพทย์และการวิจัยได้*

คำสำคัญ : ● *ทรอมบินของมนุษย์* ● *การผลิต* ● *การทำให้บริสุทธิ์* ● *พลาสมาของมนุษย์*
วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2568;35:89-96.

Introduction

Human thrombin (HT) is one of the blood coagulation factors, which transforms fibrinogen to fibrin and stops bleeding. Thus, HT has been used for anti-bleeding products, including fibrin sealant or fibrin glue. Fibrin sealant has been clinically used to stop bleeding in various applications, including dental surgery, open heart surgery, and ear, nose and throat (ENT) surgery.[1] This product was also useful for bleeding disorder patients[2]. Fibrin sealant has been mostly prepared in a small scale for using in an individual hospital operation[3,4,5].

The fibrin sealant was first produced and distributed to hospitals by National Blood Centre (NBC), Thai Red Cross Society (TRCS) since 1997. NBC's Fibrin sealant consists of 2 different solutions, including human fibrinogen and HT with concentrations of 100 IU/mL, 250 IU/mL, and 500 IU/mL. Human fibrinogen was obtained from heat-treated freeze-dried cryoprecipitate (HTFDC), a product from plasma, whereas HT was internationally imported. Since 2010, HT suppliers stopped production and could not supply HT to NBC. Therefore, fibrin sealant from NBC has been discontinued. Fibrin sealant was less accessible to patients because imported fibrin sealant was more expensive. HT production by NBC will overcome this issue and promote self-sufficiency in Thailand.

This study aims for HT production using plasma from NBC, TRCS. The production process was supported and collaborated by the Japan Blood Product Organization (JB). The process includes prothrombin complex separation, prothrombin activation, solvent detergent treatment, thrombin purification, and nanofiltration. This can achieve high quality HT and provide a material for fibrin sealant product for Thailand.

Materials and Methods

Prothrombin complex separation

Cryo-removed plasma passing quarantine and virus safety test was pooled to obtain 20 L. The pooled plasma was mixed with DEAE Sephadex (anion exchange resin), which was previously equilibrated, for 1 hour. The resin was then separated by a membrane filter using a suction pump before washing by buffer containing sodium chloride 8.7 g/L and sodium citrate 2.92 g/L for 10 minutes, 3 times. Prothrombin complex (PTC) was eluted from the resin by mixing with elution buffer containing sodium chloride 55.8 g/L and sodium citrate 2.81 g/L for 1 hour, 2 times. Crude PTC was stored at -25°C.

Prothrombin activation

PTC was thawed at 20-24°C before filtration by a 0.22 µm membrane filter. PTC was dialyzed by buffer containing sodium chloride 3.75 g/L and sodium citrate 2.50 g/L using a tangential flow filtration system with a 10 kD MW cut-off membrane at 2-5°C for 6 sample volumes. Glycine (86 g/L) was added to PTC and mixed for 10 minutes. After leaving overnight, calcium chloride (3 g/dL) was added to the mixture to activate PTC to thrombin before mixing for 10 minutes. The pH level was controlled at 6.9-7.1. Crude thrombin was filtered by a 0.22 µm membrane filter before keeping at 2-5°C for 72 hours.

Solvent detergent treatment

The crude thrombin was centrifuged at 3,500 rpm, 4°C for 15 minutes. The supernatant was collected before adding solvent detergent (SD) solution containing tri-n-butly phosphate (TNBP) and polysorbate 80 (PS80)[6] and mixing for 3 hours at 29-31°C. The pH was controlled at 5.9 to 6.1 and the conductivity was controlled at 8.00±0.5 ms/cm. The solution was filtered by a 0.22 µm membrane filter and stored below -25°C.

Human thrombin purification

SP Sephadex column was used to purify thrombin. The column was first equilibrated with a binding buffer containing sodium citrate 11.76 g/L, pH 5.9 - 6.1, conductivity 8.30-8.90 ms/cm. The crude thrombin was loaded to the column before washing with 2 washing buffers (washing buffer 1: sodium citrate 11.76 g/L, pH 5.9-6.1, conductivity 8.30-8.90 ms/cm and washing buffer 2: sodium citrate 13.23 g/L pH 6.9-7.1 conductivity 8.70-

9.30 ms/cm) not less than 5 column volumes (CV). The thrombin was eluted by an elution buffer containing sodium citrate 22.5 g/L, pH 6.6-6.8, conductivity 13.80-14.40 ms/cm. Elution fractions were collected and analyzed for thrombin titer. The fractions with more than 500 IU/mg of specific activity were pooled and dialyzed with a tangential flow filtration membrane (10 kD MW cut-off). After filtration by a 0.22 μm membrane filter, the thrombin solution was filtered by a nanofilter of 15 N.[7] The thrombin solution was mixed with L-Arginine and D-mannitol before filtration by a 0.22 μm membrane filter and stored below -25°C.

Sample analysis

Thrombin titer was assayed by coagulation analyzer following the Japanese Pharmacopoeia 17th edition[8]. Polysorbate 80 and A280 were detected by spectrophotometer. Phosphoric acid tri-n-butyl was measured by gas chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect protein components of samples.

Results

Human thrombin purification by SP Sephadex chromatography

The crude thrombin was purified by strong cation exchange chromatography, SP Sephadex. The chromatogram obtained from purification was shown in Figure 1. The fractions of 10 mL were collected during flowthrough, wash, and elution steps for thrombin analysis.

According to analysis of flowthrough and wash fractions, thrombin was significantly detected by activity test and SDS-PAGE (Figure 2). Elution was performed for 44 fractions. However, only fractions 11 to 44 found α-thrombin bands at 37 kD and β-thrombin at 26 kD on SDS-PAGE (Figure 2). The specific activity values of fractions 11 to 16 were less than 500 IU/mg which did not pass the JB criteria. Therefore, Fractions 21 to 44 were pooled and processed for the next step.

Human thrombin production from 20 L and 40 L plasma

HT production was conducted using 20 L of CRP for each batch. The production was performed for 3 consecutive batches, B001QYLT, B002QYLT and B003QYLT (Table 1). PTC was separated from CRP using DEAE Sephadex resin with titer between 43.61 and 44.95 IU/mL and volume from 407 to 425 mL. After activation and SD treatment, thrombin was purified by SP Sephadex resin with a 124 mL bed volume, and virus removal was conducted using nanofiltration. The thrombin titers of 3 batches were 12,665, 11,143 and 12,727 IU/mL, respectively. These correlated with the specific activity values and passed the criteria from JB. The TNBP and PS80 residues in 2 from 3 batches were in line with the JB criteria after purification. This indicated that SP Sephadex column was effective in

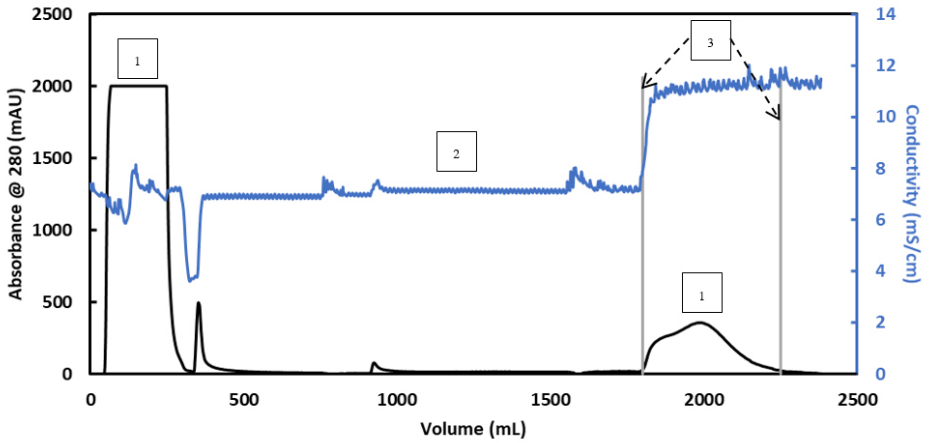

Figure 1 Chromatogram of human thrombin purification Batch B001QYLT by SP Sephadex resin. Line 1 represents the absorbance value at 280 nm (A280), and line 2 represents the conductivity value. The elution peak between line 3 was collected for SDS-PAGE analysis

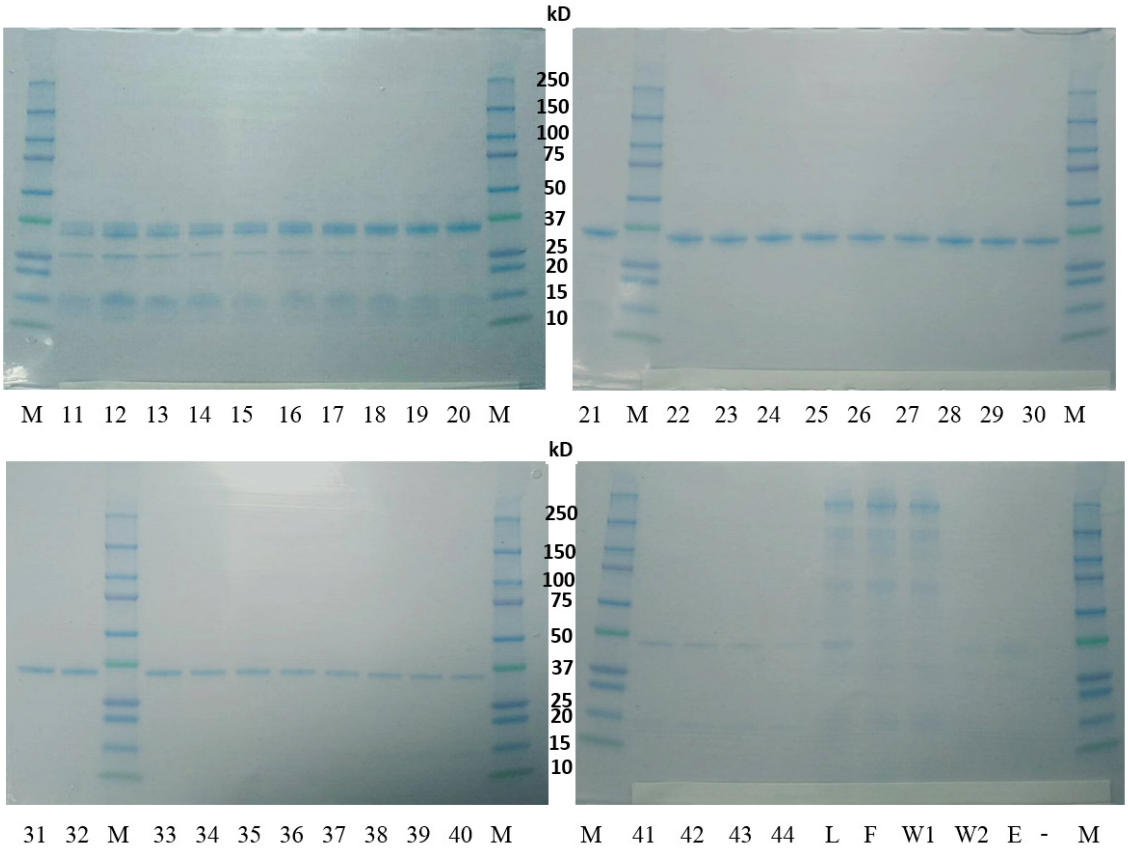

Figure 2 SDS-PAGE of fractions from thrombin purification by SP Sepharose column. Each lane represents as followed; M: Molecular weight marker, 11-44: Elution fractions, L: Loading crude thrombin, F: Flowthrough fraction, W1: Wash 1 fraction, W2: Wash 2 fraction, E: Regeneration fraction

Table 1 Analysis of thrombin bulk from 20 L of CRP after purification and nanofiltration for 3 consecutive batches

Process	Thrombin bulk (Batch no.)				
	B001QYLT	B002QYLT	B003QYLT	JB criteria	Reference
Cryo removed plasma (mL)	20,400	20,100	20,000	-	-
Eluate PTC solution (mL)	425	407	413	-	-
Titer of prothrombin (IU/mL)	44.95	43.61	44.66	-	-
Volume of thrombin bulk (mL)	41.0	23.0	31.0	-	-
pH	5.85	5.82	5.59	5.8-6.2	JB in-house
A280	8.169	7.686	8.433	≥ 6.5	JB in-house
Titer of thrombin bulk (IU/mL)	12,665	11,143	12,727	≥ 6,000	JB in-house
Specific activity (IU/mg)	1,550	1,450	1,509	≥ 500	JB in-house
TNBP residual (μg/1,000 IU)	0.1	1.25	0.7	≥ 1	JB in-house
PS80 residual (μg/1,000 IU)	5.0	6.02	8.1	≥ 10	JB in-house

Table 2 Analysis of thrombin bulk from 40 L of CRP after purification and nanofiltration for 2 consecutive batches

Process	Thrombin bulk (Batch no.)			
	C001QYLT	C002QYLT	JB criteria	Reference
Cryo removed plasma (L)	40	40	-	-
Eluate PTC solution (mL)	1,694		-	-
Titer of prothrombin (IU/mL)	46.7		-	--
Volume of thrombin bulk (mL)	73	69	-	-
pH	5.9	5.8	5.8-6.2	JB in-house
A280	8.3	7.4	≥ 6.5	JB in-house
Titer of thrombin bulk (IU/mL)	11,321	10,339	≥ 6,000	JB in-house
Specific activity (IU/mg)	1,364	1,391	≥ 500	JB in-house
TNBP residual (μg/1,000 IU)	0.0	0.2	≤ 1	JB in-house
PS80 residual (μg/1,000 IU)	5.4	9.3	≤ 10	JB in-house

removing residues from the SD treatment. In batch B003QYLT, the pH of thrombin is a bit less than the JB criteria. However, other key specifications, including titer, were still within the criteria.

The HT production was upscaled to 40 L of CRP. The production was performed for 2 lots C001QYLT and C002QYLT. After separating PTC, PTC solutions from 2 lots were combined to obtain 1,694 mL and a titer of 46.7 IU/mL. The SD treatment was performed before separating into 2 lots for thrombin purification by 294 mL of SP Sephadex resin. The results showed that pH, A280, titer, specific activity, TNBP residue, and PS80 residue values were in the JB acceptable range for both 2 lots (Table 2). This suggested that HT production from CRP can be scalable.

Discussion

The production of HT was investigated starting from 20 L of CRP for 3 batches. The process started with PTC extraction from CRP using DEAE Sephadex resin. Three batches yielded an average prothrombin titer of 44.41±0.58 IU/mL. In comparison, Fenton et al.'s large-scale human thrombin production, utilizing Cohn ethanol fractionation, PEG 6000 precipitation, and DEAE-cellulose chromatography for PTC capture, reported a higher prothrombin level of 2,320 IU/mL[9]. However, their process involved a more complex cold-ethanol fractionation requiring precise temperature and ethanol concentration control across multiple steps to obtain Cohn fraction III, followed by a PEG 6000 precipitation for impurity removal before column chromatography. Similarly, Aizawa, et al. reported a prothrombin complex titer of 76 IU/mL using a three-step chromatographic purification process involving sequential DEAE Sepharose FF and Heparin Sepharose FF columns[10]. While both Fenton, et al. and Aizawa, et al. achieved higher prothrombin titers, our method offered a distinct advantage: it employed a single-step direct adsorption of prothrombin from cryo-removed plasma using DEAE Sephadex. This streamlined approach simplified the operation and facilitated large-scale processing. After prothrombin activation, HT was purified by SP Sephadex resin. All the specifications of thrombin bulk were in the JB criteria except for TNBP residue in batch B002QYLT was higher than the criteria (1.25 μg/1,000 IU). Although the pH of one batch was lower than the criteria, the pH value was still in the acceptable range (pH 5.0-8.0) of the European Pharmacopeia Standard[11]. The JB criteria was an in-process control providing a narrow acceptable range. For TNBP residue over than the limit in batch B002QYLT, after increasing washing buffer volume from 5 CV to 6 CV as recommended by

JB technical staff and following column manufacturer's guideline (at least 5-10 CV was recommended)[12], the TNBP residue was in the acceptable range in both of the 40L-upscale batches. The average thrombin titer of the 3 batches purified using SP Sephadex resin was 12,178.33±742.34 IU/mL. A direct comparison of thrombin yield with previous reports using a Heparin Sepharose CL-6B matrix is difficult due to variations in techniques and reported units[13,14]. However, in terms of purity, SDS-PAGE analysis of the elution fractions showed a predominant thrombin band at approximately 37 kDa, with only minor additional bands. This contrasts with the loading sample, flowthrough fraction, and wash fraction, which exhibited several other bands. This observation aligns with the SDS-PAGE band of thrombin purified using a Heparin Sepharose CL-6B matrix, as reported by Ngai and Chang[14]. This indicated high purity of thrombin bulk as correlated with high specific activity values. Higher purity could be achieved by optimizing the elution step or collecting fractions only containing α-thrombin, even though the titer of thrombin might be reduced.

HT production with an initial 40 L of CRP was performed in 2 lots with a similar process. However, after PTC separation, PTC solutions from 2 lots were pooled for prothrombin activation and SD treatment to reduce time-consuming and obtain similar quality of crude thrombin bulk before purification. The crude thrombin bulk was purified in 2 lots to explore the consistency of the purification process. Both thrombin bulks achieved similar levels of quality in terms of pH, protein level, titer, specific activity, and SD impurities. The result was also tightly correlated with the thrombin production from 20 L of CRP. Thus, this HT production process showed robustness and potential for large-scale production. Although the yield might not be as high as other studies, this could be improved and should be enough for the final formulation as a component for fibrin sealant. In addition to a high-quality thrombin bulk, the production process was simple and cost-effective, which will support an affordable thrombin product in Thailand.

Conclusion

Blood Product Production Department, National Blood Centre, Thai Red Cross Society has successfully developed HT production from plasma with support from the JB. The process included PTC separation by DEAE Sephadex resin, prothrombin activation, SD treatment, thrombin purification by SP Sephadex resin, and virus removal by nanofiltration. After production in 3 lots of 20 L starting CRP and 2 lots of 40 L starting CRP, the obtained bulk HT achieved all JB criteria indicating process validity. This HT can be further processed for final formulation to meet final specifications for pharmaceutical products. The scalable production will provide sufficient HT for a fibrin sealant product, which will benefit many patients in the future. This also allows the HT to be used in other applications, such as research studies and laboratory tests.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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References

1. *Isarangkura P, Chiewsilp P, Chuansumrit A, Suwannuraks M, Keorochana SB, Attanawanich S, et al. Low cost locally prepared fibrin glue for clinical applications: reported of 145 cases. Committee of Bangkok International Hemophilia Training Center. J Med Assoc Thai. 1999;82(suppl 1):S49-56.*
2. *Suwannuraks M, Chuansmrit A, Sriudomporn N. The use of fibrin glue as an operative sealant in dental extraction in bleeding disorder patients. Haemophilia. 1999;5:106-8.*
3. *Rock G, Neurath D, Semple E, Harvey M, Freedman M. Preparation and characterization of human thrombin for use in a fibrin glue. Transfus Med. 2007;17:187-91.*
4. *Fernández-Vega-Cueto L, Persinal-Medina M, Vázquez N, Chacón M, Alfonso-Bartolozzi B, Alonso-Alonso S, et al. A simple, safe, and effective method for preparing autologous bio-based fibrin glue for ophthalmic use. Pharmaceutics. 2022;14:2325.*
5. *Kumar Vijay, Chapman John R. Whole blood thrombin: development of a process for intra-operative production of human thrombin. J Extra Corpor Technol. 2007;39:18-23.*
6. *Pinnopmanee S. Viral inactivation and viral removal for plasma products. J Transfus Med. 2019;29:145-52.*
7. *Burnouf T, Radosevich M, Goubran HA, Willkommen H. Place of nanofiltration for assuring viral safety of biologicals. Curr Nanosci. 2005;1:189-201.*
8. *Ministry of Health L and W. Japanese Pharmacopoeia. 17th ed. Ministry of Health, Labour and Welfare; 2016.*
9. *Fenton JW, Campbell WP, Harrington JC, Miller KD. Large-scale preparation and preliminary characterization of human thrombin. BBA Prot St. 1971;229:26-32.*
10. *Aizawa P, Winge S, Karlsson G. Large-scale preparation of thrombin from human plasma. Thromb Res. 2008;122:560-7.*
11. *European Directorate for the Quality of Medicines & HealthCare. Fibrin sealant kit. In: European Pharmacopoeia. 11th ed. Strasbourg Cedex: Council of Europe; 2022. p. 2763-5.*
12. *Cytiva. HiPrep™ CM FF 16/10, HiPrep DEAE FF 16/10, HiPrep Q FF 16/10, HiPrep SP FF 16/10, HiPrep Q HP 16/10, and HiPrep SP HP 16/10 prepacked columns: instructions for use. Vol. 28941323 AE V:4. 2020.*
13. *Kaetsu H, Mizuguchi J, Hamamoto T, Kamimura K, Yoshida Y, Nakagaki T, et al. Large-scale preparation of human thrombin: polyethylene glycol potentiates the Ffctor Xa-mediated activation of prothrombin. Thromb Res. 1998;90:101-9.*
14. *Ngai PK, Chang JY. A novel one-step purification of human α-thrombin after direct activation of crude prothrombin enriched from plasma. Biochem J. 1991;280:805-8.*