

Original Article

Evaluation of Factor XIII Activity Using the Ammonia Release Method with and without Iodoacetamide Sample Blanking Procedure

Pornnapa Police, Phichchapha Noikongdee, Tichayapa Phojanasenee, Kanchana Siriputtanapong, Katcharin Aryurachai, Pichika Chantrathammachart and Pantep Angchaisuksiri

Division of Hematology, Department of Medicine, Ramathibodi Hospital, Mahidol University

Abstract:

Background: The ammonia release assay is a quantitative factor XIII (FXIII) activity screening test. It is recommended to perform with a sample blank containing 1 mmol/L iodoacetamide to avoid overestimating caused by side reactions. We evaluated an ammonia release assay, Berichrome FXIII assay, on the CS-2000i analyzer with and without sample blank procedures to observe the impact of the side reactions concerning overestimating FXIII activity. **Methods:** Plasma samples from 10 suspected FXIII deficiency patients, 30 normal volunteers, 6 diluted human standard plasmas and one FXIII deficient plasma, including normal and abnormal control plasma were analyzed. All plasma samples were measured twice with and without sample blank procedure in which 1 mmol/L iodoacetamide and physiological saline was added to reagents of the assay, respectively. The results were corrected by subtracting the values with blank from the values without blank. **Results:** The means (standard deviations) of FXIII activity of normal volunteers, with and without the blank procedures, were 98.4% (19.1) and 107.3% (19.5), respectively. In the low range of FXIII activity, the values after blank subtraction were lower than that without the subtraction by 3% to 7%. Values near normal range seemed not to be affected by the side reactions. **Conclusion:** Procedure with a sample blank was recommended to avoid overestimating the results when using the ammonia release assay to detect FXIII activity.

Keywords : ● Factor XIII activity ● Ammonia release assay ● Iodoacetamide sample blank

J Hematol Transfus Med 2018;28:51-7.

Received 23 August 2017 Accepted 20 November 2017

Correspondence should be addressed to Prof. Pantep Angchaisuksiri, MD., Division of Hematology, Department of Medicine, Ramathibodi Hospital, Mahidol University 270 Rama VI Rd., Bangkok 10400 Thailand. E-mail: pantep.ang@mahidol.ac.th

นิพนธ์ต้นฉบับ

การประเมินการตรวจวัดระดับแฟคเตอร์ 13 ด้วยวิธี Ammonia Release โดยใช้และไม่ใช้วิธีการที่มี Iodoacetamide Sample Blank

พรนภา โปลิศ พิชญ์ชามา น้อยคงดี ธิชยาภา พจนเสนี กาญจนา ศิริพัฒน์พงษ์ คัชริน อายุรไชย พิจิกา จันทราธรรมชาติ และ พันธุ์เทพ อังชัยสุขศิริ

สาขาวิชาโลหิตวิทยา ภาควิชาอายุรศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

ความเป็นมา การตรวจ ammonia release เป็นการวัดระดับการทำงานของ FXIII มีการแนะนำให้ทำการทดสอบโดยมี sample blank ที่มี 1 mmol/Iodoacetamide เพื่อหลีกเลี่ยงการวัดค่าที่สูงเกินจริงซึ่งเกิดจากปฏิกิริยาข้างเคียงเราทำการประเมินการตรวจ ammonia release ด้วยชุดน้ำยา Berichrome FXIII assay ด้วยเครื่อง CS-2000i ด้วยวิธีการที่มีและไม่มี sample blank เพื่อดูผลกระทบของปฏิกิริยาข้างเคียงที่อาจทำให้ระดับของแฟคเตอร์ 13 สูงเกินความเป็นจริง **วิธีการทดลอง** ทำการตรวจวิเคราะห์พลาสมาของผู้ป่วยที่สงสัยว่าจะมีการขาด FXIII จำนวน 10 คน พลาสมาของอาสาสมัครที่เป็นคนปกติจำนวน 30 คน พลาสมามาตรฐานของคนที่ได้รับการเจือจางจำนวน 6 ระดับและพลาสมาที่ขาด FXIII รวมทั้งพลาสมาควบคุมที่ปกติและผิดปกติ สิ่งส่งตรวจทั้งหมดจะถูกทำการตรวจวัดสองครั้งด้วยวิธีการที่มีและไม่มี sample blank โดยเติม 1 mmol/Iodoacetamide และสารละลายน้ำเกลือลงไปในส่วนน้ำยาของแต่ละวิธีตามลำดับ ผลการทดสอบที่ถูกต้องได้มาจากการนำค่าที่ได้จากวิธีที่มี iodoacetamide ไปลบออกจากค่าที่ได้จากวิธีที่มีสารละลายน้ำเกลือ **ผลการทดลอง** ระดับการทำงานของ FXIII ของคนปกติจากวิธีที่มีและไม่มี sample blank มีค่าเฉลี่ย (ส่วนเบี่ยงเบนมาตรฐาน) เท่ากับ 98.4% (19.1) และ 107.3% (19.5) ตามลำดับ ในกลุ่มที่มีระดับการทำงานของ FXIII ต่ำ ค่าที่ได้หลังจากลบค่าของ sample blank ออกแล้วจะต่ำกว่าค่าที่ไม่ได้ลบด้วย sample blank โดยมีค่าแตกต่างกันประมาณร้อยละ 3-7 ในขณะที่กลุ่มที่มีระดับการทำงานของ FXIII ใกล้เคียงกับค่าปกติดูเหมือนจะไม่ได้รับผลกระทบจากปฏิกิริยาข้างเคียงที่เกิดขึ้น **สรุป** เมื่อใช้วิธีทดสอบ ammonia release ในการตรวจหาระดับการทำงานของ FXIII แนะนำให้ใช้วิธีที่มี sample blank ร่วมด้วยเพื่อหลีกเลี่ยงการรายงานค่าที่สูงเกินจริง

คำสำคัญ : ● Factor XIII activity ● Ammonia release assay ● Iodoacetamide sample blank

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2561;28:51-7.

Introduction

Factor XIII (FXIII) is a pro-transglutaminase that plays a major role in the final stage of the coagulation system. It circulates in plasma as a heterotetramer composed of two catalytic A subunits and two carrier B subunits¹. FXIII is transformed to an active transglutaminase (activated FXIII, FXIIIa) by thrombin in the presence of calcium. FXIIIa then converts loose fibrin monomers to a stable fibrin clot by cross-linking between fibrin strands and also cross-linking antipiasmin to fibrin. By this way, FXIIIa protects the clot from shear stress and increases its resistance to degradation by plasmin^{2,3}.

Congenital FXIII deficiency is a rare autosomal recessive disorder with an incidence of approximately one in two million⁴. The majority of cases are due to mutations of FXIIIA genes, whereas a few cases are found with defects in FXIIIB genes⁵. Patients with severe FXIII deficiency usually present a plasma level of FXIII activity less than 1% with spontaneous major bleeding⁶. Umbilical cord bleeding is the most frequently finding. Other symptoms include intracranial hemorrhage, muscle and subcutaneous soft tissue hematoma, impaired wound healing and recurrent spontaneous abortions^{7,8}. FXIII activity level of less than 30% is considered a moderate deficiency while activity level of more than 30% is defined as a mild disorder. Moderate FXIII deficiency is associated with mild spontaneous bleeding, whereas patients with mild FXIII deficiency are usually asymptomatic⁶.

Acquired FXIII deficiency can be associated with severe bleeding complications because of autoantibodies directed against FXIII. It has been reported in patients with autoimmune disease, most commonly with systemic lupus erythematosus, or in elderly patients with no underlying diseases. In these cases, the plasma FXIII activity is observed to be less than 20% with the majority between 0-5%. Less severe deficiency (plasma FXIII activity between 20-70%) with mild bleeding symptoms may be due to a decreased synthesis or an increased consumption of FXIII. It has been reported to be

associated with several medical conditions such as leukemia, severe liver disease, ulcerative colitis, sepsis, stroke, disseminated intravascular coagulation and major surgery^{1,9}.

The functional screening tests to diagnose FXIII deficiency consist of the clot solubility test and quantitative FXIII activity assay. The clot solubility test requires no specialized instrument and is inexpensive. Therefore, the procedure is widely used. However, the test is qualitative and can only detect severe FXIII deficiency^{10,11}. The measurement of ammonia released during transglutaminase reaction is a quantitative functional FXIII activity assay that has been recommended by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) as a first line screening test¹². The advantages of this assay include being fast, a one step true-kinetic assay with good reproducibility and easily automated. In this assay, zymogen FXIII is activated by thrombin and calcium to become transglutaminase (FXIIIa). FXIIIa catalyzes the reaction between a specific peptide substrate and the glycine ethyl ester which results in the release of ammonia. The amount of ammonia released is monitored in a glutamate dehydrogenase (GLDH) catalyzed nicotinamide adenine dinucleotide reduced form (NADH) reaction, in which ammonia is used and NADH is converted into NAD (oxidized form). The consumption of NADH is measured by the decrease of its absorbance at 340 nm. The decrease in concentration of NADH is directly proportional to FXIII activity¹³. However, NADH is consumed and ammonia produced independently of FXIIIa occurring in plasma during the assay. These side reactions do not affect the normal FXIII range, but can cause overestimating the case of severe FXIII deficiency leading to misevaluating the proper replacement therapy. To eliminate this problem the subtraction of a sample blank, in which FXIIIa is inhibited by an active side reagent such as iodoacetamide, is recommended¹⁴⁻¹⁶.

The aim of this study was to evaluate the Berichrom

FXIII assay on the CS-2000i analyzer with and without sample blank procedure to see the impact of these side reactions on the results of FXIII activity.

Materials and Methods

Plasma samples

We analyzed plasma samples of ten patients with suspected FXIII deficiency, who presented clinical symptoms of bleeding diathesis and normal coagulograms. Their mean age was 26.5 ± 21.1 years (range 2-59 years). Four were younger than 18 years old; two were males and eight were females. These samples were sent to our laboratory for factor XIII screening using the clot solubility test. Thirty healthy volunteers consisted of 18 males and 12 females, and their mean age was 34.2 ± 10.7 years (range 22-58 years). Blood samples were collected in vacutainer tubes containing one part 0.109 mM/L sodium citrate (9:1 ratio) and were centrifuged at 1,500 g for 20 minutes at 4°C. Plasma was analyzed immediately or kept frozen at -80°C until assayed. Ethics approval was obtained from the Committee on Human Rights Related to Research Involving Human Subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand.

Reagents and instrument

The Berichrome FXIII chromogenic assay, standard human plasma, normal control plasma (NCP) and pathological control plasma (PCP) including the CS-2000i coagulation analyzer were from Sysmex Corporation, Japan. Iodoacetamide was from Sigma, Saint Louis, MO, USA. FXIII deficient plasma was from Hyphen BioMed, Rue d'Eragny, Neuville-Sur-Oise, France.

Methods

FXIII activity was measured using the Berichrome FXIII chromogenic assay on the CS-2000i coagulation analyzer. All plasma samples were assayed twice with and without sample blank procedure. Two standard curves were constructed for sample processing. In the first procedure, iodoacetamide with final concentration of 1 mmol/L was added to activator and detection reagents of the Berichrome FXIII assay before testing,

whereas 0.155 mol/L normal sodium chloride (0.9% NSS) was added in the same manner in the second procedure. Plasma samples were processed on the CS-2000i analyzer. FXIII activity results were corrected by subtracting the values of the iodoacetamide procedure from the values of the sodium chloride procedure.

To determine the influence of the side reactions on FXIII activity results, especially in low ranges of factor XIII activity, we diluted standard human plasma (FXIII activity 106%) with FXIII deficient plasma in dilutions of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 to represent low levels of FXIII activity. Severe FXIII deficient plasma was also used as 0% activity. To increase preciseness, within run and between run precisions were analyzed on normal and abnormal control plasma. The within run assay was processed in ten samples of control plasma within one day, while between run assay of each sample of plasma was assessed in ten consecutive days.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows. The data were expressed as mean, standard deviation (SD), range (mean $\pm$ 2SD) and coefficient of variation (CV). The results with and without sample blank procedures of normal volunteer plasma were evaluated using independent t-test analysis. Significant level was set at p-value < 0.05.

Results

Mean $\pm$ 2SD value of FXIII activity of 30 normal volunteers performing without blank procedure was $107.3 \pm 39.0\%$ (range 68.3-146.3%). The values with blank correction were $98.4 \pm 38.2.6\%$ (range 60.2-136.6%). The mean values of the normal volunteers obtained from both procedures did not significantly differ ($p = 0.96$, 95%CI: -18.9-1.1). Results of ten patient's samples with and without blank subtraction are shown in Table 1. Three samples (samples 1-3) had FXIII activities below the normal range. Noncorrected and corrected values were 5.4% and 0.0%, for sample 1, 42.6% and 31.8% for sample 2 and 53.5% and 46.1% for sample 3, respec-

Table 1 Results of FXIII activity with and without blank correction in 10 plasma samples

Samples from 10 patients with bleeding diathesis	FXIII activity (%)	
	Noncorrected	Corrected
1 (ecchymosis)	5.4	0.0
2 (bleeding from tracheostomy)	42.6	31.8
3 (subdural hemorrhage)	53.5	46.1
4 (intracerebral hemorrhage)	84.9	81.3
5 (epistaxis)	94.5	87.2
6 (epistaxis, hypermenorrhea)	114.2	107.9
7 (abnormal bleeding after surgery)	127.2	120.5
8 (epistaxis)	140.9	134.7
9 (abnormal bleeding after trauma)	150.3	139.3
10 (abnormal bleeding after surgery)	151.2	148.5

Table 2 Results of FXIII activity with and without blank correction in standard human plasma dilutions and in FXIII deficient plasma

Samples	FXIII activity (%)	
	Noncorrected	Corrected
1. FXIII deficient plasma	7.1	0
2. Dilution 1:160	7.7	0
3. Dilution 1:80	8.8	1.3
4. Dilution 1:40	10.0	4.0
5. Dilution 1:20	15.2	11.2
6. Dilution 1:10	16.4	13.7
7. Dilution 1:5	28.5	24.9

FXIII activity of standard human plasma = 106%

Table 3 Precision study of the two procedures obtained from normal and abnormal control plasma

Parameters		Normal control plasma		Abnormal control plasma	
		Noncorrected	Corrected	Noncorrected	Corrected
Within run precision	Mean	97.4	97.2	35.6	35.3
	CV (%)	4.5	5.7	6.3	6.7
Between run precision	Mean	105.4	103.8	35.9	33.9
	CV (%)	3.6	4.1	5.2	6.4

CV = coefficient of variation

tively. Patients 1 and 2 were diagnosed with acquired FXIII deficiency, while patient 3 had subdural hemorrhage postcardiac arrest. The diluted standard human plasma in dilutions of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160, and FXIII deficient plasma were analyzed to determine the overestimation of the Berichrome FXIII assay, es-

pecially in the low range of factor activity. Table 2 demonstrates that FXIII activity values with blank subtraction were approximately 3% to 7% lower in all dilutions than those of no blank correction including the FXIII deficient plasma sample. The precision study of the two procedures is shown in Table 3. Means with

coefficient of variation (CV) of normal control plasma performed with and without sample blank procedures were 97.4 (4.5) and 97.2 (5.7), respectively, for within run assay, and 105.4 (3.6) and 103.8 (4.1), respectively, for between run assay. The values of abnormal control plasma obtained from both procedures were 35.6 (6.3) and 35.3 (6.7), respectively for within run assay, and 35.9 (5.2) and 33.9 (6.4), respectively, for between run assay.

Discussion

FXIII is an essential component of the blood coagulation system. Its deficiency is associated with bleeding diathesis. Congenital FXIII deficiency usually presents spontaneous, delayed or recurrent bleeding symptoms. In the case of severe deficiency, untreated patients may have spontaneous intracranial hemorrhage, which is the major cause of death. Severe bleeding may also arise by the presence of autoantibodies against FXIII resulting in acquired FXIII deficiency⁶⁻⁹.

The measurement of ammonia released during the transglutaminase reaction of FXIII is recommended to be the first line to screen for FXIII deficiency by the SSC of the ISTH¹². Although this assay offers several advantages, problems may occur due to the presence of enzymes using NADH and/or FXIII independent of the ammonia produced in plasma during the assay. These side reactions can cause an overestimation of FXIII activity, especially in the low activity range, resulting in less severe FXIII deficiency and incorrect evaluation of the proper replacement therapy¹⁴⁻¹⁶.

In the present study, we evaluated the Berichrom FXIII assay on the CS-2000i analyzer with and without sample blank procedure. Both procedures provided good precision with low CV levels (< 10%) of normal and abnormal control plasma within run and between run assays. The reference intervals of FXIII activity obtained from 30 normal volunteers with and without blank subtraction were 60-137% and 68-146%, respectively, compared with that of the manufacturer (70-140%).

The influence of the side reactions on FXIII activity was displayed in patient's samples by low FXIII activity. Patient sample 1 clearly demonstrated that the noncorrected value was approximately five times higher than the corrected one (5.4% vs. 0.0%). The difference of the two values in samples 2 and 3 was 10.8% (42.6% vs. 31.8%) and 7.4% (53.5% vs. 46.1%), respectively. The values near normal range were unaffected as they seemed to have similar values in both procedures. We used diluted standard human plasma and FXIII deficient plasma as a representative of low FXIII activity samples to determine the overestimation of the Berichrom FXIII assay. The results confirmed the influence of side reactions on the assay because the activity values without blank subtraction were greater than the subtracted ones of all dilutions (approximately 3%-7%). Some studies have reported that when the values with the iodoacetamide blank were not subtracted, FXIII activities were overestimated by 5%¹⁴. The experiment from Lawrie et al.¹⁶ demonstrated that the overestimation of the Berichrom FXIII assay did not affect the samples with FXIII activity values near normal ranges but caused misleading results at low levels of the values (below 10%). The study from Cini et al.¹⁷ using the Berichrom FXIII assay also reported similar results. In this study, the authors divided plasma samples in three groups, FXIII values < 20%, 20%-70% and > 70% and found that overestimation was more significant in the group of values below 20%.

In conclusion, our study of the Berichrom FXIII assay with and without sample blank procedures displayed a good precision regarding the procedures. The assay was fast, precise and easily automated. The results confirmed a problem involving side reactions on the FXIII activity leading to an overestimation of the assay, especially in samples with low levels of the factor. The use of iodoacetamide sample blank procedures is recommended to avoid this obstacle. Eliminating the overestimation will facilitate better clinical management of patients.

References

1. Muszbek L, Adany R, Mikkola H. Novel aspects of blood coagulation factor XIII. I. Structure, distribution, activation and function. *Crit Rev Clin Lab Sci*. 1996;33:357-421.
2. Bereczky Z, Katona E, Muszbek L. Fibrin stabilization (factor XIII), fibrin structure and thrombosis. *Pathophysiol Haemost Thromb*. 2003;33:430-7.
3. Mosesson MW, Siebenlist KR, Hernandez I, Lee KN, Christiansen VJ, McKee PA. Evidence that alpha2-antiplasmin becomes covalently ligated to plasma fibrinogen in the circulation: a new role for plasma factor XIII in fibrinolysis regulation. *J Thromb Haemost*. 2008;6:1565-70.
4. Peyvandi F, Duga S, Akhavan S, Mannucci PM. Rare coagulation deficiencies. *Hemophilia*. 2002;8:308-21.
5. Biswas A, Ivaskevicius V, Seitz R, Thomas A, Oldenburg J. An update of the mutation profile of factor 13 A and B genes. *Blood Rev*. 2011;25:193-204.
6. Peyvandi F, Di Michele D, Bolton-Maggs PHB, Lee CA, Tripodi A, Srivastava A, for the Project on Consensus Definitions in Rare Bleeding Disorders of the Factor VIII/Factor IX Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. Classification of rare bleeding disorders (RBDs) based on the association between coagulation factor activity and clinical bleeding severity. *J Thromb Haemost*. 2012;10:1938-43.
7. Lak M, Peyvandi F, Ali Sharifian A, Karimi K, Mannucci PM. Pattern of symptoms in 93 Iranian patients with severe factor XIII deficiency. *J Thromb Haemost*. 2003;1:1852-3.
8. Naderi M, Karimi M, Hosseini MS, Moradi E, Shamsizadeh M, Dorgalaleh A. Long term follow up on a large group of patients with congenital factor XIII deficiency treated prophylactically with fibrogammin P. *Iranian J Phar Res*. 2016;15:635-40.
9. Ichinose A, Souri M; Japanese Collaborative Research Group on Acquired Haemorrhaphilia Due to Factor XIII deficiency. As many as 12 cases with haemorrhagic acquired factor XIII deficiency due to its inhibitors were recently found in Japan. *Thromb Haemost*. 2011;105:925-7.
10. Jeanings I, Kitchen S, Woods TA, Preston FE. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEOAS study. *J Thromb haemost*. 2003;1:2603-8.
11. Katona E, Penzes K, Molnar E, Muszbek L. Measurement of factor XIII activity in plasma. *Clin Chem Lab Med*. 2012;50:1191-202.
12. Kohler HP, Ichinose A, Seitz R, Ariens RAS, Muszbek L; on behalf of the Factor XIII and Fibrinogen SSC Subcommittee of the ISTH. Diagnosis and classification of factor XIII deficiencies. *J Thromb Haemost*. 2011;9:1404-6.
13. Muszbek L, Polgar J, Fesus L. Kinetic determination of blood coagulation Factor XIII in plasma. *Clin Chem*. 1985;31:35-40.
14. Karpati L, Penke B, Katona E, Balogh I, Vamosi G, Muszbek L. A modified, optimized kinetic photometric assay for the determination of blood coagulation factor XIII activity in plasma. *Clin Chem*. 2000;46:1946-55.
15. Ajzner E, Muszbek L. Prophylactic and perioperative replacement therapy for acquired factor XIII deficiency: a rebuttal. *J Thromb Haemost*. 2004;2:2075-7.
16. Lawrie AS, Green L, Mackie IJ, Leisner R, Machin SJ, Peyvandi F. Factor XIII - an under diagnosed deficiency - are we using the right assays? *J Thromb Haemost*. 2010;8:2478-82.
17. Cini M, Legnani C, Frascaro M, Pancani C, Cappelli C, Rodorigo G, Valdre L, Cosmi B. Measurement of factor XIII (FXIII) activity by an automatic ammonia release assay using iodiacetamide blank-procedure: no more overestimation in the low activity range and better detection of severe FXIII deficiencies. *Clin Chem Lab Med*. 2016;54:805-9.

