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# Evaluation of Factor XIII Activity Using Berichrom FXIII Chromogenic Assay on the Fully-Automated Coagulation Analyzer CS-2000i - Based on Comparisons with Universal Biochemical Analyzer and Antigen Method -

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*The fully-automated coagulation analyzer CS-2000i (hereinafter CS-2000i; Sysmex Corporation, Kobe, Japan) is capable of monitoring reactions using four measuring technologies - clotting, chromogenic, immunologic and agglutination methods. The fundamental performance and precision of the Berichrom FXIII chromogenic ammonia release assay on this analyzer is evaluated and reported here. High reproducibility of this assay (Control Plasma N: mean 83.6%, CV 1.8% ; Control Plasma P: mean 27.3%, CV 4.2%) and the acceptable linearity studies using pooled plasma (14.4-147%) were obtained. The on-board stability of reagents was demonstrated. Not only the interference material (Interference Check A Plus) but also ammonia (acceptable range of concentration described in the product document) had no influence on the Berichrom FXIII assay. With this reagent, there seems to be no difference between analyzers (CS-2000i and Hitachi H-7180) and there was good correlation with the FXIII-A subunit antigen. This study suggests that the basic performance of the FXIII activity assay on CS-2000i is favorable and useful as a diagnostic test.*

**Key Words** Fully-Automated Coagulation Analyzer, CS-2000i, Berichrom FXIII, Factor XIII Activity

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## INTRODUCTION

Blood coagulation factor XIII (FXIII), also known as fibrin stabilizing factor, is a transglutaminase precursor that acts at the final stage of the blood coagulation reaction. It plays an important role in the completion and maintenance of hemostasis and in wound healing. *In vivo*, FXIII provides thrombi with resistance against clot lysis by physical force or plasmin, by inducing cross-linking between fibrin molecules or between fibrin and  $\alpha$ 2-plasmin inhibitor. Thus, congenital and severe acquired FXIII deficiency, in which FXIII activity is reduced or absent because of a disorder in FXIII production or its excessive consumption, cause abnormal bleeding and abnormality in wound healing. When patients present with such clinical symptoms, a FXIII deficiency is suspected and laboratory assessment should be carried out for FXIII activity. This assay is also very useful for determining whether there is a need to administer FXIII concentrates and for monitoring the efficacy of therapy. As for measuring FXIII activity, two methods for the measurement of FXIII activity are

available at the bedside in Japan. One is the synthetic substrate (chromogenic) method, and the other is the antigen measurement method, in which the amount of the FXIII-A subunit having the enzymatic activity is determined immunologically using a specific antibody. However, since autoantibody for FXIII (as it is called FXIII inhibitor) is present in some diseases, it is more important to measure the enzymic activity rather than the FXIII-A subunit antigen level<sup>1)</sup>. Berichrom FXIII (Siemens Healthcare Diagnostics, Marburg, Germany) is a commercially available kit for an easy method to measure the amount of ammonia released from a synthetic substrate by the action of FXIII to determine the FXIII activity. The fully automated blood coagulation analyzer CS-2000i (hereinafter referred to as "CS-2100i") developed by Sysmex is capable of measuring FXIII activity easily using Berichrom FXIII, by detection at 340 nm with the multi-wavelength detection capability of the analyzer<sup>2,3)</sup>. In this study, we examined the basic performance of CS-2000i in measuring FXIII activity, and compared these results with those obtained from another analyzer, and with results from the FXIII-A subunit antigen method.

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## MATERIALS AND METHODS

Prior approval for the study was obtained from the University of Tokyo, Graduate School of Medicine Research Ethics Committee.

### 1. Analysis Systems and Reagents

The reagents used were Berichrom FXIII for measuring FXIII activity by the synthetic substrate method and LPIA FXIII (Mitsubishi Chemical Medience Corp, Tokyo, Japan) for determining the FXIII-A subunit antigen level by latex immunoassay. The automated instruments used for measuring FXIII activity were CS-2000*i* and Hitachi automatic analyzer 7180 (H-7180) (Hitachi High-Technologies, Tokyo, Japan). LPIA-A700 analyzer (LPIA) (Mitsubishi Chemical Medience Corp, Tokyo, Japan) was used for measuring the FXIII-A subunit antigen. The measurement principle of FXIII activity using Berichrom FXIII is described in the product insert of Berichrom FXIII or references<sup>1,3)</sup>. Briefly, FXIII activated by thrombin in the reagent reacts with the synthetic substrate to release ammonia. The ammonia released oxidizes NADH to NAD. The difference in absorbance (delta optical density; dOD), the reduction in the absorbance of each measurement point at 340 nm due to this oxidation, is measured to determine FXIII activity. We also used the following reagents: Control Plasma N, Control Plasma P and Owren's Veronal Buffer (OVB) from Siemens Healthcare Diagnostics (Marburg, Germany), factor XIII-deficient plasma from George King Bio-Medical (Overland Park, KS), Interference Check A Plus from Sysmex Corporation (Kobe, Japan), and ammonia water from Japanese Pharmacopoeia (Tokyo, Japan).

### 2. Within-Run Reproducibility

The within-run reproducibility was determined by performing 10 replicate measurements on each of the coagulation control plasmas, Control Plasma N and P, and factor XIII-deficient plasma.

### 3. Linearity

Pooled plasma samples from individual subjects were prepared by mixing samples with high FXIII activity. A 10-point dilution series was prepared from this stock plasma using Owren's Veronal Buffer (OVB) as the diluent. OVB was used as the 0% sample. Two replicate measurements were made on each sample of the dilution series for the linearity study.

### 4. Reagent On Board stability

The same vials of Reagent 1 and Reagent 2 of Berichrom FXIII test kit were set for 9 hours from 9:00 to 18:00 in the opened condition inside the refrigerated reagent chamber of the CS-2000*i* analyzer for 5 days. Control Plasmas N and P were prepared freshly each day before use.

### 5. Influence of Interfering Substances Against FXIII Activity

The influence of interfering substances was studied using Interference Check A Plus with two types of pooled plasmas (FXIII activity 25% and 93%). Ammonia water was also used as an interfering substance with the plasma samples with FXIII activity 93%.

### 6. Test for Detection Sensitivity

A dilution series (1/2, 1/4 and 1/8) was prepared from factor XIII deficient plasma using OVB as a diluent. OVB was used as the 0% sample. Five replicate measurements were performed with each diluted sample. The lowest concentration at which the dOD value of the mean minus 2 standard deviations (SD) did not overlap with that of the mean plus 2SD of the 0% sample was determined.

### 7. Influence of Fibrinogen Levels on FXIII Activity

The product insert of the reagents mentions the possibility of underestimating FXIII activity at fibrinogen concentrations < 80 mg/dL and > 600 mg/dL. Therefore, measurements were made using plasma specimens with  $\geq$  600 mg/dL of fibrinogen (as it is called "original sample" of high fibrinogen level) and a 1/2 re-dilution was made automatically by the CS-2000*i*; measurements were made using plasma specimens with  $\leq$  80 mg/dL of fibrinogen ("original sample" of low fibrinogen level) and automatic 3/2 dilution of the sample was made by the CS-2000*i* to increase the level of fibrinogen more than the default setting (doubling dilution). In general, the sample was diluted 2-fold automatically before the measurements (*Table 1*). Consequently, the 1/2 re-dilution of the samples with high fibrinogen level means 4-fold dilution, and 3/2 dilution of the samples with low fibrinogen level means 1.5-fold dilution. The measured FXIII values were compared, and the influence of high and low fibrinogen concentrations on the results was confirmed.

### 8. Verification of the Protocol with a Biochemical Analyzer

Since a synthetic substrate is used, Berichrom FXIII measurements can also be obtained with a general-purpose automatic biochemistry analyzer. To investigate the influence of the FXIII activity by the differences between analyzers, we analyzed Berichrom FXIII activity on H-7180.

### 9. Correlation

The correlation between FXIII activity measured by the two analyzers, CS-2000*i* and H-7180, using Berichrom FXIII and the correlation of these results with those from the antigen method (CS-2000*i* vs. LPIA and H-7180 vs. LPIA) were examined using plasma samples from 76 patients. These plasmas from both hospitalized patients and outpatients submitted for coagulation testing were used for this study.

## RESULTS

### 1. Within-Run Reproducibility

The within-run reproducibility of FXIII activity measured by CS-2000i using Berichrom FXIII for the Control Plasma N was  $83.6 \pm 1.5\%$  (mean  $\pm$  SD) with a coefficient of variation (CV) of 1.8%. The corresponding values were  $27.3 \pm 1.1\%$  and 4.2% for Control Plasma P and  $6.1 \pm 1.0\%$  and 16.1% for factor XIII deficient plasma.

### 2. Linearity

Measurements of the 10-point dilution series of pooled plasma showed linearity in the range of 14.4% to 147%

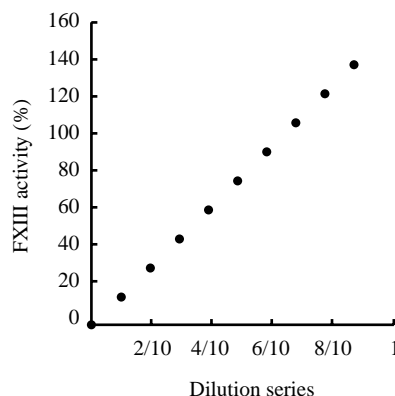
with the line passing through the origin (including the 0% sample) (*Fig. 1*).

### 3. Reagent On Board Stability

The results of both the Control Plasmas N and P showed almost stable results for up to 5 days with CVs of 1.6% and 2.9%, respectively. These results suggest that there is no degradation of the Berichrom FXIII reagents during the test period (*Table 2*).

### 4. Influence of Interfering Substances Against FXIII Activity

The measured values of two types of pooled plasma samples with different concentrations showed no effect of turbidity up to 1,500 formazin turbidity units, and no



*Fig. 1* Linearity

The pooled plasma sample with high FXIII activity was used as the stock plasma (Dilution series 1) and a dilution series was prepared using Owen's Veronal Buffer (OVB) as the diluent. The buffer alone was used as the sample with 0% FXIII activity (Dilution series 0). Two replicate measurements were made with each diluted sample.

*Table 1* Comparison of Berichrom FXIII Parameters

Parameters	F-XIII (Berichrom FXIII)	
	CS-2000i	HITACHI-7180
Analyzer	CS-2000i	HITACHI-7180
Method of analysis	Rate	Rate A
Photometric measurement points	300 (sec) to 600 (sec)	30 to 34
Wavelength (sub/main) (nm)	340	660/340
Sample volume (μL)	20	12
Volume of diluent (μL)	20	No dilution
Volume of R1 (Activator) (μL)	100	120
Volume of R2 (Substrate) (μL)	100	80
Calibration curve	Connected line	Straight line
Calibration points	5 (automatic dilution)	2

For analysis with the CS-2000i (following the instructions of the package insert), NADH Reagent was dissolved in 5 mL of purified water and then added to the FXIII activator for use as R1.

For analysis with H-7180, NADH Reagent was dissolved in 8 mL of purified water and the entire amount was then added to the FXIII activator for use as R1.

Detection Reagent (include substrate) for both analyzers was dissolved in 5 mL of purified water to prepare R2.

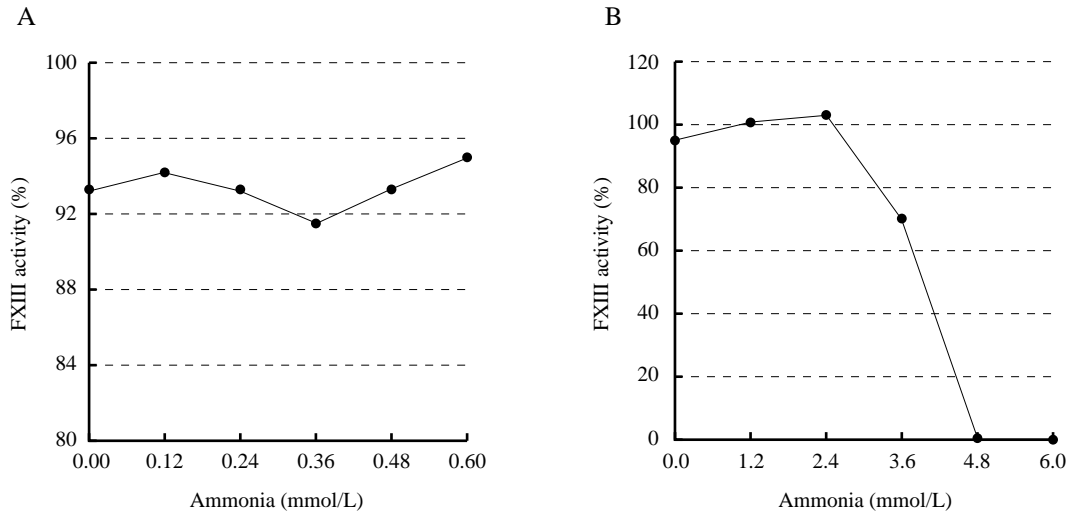
effect of hemolysis up to 496 mg/dL of hemoglobin. Bilirubin C and bilirubin F also had no effect up to 201 mg/dL and 192 mg/dL respectively. No effect of ammonia was seen up to 0.6 mmol/L (**Fig. 2-A**). There was a positive error of 8% (95-103%) with ammonia concentration of 0.6-2.4 mmol/L (**Fig. 2-B**). Ammonia concentration > 3.6 mmol/L had a lowering effect on the FXIII activity and the activity became almost 0 at 4.8 mmol/L and above.

### 5. Minimum Detectable Sensitivity

The minimum detectable sensitivity as the lowest concentration of the dilution series of FXIII deficient plasma corresponded to FXIII activity of 1.94% (**Fig. 3**).

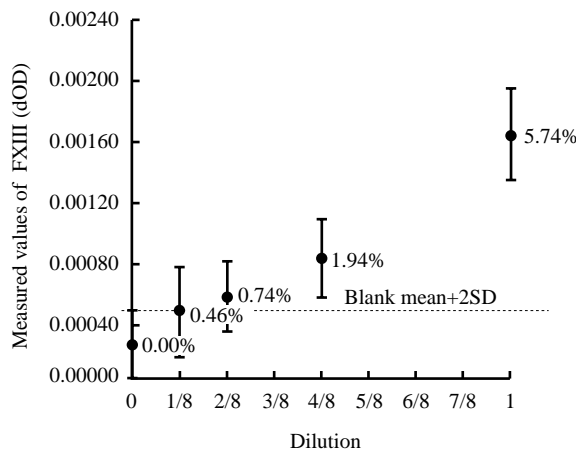
### 6. Influence of Fibrinogen Level on FXIII Activity

Plasma samples with high fibrinogen levels from 9



**Fig. 2** Influence of Interfering Substance (Ammonia) Against FXIII Activity

Ammonia water was added as an interfering substance to pooled plasma samples (FXIII activity 93%) and then the FXIII activity in the plasmas was determined. (A) With low ammonia concentration (0-0.6 mmol/L), (B) With high ammonia concentration (0-6.0 mmol/L).



**Fig. 3** Minimum Detectable Sensitivity

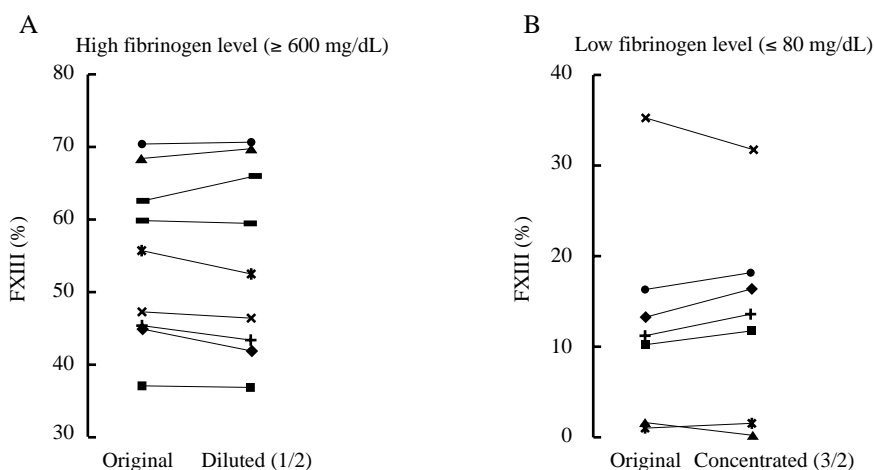
A dilution series of samples was prepared using factor XIII deficient plasma (Dilution series 1) and OVB (Dilution series 0), and the minimum detectable sensitivity for dOD was estimated. The numerical values in the Fig are the means of FXIII activity (%) obtained from dOD. Filled circles with error bars represent mean and 2SD of five independent experiments.

patients submitted for coagulation testing in our clinical laboratory were selected and their undiluted (original sample) and OVB-diluted samples were assessed for FXIII activity. No difference was shown between two kinds of methods and the means of FXIII activity were 54.7% and 54.2% respectively (**Fig. 4-A**). For 7 samples with low fibrinogen levels, there was no difference in the mean values of FXIII activity between the original and 3/2 times concentrated samples (12.6% and 13.3% respectively) (**Fig. 4-B**).

### 7. Verification of Protocol with a Biochemical Analyzer

We investigated whether it was possible to measure FXIII activity using Berichrom FXIII on H-7180, and

whether the measured results agreed with those of CS-2000i. For CS-2000i, there was no problem with setting up Berichrom FXIII as a monitoring test for FXIII activity according to the instructions of its package insert. However, in the case of using H-7180, it was difficult to set up this reagent with the standard reconstitution method described in the package insert. Since the absorbance levels of these prepared reagents exceeded the control limit of the analyzer, we thought this was the reason why the absorbance was unreliable and the calibration curve could not be prepared. Therefore, we tried to make a new protocol for H-7180 to assess FXIII activity using Berichrom FXIII. NADH Reagent was dissolved in 8 mL of purified water which is more dilute than standard method and the entire volume of this solution was added to the FXIII activator (Berichrom



**Fig. 4** Influence of High and Low Fibrinogen Levels On FXIII Activity

(A) Plasma samples with high fibrinogen levels of 9 patients were assessed for FXIII activity using two kinds of setting of CS-2000i. Their undiluted and diluted samples (1:1 with OVB) were analyzed (t-test for difference between the two groups:  $p = 0.48$ ) using the ordinary method. (B) Plasmas of 7 samples with low fibrinogen levels were assessed for FXIII activity with or without setting for making plasma concentrated (t-test:  $p = 0.45$ ).

**Table 2** Stability of Reagents on Board

Coagulation control plasma N									
	Day-1	Day-2	Day-3	Day-4	Day-5				
1	81.9	81.1	78.9	82.8	82.8	MEAN	SD	CV	
2	83.7	80.2	84.1	85.4	80.2				
MEAN	82.8	80.65	81.5	84.1	81.5	82.11	1.352	1.647	

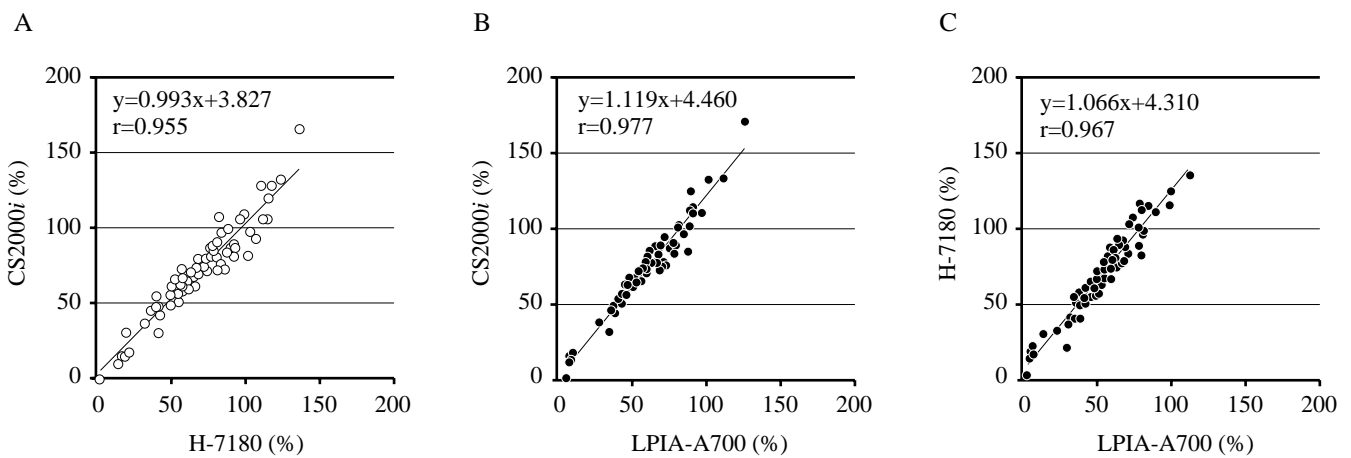
Coagulation control plasma P									
	Day-1	Day-2	Day-3	Day-4	Day-5				
1	29.1	29.1	26.8	24.6	27.3	MEAN	SD	CV	
2	27.3	25.5	25.5	28.6	27.7				
MEAN	28.2	27.3	26.15	26.6	27.5	27.15	0.798	2.941	

FXIII kit reagent) for use as R1. In an attempt to avoid the influence of the endogenous ammonia and LDH on the NADH, we delayed the beginning of the photometric points, and changed to between 30 to 34 measurement points (corresponding to from about 5 to 10 minutes after adding the second reagent) in the later stages of the reaction (**Table 2**).

### 8. Correlation

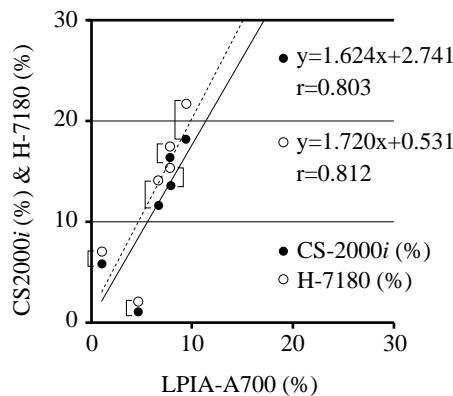
FXIII activity using Berichrom FXIII determined by CS-2000i and H-7180 showed good correlation, with the slope of the regression line at 0.993 and correlation coefficient 0.955, demonstrating that there was no

significant difference between the analyzers (**Fig. 5-A**). The correlation between FXIII activity measured with each analyzer and the FXIII-A subunit antigen level determined by the LPIA method was also good. The slope was 1.119 and correlation coefficient 0.977 between CS-2000i and LPIA (**Fig. 5-B**); and 1.066 and 0.967 respectively between H-7180 and LPIA (**Fig. 5-C**). With a similar correlation using samples with  $\leq 20\%$  FXIII-A subunit antigen, the slope was 1.624 and correlation coefficient 0.803 between CS-2000i and LPIA; and 1.720 and 0.812 respectively between H-7180 and LPIA (**Fig. 6**). Thus, the FXIII activity assessed by Berichrom FXIII was higher than that assessed by the FXIII-A subunit antigen level.



**Fig. 5** Correlation of FXIII Activity Between Different Analyzers and Methods (plasma from 76 patients)

(A) Correlation of results between CS-2000i and H-7180 obtained using Berichrom FXIII; (B) Correlation between FXIII activity (CS-2000i) and FXIII-A subunit antigen (LPIA-A700); (C) Correlation between FXIII activity (H-7180) and FXIII-A subunit antigen (LPIA-A700)



**Fig. 6** Correlation of FXIII Measurements Between Different Analyzers and Methods (plasma from 6 patients with low FXIII-A subunit antigen levels)

In this scatter plot with two regression lines, the Y-axis represents FXIII activity measured by H-7180 (○, broken line) or by CS-2000i (●, solid line); the X-axis represents the FXIII-A subunit antigen level (LPIA-A700). Each pair of symbols (○ and ●) connected by a square bracket represents single sample from identical patient.

## DISCUSSION

We evaluated the basic performance of the CS-2000i, a fully automated blood coagulation analyzer, in measuring FXIII activity using Berichrom FXIII, and compared the results with those obtained from another analyzer (a biochemical analyzer), and with the results of a FXIII-A subunit antigen method.

Basic parameters of performance, such as the within-run reproducibility, stability of reagents on board, linearity of the results, influence of interfering substances (Interference Check A Plus), and influence of fibrinogen, were satisfactory in the measurements made by the CS-2000i. However, an important requirement is being able to measure low levels of FXIII accurately for diagnosing FXIII deficiency. It is reported<sup>1,3,4)</sup> that presence of endogenous enzymes in plasma caused positive error, and poor sensitivity in the low level of FXIII, especially less than 5%, since those enzymes were contributing to NADH consumption or ammonia production. Indeed in our investigations, using OVB as the 0% sample, it was possible to obtain good linearity in the physiological range of FXIII activity, and to detect a fairly low level (1.94%) of FXIII activity. However, in case of assessment of FXIII activity using FXIII deficient plasma (displayed FXIII activity; 1% or less and ) without dilution, FXIII activity analyzed by both CS-2000i or H-7180 was 6-7% in despite of undetectable level of FXIII-A subunit antigen (< 1-2%) . The limit of accuracy in FXIII activity measurement is considered to be 5% or more<sup>5)</sup>. Thus, we then studied the correlation between the

FXIII activity using Berichrom FXIII and the FXIII-A subunit antigen level using plasmas with less than 20% antigen level. The results reveal that the activity levels measured by the two analyzers were higher than the antigen levels. As reported earlier<sup>6)</sup>, it may be necessary to perform some adjustment such as subtracting a blank value obtained from plasma sample spiked with iodoacetamide. Therefore, we think that it is necessary to make public announcement of characteristic features of this reagent, and to develop highly sensitive analytical reagents without preparing blanks for measurements.

The influence of ammonia as a negative error on CS-2000i measurements occurred from 3.6 mmol/L onwards, as reported by Fickenscher<sup>4)</sup>. There was no effect up to 0.6 mmol/L, although a positive error occurred in the concentration range of 0.6-2.4 mmol/L, as reported by Kawaguchi et al.<sup>7)</sup>. They hypothesized that the measurement points might play an important role in such error, since it took some time to disperse added ammonia in this measurement system. It is, however, not clear whether the excess NADH in the reagent was sufficient to process the endogenous enzyme that affects this assay. We assumed that the suitable time for photometric measurements was between 300 and 600 seconds after addition of the reagent. Actually in CS-2000i, this timing appears to be important in avoiding the influence of ammonia on the measured value.

As reported earlier<sup>7)</sup>, Berichrom FXIII does not require a special analyzer and can be used with a general-purpose automatic analyzer. Compared with using CS-2000i, we therefore assessed Berichrom FXIII on H-7180 to investigate the accuracy of the FXIII activity

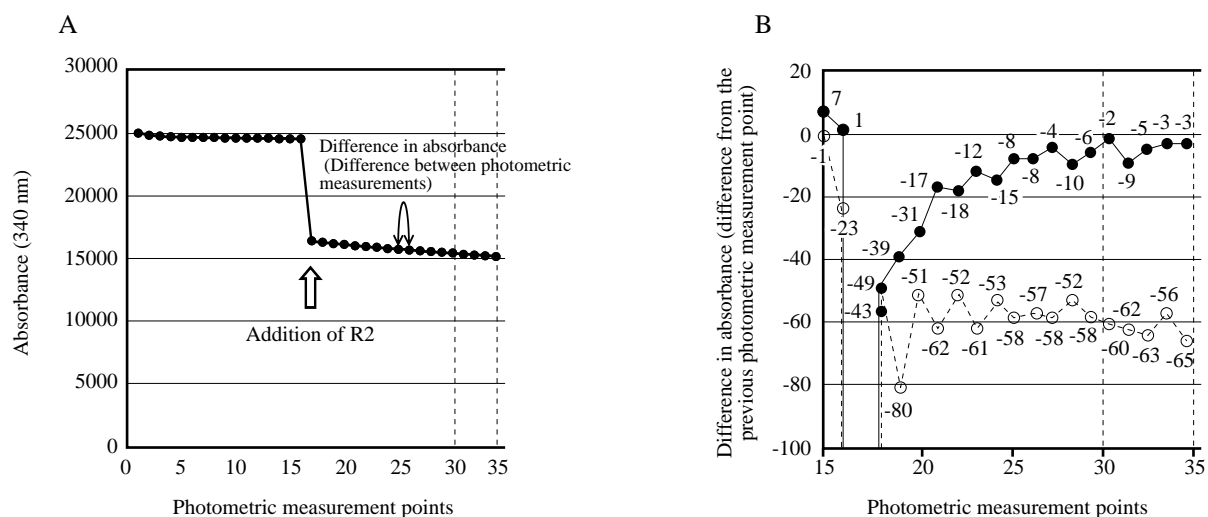


Fig. 7 Absorbance Measured By H-7180 Using Berichrom FXIII

The amount of change in absorbance was assayed by rate assay at a wavelength of 340 nm using H-7180 between 30 to 34 photometric assay points (vertical broken lines). (A) The time course of absorbance measured with physiological saline used as the sample with 0% FXIII activity. (B) Graph magnified view of (A) shows the difference in the absorbance change between the two measurement points. Physiological saline is represented by the solid line and the calibrator is (displayed FXIII activity 102%) as the broken line.

measurements. There is already a report<sup>8)</sup> on the protocol for mixing R1 (NADH and FXIII activator reagent) and R2 (substrate) for analysis by the Hitachi automatic analyzer 7170 (Hitachi High-Technologies), a different model of the same series as H-7180. However, the stability of the reagents decreases quickly after R1 and R2 are mixed. Hence, in this study we tried to develop a protocol where R1 and R2 would not be mixed. Because abnormal absorbance error occurred in using the normal protocol for dissolving these reagents, we examined further this protocol and finally obtained stable analysis results by dissolving NADH in 8 mL of purified water and adding the entire amount into the FXIII activator reagent to prepare R1. We also examined the time course of the reaction with H-7180 (*Fig. 7-A*), and the photometric points were determined by reference to the report<sup>9)</sup> previously. The measurement point allowed for a 5 minutes lag time for eliminating endogenous ammonia in the plasma with an excess of NADH. At the initial measurement stage, the change in absorbance has a sharp slope, but the actual measurement time was set at 30-34 measurement points where the difference between successive measurement points was small. At the initial measurement stage, the change in absorbance (dOD) has a steep slope. In contrast, it is essential to set the actual measurement time during undistorted and stable monitoring. Since dOD represents the level of FXIII activity, it seems that this error could have a major impact on its value and could be a large margin of error, particularly in samples with low activity (*Fig. 7-B*). Consequently, those points were set at 30 to 34 measurement points where the difference in absorbance was small between successive measurement points. FXIII activity measured using this protocol showed good correlation between measurements by CS-2000i and the other analyzer.

It is natural that FXIII activity and FXIII-A subunit antigen showed good correlation, while the FXIII-A subunit antigen method generally gave lower values as reported earlier<sup>3)</sup>. This difference in the measured values was greater in samples with low FXIII-A subunit antigen levels of less than 20%. Although the reasons for this difference have not yet been confirmed, that could be the positive error due to the characteristics of the FXIII activity assay reagents, and be attributed to influence of the endogenous enzyme. Also, there is the difference of the measurement principle between the two assays and the range of FXIII activity. FXIII-A subunit antigen reagent measures the FXIII-A subunit immunologically as a FXIII enzymatic activity. FXIII-A subunit antigen reagent allows its enzymatic activity as an alternative in the range of 10-140% to be determined (use by LPIA-NV7; described in the product insert). Further investigations are necessary to develop a method to obtain the true values for FXIII activity, especially in the low antigen range.

FXIII is an important factor in the hemostatic mechanism *in vivo*. However it is impossible to be assessed by

ordinary screening tests like PT and APTT. Thus, FXIII deficiency has to be ruled out in some cases of unexplained abnormal bleeding, and it is essential to evaluate FXIII activity directly. Nonetheless, there are difficult hurdles to diagnose this condition, since only very few hospital laboratories measure FXIII activity routinely. The Berichrom FXIII used in the present study is a commercially available reagent for FXIII activity and is currently used worldwide<sup>3,5)</sup>. Our study showed that FXIII activity using that reagent could be determined with an ordinary biochemical analyzer that is capable of measuring absorbance at 340 nm. Taken together, it is hoped that this would make the measurement of FXIII activity possible in any clinical laboratory.

## CONCLUSION

The basic performance of CS-2000i in FXIII activity measurement was sufficient and useful for routine FXIII activity assay.

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