Activated partial thromboplastin time-based clot waveform analysis enables measurement of very low levels of factor IX activity in patients with severe hemophilia B

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Running head: CWA measurement of very low level of FIX

Type of manuscript: Original article

Abstract words; 197 words, **Manuscript words;** 3,208 words,

Numbers of reference 29, **Table** 3, **Figure** 3, and **supplemental data** 0

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Abstract

The precise measurement of very low levels of factor IX activity (FIX:C <1 IU/dL) is essential for understanding clinical severity and risk of inhibitor development in patients with severe hemophilia B (Pw-SHB). However, such measurement sensitivity has not yet been achieved. We aimed to establish a measurement method using clot waveform analysis (CWA). Residual FIX:C by adding anti-FIX monoclonal antibody, FIX:C by adding recombinant (r)FIX to the commercial Pw-SHB plasmas, and FIX:C in our Pw-SHB were determined by CS-2000i™/CS-2400™, followed by analysis of CWA parameters. The presence of anti-FIX antibody in the commercial Pw-SHB plasmas significantly decreased coagulation potential compared to its absence. The addition of rFIX to these innate plasma samples produced significant changes in three parameters upon adding FIX:C at 0.1–1 IU/dL, supporting the presence of trace FIX:C in Pw-SHB. Therefore, appropriate FIX-depleted plasma containing minimum residual FIX:C was chosen from reference curves of FIX:C (0.01–1 IU/dL). Among patients with untreated Pw-SHB, two had FIX:C 0.6–0.7 IU/dL and two had lower than detectable levels using FIX-depleted plasma. One of the latter had detectable trough levels post-rFIX administration. In conclusion, CWA enabled measurement of very low levels of FIX:C using appropriate FIX-deficient plasma.

Key words; hemophilia B, factor IX, severity, clot waveform analysis, activity

Introduction

Hemophilia A (HA) and hemophilia B (HB) are caused by a deficiency or defect in factor (F)VIII and FIX procoagulant protein, respectively. The clinical severities in HA and HB patients are based on the FVIII and FIX activity (FVIII:C and FIX:C) levels obtained by a one-stage clotting assay and are classified into three categories: severe (<1 IU/dL), moderate (1≤, <5 IU/dL), and mild type (5≤, <40 IU/dL) [1]. The introduction of regular prophylaxis using clotting factor products to prevent repeated joint and/or intramuscular bleeding in these patients has dramatically improved the quality of life of severely affected patients [2, 3].

In both types of hemophilia, although both clotting factor activities <1 IU/dL define severe deficiency, differences in the clinical phenotypes are often seen in individuals with similar levels of activity [4, 5]. Some reasons are considered to influence these observations. One reason is the difference in hemophilia related to the *F8* and *F9* gene mutation types. The majority of HB patients are reported as having missense mutations associated with mild to moderate clinical severity [6-8], supporting the presence of low levels of FIX:C. Furthermore, several severe HB patients appear to express very low levels of FIX:C, in contrast to severe HA patients associated with null mutations of *F8* containing the intron 22 inversion in approximately half of these patients [9, 10]. Severe HB patients require joint surgery less than severe HA patients [11, 12], indicating that the clinical manifestations of FIX defects appear likely to be relatively mild compared to FVIII defects.

Another reason may be the measurement sensitivity of very low levels of FVIII:C and FIX:C by activated partial thromboplastin time (APTT) conventional assays. We successfully established the measurement of very low levels of FVIII:C (0.2 IU/dL for the lowest limit) by APTT-based clot waveform analysis (CWA) using an MDA-II® instrument [13, 14]. In addition, we demonstrated that even the presence of similar FVIII:C <1 IU/dL affected the clot waveform patterns in severe HA patients [15, 16], and contributed to distinguishing the different clinical phenotypes among severe types. Considering the treatment and the risk of inhibitor development for hemophilia patients, it is important to measure very low levels of clotting factor activity precisely, and deeply understand the clinical phenotype of patients with severe hemophilia in clinical practice.

However, the measurement of very low levels of FIX:C in patients with severe HB remains to be established from the point of view of measurement sensitivity. In the present study, we attempted to establish the precise measurement of very low range of FIX:C levels in patients with severe HB patients by APTT-based CWA using the widely-spread CS series instrument (Sysmex Corp, Kobe, Japan).

Materials and Methods

Ethics: This study was approved by the Medical Research Ethics Committee of Nara Medical University (No. 2503), and blood samples were obtained after obtaining informed consent following local ethical guidelines.

Reagents: The rFIX preparations (Benefix®; Pfizer, New York, NY), plasma of FIX-deficient patients, plasma of FVIII-deficient patients (George-King Inc; Overland Park, KS), FIX-depleted plasma (Sysmex, SIEMENS; Munchen, Germany, HYPHEN BioMed; Neuville-sur-Oise, France), Coagtrol®, and Thrombocheck®APTT-SLA kit (Sysmex) were purchased from the indicated vendors. Recombinant monoclonal IgG antibody to the γ-carboxyglutamic acid (Gla) domain of human FIX/FIXa was expressed in Expi293-F™ cells (Thermo Fisher Scientific Japan, Tokyo, Japan) and purified using protein A Sepharose. Its variable regions were derived from the antibody described in a previously published article [17]. An anti-FVIII polyclonal antibody was purified using a protein G Sepharose from the plasma obtained from a patient with severe HA and a high-titer inhibitor [18].

Patients: Severe HB patients aged 2–46 years, without (n=3) and with an inhibitor (n=1) who were admitted to our hospital participated in the present study.

Blood samples: Whole blood was obtained by venipuncture from patients and healthy volunteers (n=20; men:women=3:1, age ranging from 23–49 years) after obtaining informed consent following local ethical guidelines. The samples were placed in test tubes containing a 1:9 volume of 3.2% (w/v) trisodium citrate without a corn trypsin inhibitor. None of the study subjects had taken any other medication that might have influenced the platelet or coagulation function 1 week prior to blood sampling. Platelet-poor plasma was obtained after the centrifugation of citrated whole blood for 15 min at 1,500 *g*. All plasma samples were stored at -80 °C and were thawed at 37 °C immediately prior to the APTT assay.

FIX:C assay: FIX:C was measured by an APTT-based one-stage clotting assay using FIX-deficient plasma and Thrombocheck APTT-SLA (Sysmex) on the CS-2000i™ and CS-2400™ (Sysmex). The FIX inhibitor titers were determined using the Bethesda assay [19]. The incubation reaction with the commercial plasma or patient's plasma and an anti-FIX antibody or anti-FVIII antibody were performed at 37 °C for 10 min.

Clot waveform analysis (CWA): CWA was performed on a CS-2000i™/CS-2400™ (Sysmex) using the APTT-trigger reagent [15, 16]. This automated coagulation analyzer detects the intensity of transmitted light every 0.1 s at 660 nm wavelength in the APTT assays. The obtained clot waveforms were computer-processed using a commercial kinetic algorithm [15, 16]. The horizontal axis shows the time (s), and the vertical axis shows the transmittance (%), defined as the intensity of transmitted light from the pre-coagulation to the post-coagulation phase. The clot time (CT) is determined as the time to the point where the transmittance reduces to a predefined level. The first derivative of the transmittance (dT/dt) reflects the coagulation velocity at each time point. The minimum value of the first derivative (min1) was calculated as an indicator of the maximum coagulation velocity. As the minimum of min1 was derived from negative changes, the data were expressed as |min1|. The second derivative of the transmittance data (d^2T/dt^2) reflects the acceleration of the reaction at any given time point; additionally, the maximum coagulation acceleration (|min2|) was calculated from the second derivative curve.

In addition, the transmittance in the post-coagulation phase is influenced by the fibrinogen concentration and fibrin clot density; however, in our modified CWA analyses, the minimum transmittance (0%) was also set at the immediate post-coagulation phase (adjusted-CWA) [20]. Ad|min1| and Ad|min2| were defined as |min1| and |min2| of the adjusted clot waveform, respectively.

***Data analysis*:** Data analysis was performed using Microsoft Excel. The analysis of variance (ANOVA) test and multiple comparison analysis tests, including the Tukey and Dunnett tests, were performed. Significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism (version 4.0; GraphPad Software, Inc., San Diego, CA).

Results

Comparison of the coagulation function by CWA between the plasma of severe HB and HA patients

First, to investigate whether very low levels of FIX:C were present in the plasma obtained from patients with severe HB (FIX:C <1 IU/dL measured by one-stage clotting assay), coagulation potentials in commercial severe HB plasma (n=6) preincubated with an anti-FIX monoclonal antibody (f.c. 67.8 μg/ml) were assessed by an APTT-based CWA with CS-2000i™. The CWA parameters before and after the addition of anti-FIX antibody were compared. These parameters were also compared with those in the plasma from patients with severe HB inhibitor (n=3; 0.5, 2.4, and 3.2 BU/mL). The addition of this antibody in plasma samples with severe type demonstrated that the clot times were prolonged and the |min1| and |min2| values decreased significantly compared to their absence. The parameters with the addition of antibodies were comparable to those in HB inhibitor plasmas (**Figure 1A**). These results indicated that the plasma from patients with severe HB contained very low levels of FIX:C; additionally, FIX-complete defect plasma could be distinguished among the plasmas of patients with severe HB.

Similarly, to investigate whether severe HA patients (FVIII:C <1 IU/dL) presented very low levels of FVIII:C, the coagulation potentials in commercial severe HA plasma (n=6) added an anti-FVIII polyclonal antibody (f.c. 4.3 μg/ml) and in the plasma from HA inhibitor patients (n=4; 9.0, 18, 60, and 107 BU/mL) were evaluated repeatedly by an APTT-based CWA under same conditions. Unlike the severe HB plasma, these parameters changed only slightly before and after the addition of an anti-FVIII antibody and were comparable to the HA inhibitor plasmas, supporting that severe HA plasma samples used in this study contained little residual FVIII:C (**Figure 1B**).

Furthermore, to examine the contribution of FVIII and FIX on the coagulation function in each plasma inhibitor, HB inhibitor plasma, or HA inhibitor plasma was preincubated with an anti-FVIII antibody or anti-FIX antibody, respectively, prior to the measurement. The addition of anti-FVIII antibody to HB inhibitor plasma did not significantly affect the parameters, while the addition of anti-FIX antibody to the HA inhibitor plasma prolonged the clot time and decreased |min1| and |min2| significantly, showing a further reduction of coagulation potentials (**Figure 1A and 1B**). This result confirmed the essential contribution of FIX in the coagulation function, and that the HB-complete deficient plasma possessed a lower coagulation function than HA-complete deficient plasma.

Estimation of residual FIX:C levels in severe HB patients' plasmas

To estimate the very low residual levels of FIX:C in the plasma of patients with severe HB, serially diluted rFIX preparations (0, 0.001, 0.01, 0.1, 1, 10, and 100 IU/dL) were added to the plasma of commercial severe HB patients (n=6), followed by APTT-CWA and analyses of parameters. Severe HB plasma with an anti-FIX antibody (as FIX-complete deficient plasma) was also prepared. The mean clot time value was shortened and the |min1| value increased significantly with the addition of at least 0.1 IU/dL of rFIX significantly. The mean of the |min2| value also showed a significant increase with the addition of at least 1 IU/dL of rFIX (**Figure 2**). In all samples, all the parameter values converged with the addition of an anti-FIX antibody. Notably, the concentration of spiked rFIX at which the parameter values clearly changed had a large inter-individual variation between 0.01 to 1 IU/dL, indicating that these severe HB plasma samples contained very low residual levels of FIX:C, approximately 0.01–1 IU/dL.

Establishment of a method for the measurement of very low levels of FIX:C by APTT-based CWA

Subsequently, we attempted to establish a method to measure very low levels of FIX:C using an APTT-based CWA using CS-2400™. The aforementioned results suggest the importance of utilizing FIX-complete deficient plasma to precisely measure very low levels of FIX:C. Therefore, we focused on the commercial FIX-deficient plasma created by the adsorption of FIX (FIX-depleted plasma). A total of 10 different lots of FIX-depleted plasma (five lots in Sysmex, four lots in SIEMENS, and one lot in HYPHEN) were used. An anti-FIX antibody was added to each sample of FIX-depleted plasma, followed by APTT-CWA measurement. The minimum rate of change in the parameters before and after the addition of the antibody was regarded as the presence of the minimum residual FIX activity. The rate of change was calculated as follows; "(|Parameters before the addition of antibody − Parameters after the addition of antibody|)/(Parameter before the addition of antibody)". The rate of change in the FIX-depleted plasma used is summarized in **Table 1**, and Sysmex lot #2 plasma showed the minimum rates of change in all parameters, that is, the presence of minimum residual activity.

Serially diluted normal plasma (Coagtrol N®: FIX:C 103 IU/dL) containing FIX:C levels ranging from 0 to 1 IU/dL (0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 IU/dL) were prepared as reference samples. The APTT-based CWA for FIX:C was performed using FIX-depleted plasma (Sysmex lot #2) that contained the minimum residual activity. The reference sample plasmas were pre-diluted 20-fold with imidazole buffer and mixed with equal amounts of FIX-depleted plasma and APTT reagent. After incubation for 3 min at 37 °C, an equal amount of $CaCl_2$ was added, and the measurement was started. The obtained parameters were used to create a reference curve for very low FIX:C levels. The reference curves were set in the three ranges of FIX:C 0.1–1 IU/dL, 0.05–1 IU/dL, and 0.01–1 IU/dL; additionally, the R^2 values in the reference curve for each parameter were determined (**Table 2**). The clot time was highly correlated in the range of 0.1−1 IU/dL (**Figure 3**), and the |min1|, Ad|min1|, and |min2| parameters were highly correlated in the range of FIX:C 0.01–1 IU/dL, indicating the usefulness of these parameters. Among all parameters, the R^2 value in Ad|min2| was the closest to 1, suggesting that Ad|min2| appeared to be the most accurate parameter for measuring very low levels of FIX:C (**Figure 3**).

Very low levels of FIX:C assessment in our severe HB patients in clinical practice

A total of eight plasma samples from four severe HB patients (FIX:C <1 IU/dL measured by one-stage clotting assay) who visited our hospital were assessed for their FIX:C values with the reference curves using the above-chosen FIX-depleted plasma by CWA. The patients' plasma included three samples from one inhibitor case, two samples from three untreated HB non-inhibitor cases, and one case after rFIX (Benefix®) administration for pharmacokinetic evaluation.

The results of the FIX:C measurements for all samples are summarized in **Table 3**. The FIX:C levels in one inhibitor patient (case 1) were below the detectable limit (<0.01 IU/dL) in all three samples. Two non-inhibitor patients (cases 2 and 3) showed the innate FIX:C levels of 0.62 and 0.71 IU/dL, assessed by the Ad|min2|. After 8 days of rFIX infusion at 39 IU/kg, and after 7 days of rFIX infusion at 78 IU/kg in one non-inhibitor patient (case 4), the trough values of the patient were 0.2–0.3 IU/dL and 0.5–0.6 IU/dL, respectively, supporting the finding that very low levels of FIX:C could be assessed by the Ad|min2|.

Discussion

In the present study, we observed some characteristics of plasma obtained from patients with severe HB. From the assessment of the plasma of patients with severe hemophilia A and B by the APTT-CWA, the addition of an anti-FIX antibody to the FVIII inhibitor plasmas further decreased the coagulation potentials, while the addition of an anti-FVIII antibody to the FIX inhibitor plasmas did not. These findings indicate that the presence of FIX, even in the absence of complete FVIII, exerted some effect on coagulation function; however, the complete absence of FIX did not exert any coagulation function [21]. Activated FVIII (FVIIIa) is a cofactor of FIXa for FX activation, and FIXa functions as an enzyme on phospholipid membranes [21]. The presence of FIXa can activate FX, albeit very slowly, even in the absence of FVIIIa. Our results supported that HB complete-deficient patients possessed lesser coagulation function than HA complete-deficient patients, based on the functional differences of FIX and FVIII molecules.

From a clinical point of view, there have been some reports that the clinical phenotypes in severe HB appears to be mild compared to that of severe HA [4, 5, 11, 12]. The present study also showed that the CWA parameters in plasma from patients with severe HA showed little change even with the addition of an anti-FVIII antibody to their plasma, comparable to the HA inhibitor patients, indicating that the FVIII:C levels in several cases with severe HA appeared to be completely or almost completely defective. However, the CWA parameters in severe HB plasma samples showed a further decrease in the coagulation potential by the addition of an anti-FIX antibody to the plasma. These results demonstrated that the presence of very low levels of FIX:C in patients with severe HB might contribute to their mild clinical phenotypes. This difference appears to be due to the difference in causative gene mutations, as it is known that null mutations in severe HA and non-null mutations in severe HB are more common [6-10]. Thus, trace residual FIX:C could be present in the plasma of patients with severe HB. Therefore, when the FIX-deficient plasma used to prepare the reference curve contained trace residual FIX:C levels, it would be difficult to precisely assess very low levels of FIX:C. We need to be careful as it is important to choose the FIX-deficient plasma with as little residual activity as possible for the measurement of very low levels of FIX:C.

In the present study, we utilized artificial FIX-depleted plasma to choose the appropriate plasma containing minimum or no residual activity. From the reference curve we developed, Ad|min2| was the most useful parameter for very low levels of FIX measurement. The |min2| reflects the coagulation acceleration on the clot waveform, which corresponds to the amplification and propagation phases during the coagulation process. Endogenous tenase activity as its central role is governed by the presence of FVIIIa and FIXa [21]. This result was consistent with our previous report that |min2| was sensitive to FVIII:C and FIX:C measurements [13, 14]. In addition, it is known that the fibrinogen concentration in plasma affects the fibrin permeability in the coagulation waveform [20, 22]. We developed an adjusted method to eliminate the influence of fibrinogen as much as possible [20]; additionally, it is reasonable that the Ad|min2| parameter was more effective for measuring very low levels of FIX:C. It is known that coagulation factor deficiencies may alter the diameter and density of fibrin clot (make fibrin fiber thicker) compared to normal fibrin fiber (thinner), resulting in more decreased transmittance than normal plasma [23]. Therefore, |min1| and |min2| without the adjustment might be over-estimated due to excessive changes of transmittance. Although it is not clear how much this adjustment corrects the impaired fibrin's quality in FIX-deficient plasma, it is important to minimize the influence of other factors when assessing the FIX:C level in the patient's plasma, as in this case. Our results showed that the adjusted parameters (Ad|min1| and Ad|min2|) allowed for more accurate measurement of the FIX:C range at very low levels.

Matsumoto et al. [14] previously reported a measurement limit of 0.2 IU/dL for a trace level of FIX:C in aPTT-based CWA using MDA-II®. Compared to the previous report, the present study had two advantages by using i) optimal FIX-depleted commercial plasma and ii) parameters adjusting the coagulation waveform such as Ad|min1| and Ad|min2|. Consequently, we successfully evaluated the FIX:C at an extremely trace level at 0.01 IU/dL for the lowest limit. This assessment could allow us to measure the innate FIX:C level and residual FIX:C for hemostatic management precisely and in addition predict the null mutations carrying for HB patients.

In clinical practice, the ability to measure very low levels of FIX:C provides a precise assessment of the FIX:C levels in patients with severe HB and non-inhibitor, as well as to measure the trough levels of FIX:C after FIX concentrated infusion (*see* Table 3). In addition, the ability to determine very low levels of residual activity would sufficiently influence the treatment decisions, such as the dosage, dosing interval, and FIX product.

Inhibitor development is one of the major issues in hemophilia treatment. The incidence rates are lower in HB (3–5%) than in HA (20–30%) [24-29]. The reason for the lower incidence of inhibitors in HB remains unclear; however, it occurs mainly in severe cases. Large deletions and nonsense mutations have been reported to account for approximately 80% of inhibitor cases in HB [30], suggesting a causal relationship between large gene deletions and FIX inhibitor development. Therefore, we would like to mention that the identification of complete defects in plasma FIX by measuring trace amounts of FIX:C would be very meaningful in predicting the risk of developing inhibitors.

Acknowledgements

We thank Ms. Tomoko Matsumoto (Nara Medical University) for her technical support.

Authorships

AN. performed all experiments, analyzed the data, interpreted the data, made the figures, and wrote the manuscript. **K.O.** designed the experiments, provided clinical support, analyzed the data, interpreted the data, edited the manuscript, and approved the submission of the first version. **KM.** designed the experiments and interpreted the data. **N.H.** interpreted the data. **M.T.** provided clinical support. **K.N.** designed the experiments, provided clinical support, interpreted the data, made the figures, and wrote and edited the manuscript.

Conflict of interest statements

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Comparison of the coagulation potential values obtained from APTT-based CWA of plasma from severe HB and severe HA patients

(**A**) Commercially available plasma samples of patients with severe HB (n=6) that reacted with an anti-FIX antibody, HB inhibitor patients' plasma (n=3), and those that reacted with an anti-FVIII antibody were measured by APTT-CWA. (**B**) Commercially available plasma samples of patients with severe HA (n=6) that reacted with an anti-FVIII antibody, HA inhibitor patients' plasma samples (n=4), and those samples that reacted with an anti-FIX antibody, were measured by APTT-CWA. The obtained waveforms were analyzed to calculate the parameters. |min1|; maximum coagulation velocity |min2|; maximum coagulation acceleration *: P <0.05, **: P <0.01, ***: P <0.001

Figure 2 Estimation of very low residual levels of FIX:C levels in the plasma of severe HB patients

The plasma of patients with severe HB with the addition of rFIX product (FIX:C 0–100 IU/dL) or with an anti-FIX antibody was performed using APTT-CWA, followed by parameter analyses. |min1|: maximum coagulation velocity, |min2|: maximum coagulation acceleration. *: P <0.05, **: P <0.01, ***: P <0.001

Figure 3. Reference curve for very low levels of FIX:C

Samples containing the FIX:C level within 0–1 IU/dL were prepared by serial dilutions of commercial normal plasma. APTT-CWA for samples with very low levels of FIX:C was performed using the FIX-depleted plasma, which was assessed to have the minimum residual FIX:C, followed by the parameter analyses. 0.1–1 IU/dL, 0.05–1 IU/dL, and 0.01–1 IU/dL FIX:C levels were used as the reference curves. The relationship (R^2) between the reference curve and the representative parameters, clot time, and Ad|min2| are shown.

Table 1. Rate of change of CWA parameters before and after the addition of anti-FIX monoclonal antibody in the FIX-deficient (depleted) plasma

	CWA parameters				
	Clot time	|min1|	Ad|min1|	|min2|	Ad|min2|
Sysmex #1	0.1014	0.2058	0.2401	0.2796	0.3106
#2	**0.0339**	**0.0800**	**0.0970**	**0.1006**	**0.1172**
#3	0.0607	0.1198	0.1480	0.1522	0.1793
#4	0.0372	0.1114	0.1181	0.1212	0.1279
#5	0.2859	0.5013	0.5457	0.5744	0.6123
SIEMENS#1	0.1716	0.8846	0.8977	0.3356	0.4114
#2	1.2997	0.8782	0.8983	0.6630	0.7195
#3	0.1565	0.8620	0.8775	0.3404	0.4153
#4	2.0627	0.8530	0.8739	0.5691	0.6309
HYPHEN#1	0.3528	0.4731	0.4616	2.3704	2.4414

The data indicate the change rate of parameters before and after the addition of anti-FIX antibody. This change rate was calculated by (|Parameter before the addition of anti-FIX antibody − Parameter after the addition of anti-FIX antibody|) / (Parameter before the addition of anti-FIX antibody). The minimum rate of change, *i.e*, the presence of minimum residual FIX activity is shown in bold type. CWA; clot waveform analysis, FIX; factor IX

Table 2. R^2 value on the reference curve for each CWA parameter

Parameters	Reference range of FIX:C		
	0.01 − 1.0 (IU/dL)	0.05 − 1.0 (IU/dL)	0.1 − 1.0 (IU/dL)
Clot time	0.8119	0.9523	**0.9862**
|min1|	**0.9718**	0.9704	0.9701
Ad|min1|	0.9814	0.9809	**0.9827**
|min2|	**0.9829**	0.9820	0.9796
Ad|min2|	**0.9895**	0.9891	0.9886

The R^2 values were compared among each parameter, and the highest correlation values are shown in bold type. CWA: clot waveform analysis, FIX:C; factor IX activity

Table 3. Measurements of very low concentrations of FIX:C using the selected FIX-deficient plasma in severe HB patients with or without inhibitor

Parameter	Reference curve of FIX:C	Severe HB with inhibitor$			Severe HB without inhibitor				
		Case 1 #			Case 2	Case 3	Case 4 *		
		1	2	3	Untreated		Untreated	Post 8 day (39 IU/kg)	Post 7 day (78 IU/kg)
	IU/dL	*IU/dL*			*IU/dL*		*IU/dL*		
Clot time	0.1 − 1.0	<0.1	<0.1	<0.1	0.62	0.28	<0.1	0.20	0.60
|min1|	0.01 − 1.0	<0.01	0.04	<0.01	0.68	0.78	<0.01	0.31	0.61
Ad|min1|	0.1 − 1.0	<0.1	<0.1	<0.1	0.60	0.70	<0.1	0.23	0.56
|min2|	0.01 − 1.0	<0.01	0.04	<0.01	0.68	0.77	<0.01	0.21	0.52
Ad|min2|	0.01 − 1.0	<0.01	<0.01	<0.01	0.62	0.71	<0.01	0.19	0.50

#: Case 1 shows the measurement of samples obtained from three different 3 days. $: FIX inhibitor titer; 1–3; 3.2, 0.53, 6.53 BU/mL, respectively

*: Case 4 received the rFIX infusion at 39 IU/kg and the rFIX infusion at 78 IU/kg, followed by measuring the rough levels of FIX:C at 8 and 7 days after administration. FIX:C; factor IX activity, HB; hemophilia B

Figure 1

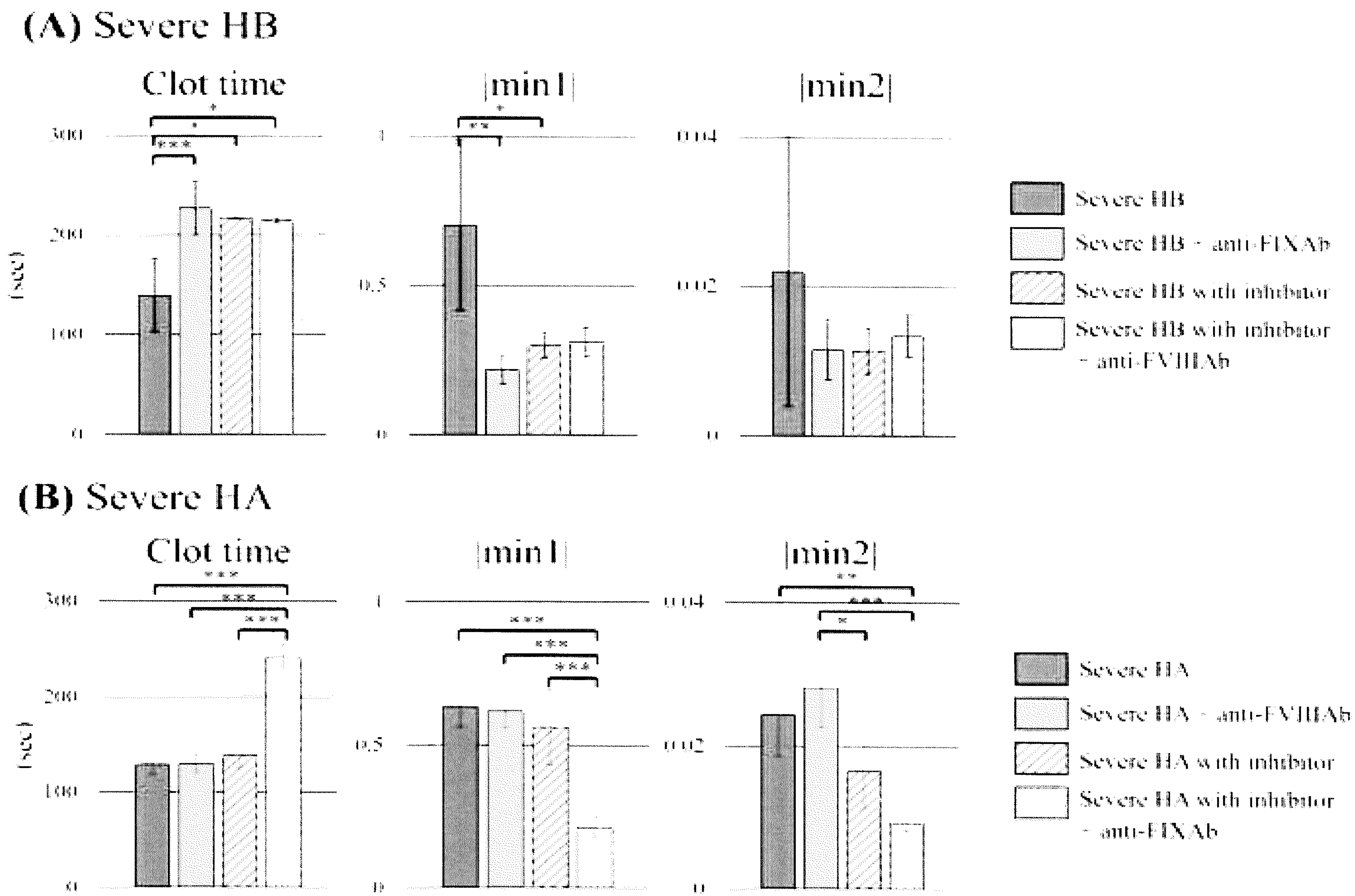

Figure 1

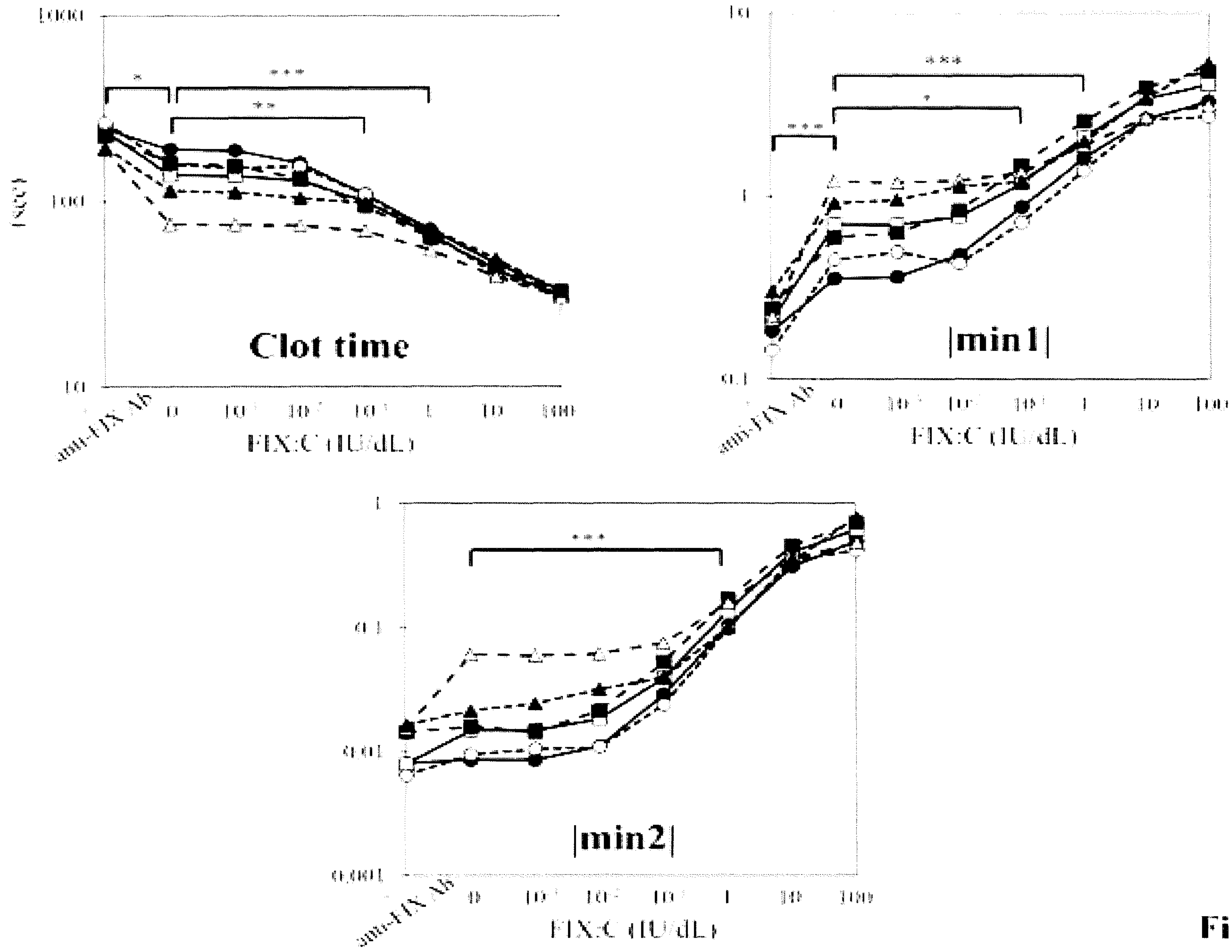
Clot time
(sec)
|min1|
|min2|
FIX:C (IU/dL)
FIX:C (IU/dL)
FIX:C (IU/dL)
anti-FIX Ab

Figure 2

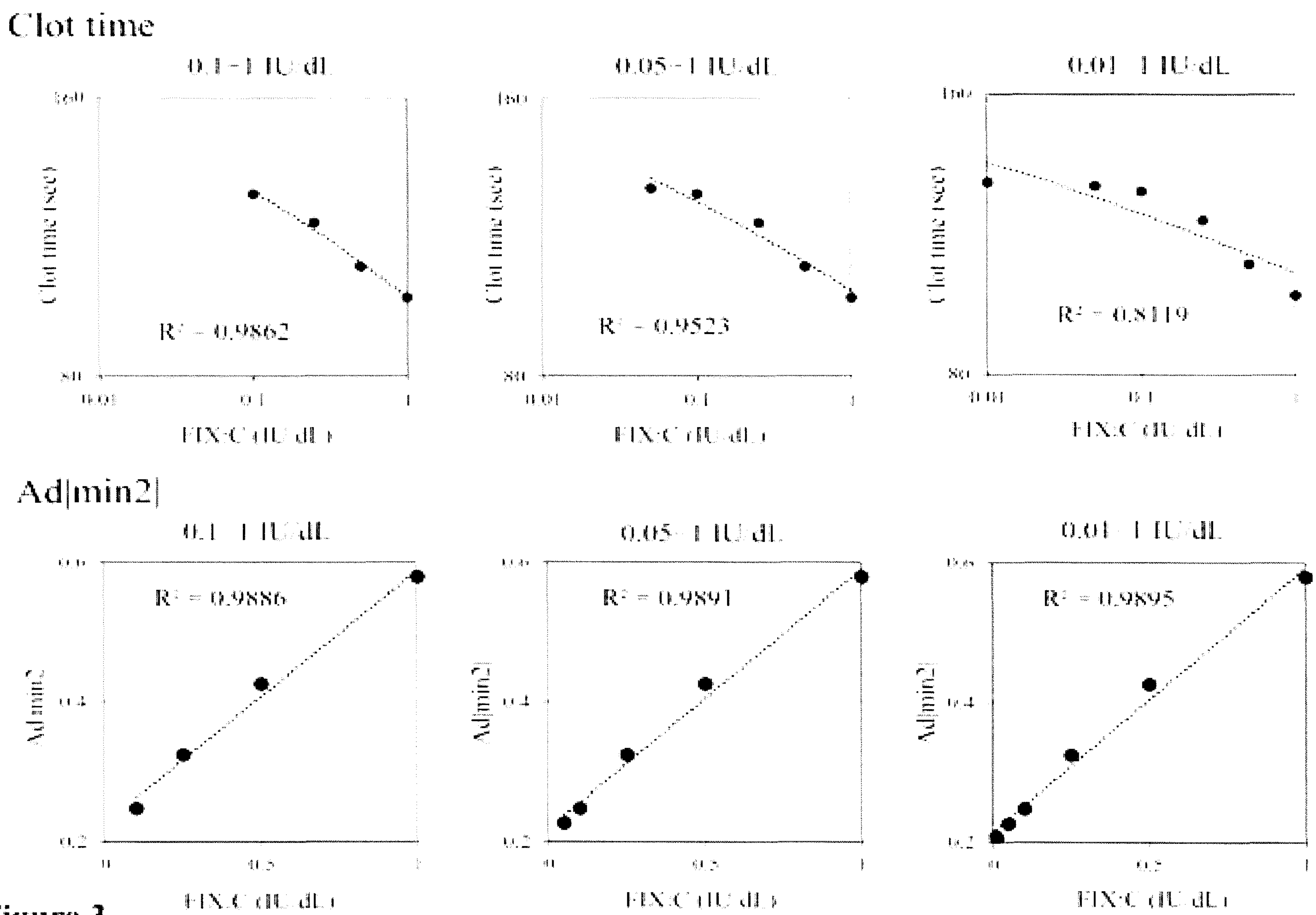
Clot time
0.1 - 1 IU/dL
0.05 - 1 IU/dL
0.01 - 1 IU/dL
Clot time (sec)
R² = 0.9862
R² = 0.9523
R² = 0.8119
FIX:C (IU/dL)
Ad|min2|
R² = 0.9886
R² = 0.9891
R² = 0.9895
Ad|min2|

Figure 3