Optimization of the Mechanism for Measuring Ristocetin Cofactor Activity (VWF:RCo) with the Fully Automated Blood Coagulation Analyzer Sysmex® CS-2000*i*

Nobuo ARAI, Kae MUKAIDE, Toru SUGIYAMA and Kunihiro FUNAKOSHI

Scientific Affairs, Sysmex Corporation, 1-3-2 Murotani, Nishi-ku, Kobe 651-2241, Japan

To make it capable of VWF:RCo measurements, we provided a built-in mixing function in the CS-2000i, comprising a reaction cuvette in which a magnetic stirrer bar is inserted. The shape (diameter and length) of the stirrer bar and the stirring speed have major impact on data in this measurement. We conducted various investigations for optimizing the assay conditions to obtain good quality data. After comprehensive evaluation including reproducibility and the slope of the calibration curve, we set the stirrer bar diameter at 1.2 mm and length at 3.8 mm and stirring speed at 900 rpm as the assay conditions. Assay results obtained with two levels of control plasma showed good within-run reproducibility, the CV being 4.4% for normal plasma and 3.3% for pathological plasma. When the sample dilution was changed before measurement in the very low and very high activity ranges, dilution linearity was shown in the 5 - 330% VWF:RCo activity range. The detection limit determined with low VWF:RCo activity samples was found to be 5% activity. The results obtained with the CS-2000i were correlated with those of the manual method. The two were especially close in the low activity range.
We believe that the CS-2000i, capable of measuring VWF:Ag, FVIII:C and VWF:RCo, can provide useful information for VWD diagnosis.

Key Words Fully Automated Blood Coagulation Analyzer CS-2000*i*, VWF, VWF:RCo

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INTRODUCTION

von Willebrand disease (VWD) is a hemorrhagic disease caused by a quantitative or qualitative abnormality in von Willebrand factor (VWF), arising from a genetic abnormality. Persons with this disease are known to have low or missing factor VIII ristocetin cofactor activity (VWF:RCo), in the high molecular weight part of the coagulation factor VIII molecule[1-3]. The measurement of activity (function) and the measurement of the antigen level are available as methods of quantifying VWF. The bleeding time and platelet aggregation have been used for a long time for measuring VWF function. However, these methods are low in specificity and difficult to quantify. Therefore, VWF:RCo and ristocetin-induced platelet aggregation (RIPA) are now more widely used. Immunoelectrophoresis using antihuman VWF polyclonal antibody is available for measuring the VWF antigen level. This method is also being replaced by a simpler method, the latex aggregation method, wherein latex particles coated with polyclonal antibodies are used[4,5]. VWF:RCo measurement is a highly sensitive secondary screening test wherein VWF is measured through its biological activity of aggregating platelets in the presence of ristocetin. The test is conducted manually or with a platelet aggregometer (or certain types of blood coagulation analyzers). So far it has not been carried out with general purpose coagulation analyzers. While developing the fully automated blood coagulation analyzer, the Sysmex® CS-2000*i* (hereinafter CS-2000*i*) we provided a stirring mechanism in the detector unit to provide VWF:RCo assay capability in a general purpose coagulation analyzer[6]. We shall report here our investigations on the optimization of the stirring mechanism in the CS-2000*i*.

ASSAY PRINCIPLE

The VWF:RCo reagent is a lyophilized product containing stabilized human platelets, ristocetin, and EDTA. The assay makes use of the fact that VWF in the plasma, together with the ristocetin and stabilized human platelets in the reagent, cause platelet aggregation. The change in turbidity of the reaction mixture caused by this aggregation is measured photometrically (***Fig. 1***), and the measured value is expressed as percent activity in relation to a calibration curve prepared with normal plasma. In the CS-2000*i*, a stirring mechanism, in which a stirrer continuously mixes the sample and reagent, is built into the detector unit (***Fig. 2***).

MATERIALS AND METHODS

1. Analyzer

Fully automated blood coagulation analyzer Sysmex® CS-2000*i*

2. Reagents

1) BC von Willebrand reagent (SIEMENS)
2) Standard Human Plasma (SIEMENS)
3) Control Plasma N (SIEMENS)
4) Control Plasma P (SIEMENS)

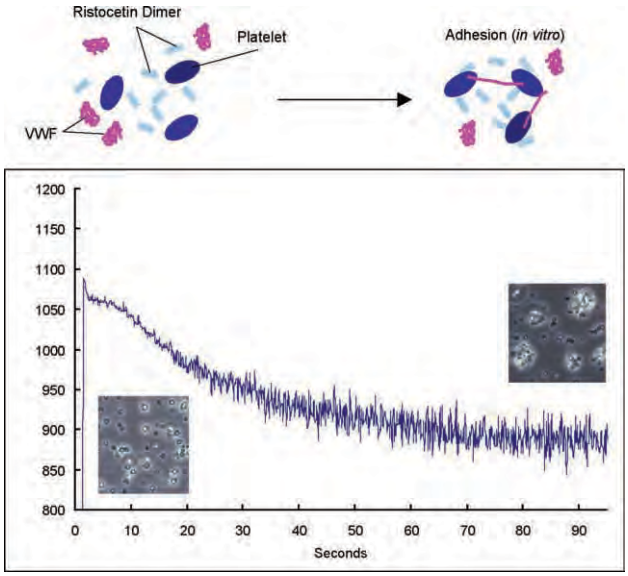


Fig. 1 *VWF:RCo Assay Principle*

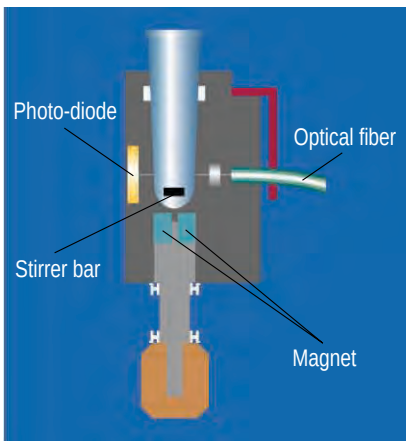


Fig. 2 *Cuvette Mixing System*

3. Methods

Firstly, the optimum shape of the stirrer bar was selected in trials with stirrer bars of different diameter and length and then the optimum stirring speed determined. Basic examination was conducted with these optimum settings.

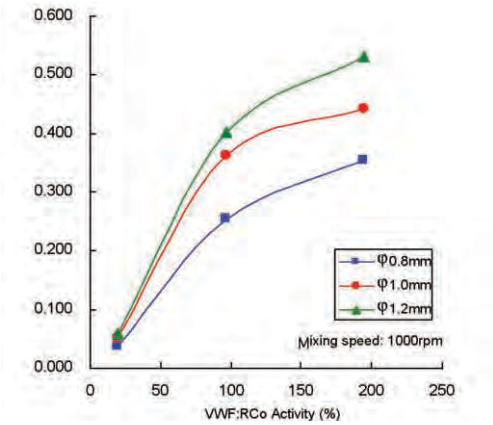
SETTING OF ASSAY CONDITIONS

1. Diameter of stirrer bar

Calibration curves obtained with stirrer bars of three different diameters (0.8, 1.0, and 1.2 mm) were compared. The rate of change in absorbance (dOD/min) tended to increase with increase in the diameter. Therefore we decided to use a 1.2 mm diameter stirrer bar for which the dOD/min was highest at each level of activity tested (***Fig. 3***).

2. Length of stirrer bar

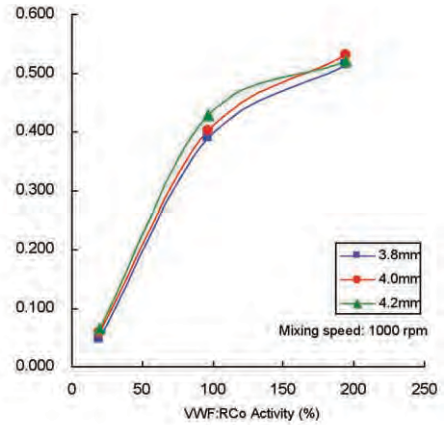
Calibration curves obtained with stirrer bars of three different lengths (3.8, 4.0 and 4.2 mm) at 1,000 rpm (rotations per minute) were compared to decide the optimum length. The dOD/min was about the same with no major differences among the three lengths (***Fig. 4***).
After comprehensive evaluation including



n = 5

Diameter	dOD/min			
	VWF: RCo	19.7%	97%	194%
φ 0.8mm	Mean	0.0365	0.2552	0.3531
	%CV	19.9	5.6	7.1
φ 1.0mm	Mean	0.0517	0.3621	0.4432
	%CV	15.3	7.8	8.3
φ 1.2mm	Mean	0.0581	0.4023	0.5313
	%CV	5.3	4.2	8.9

Fig. 3 *Evaluation of Diameter of Stirrer Bar*



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Length	dOD/min			
	VWF: RCo	19.7%	97%	194%
3.8mm	Mean	0.0474	0.3887	0.5134
	%CV	4.0	4.0	6.9
4.0mm	Mean	0.0581	0.4023	0.5313
	%CV	5.3	4.2	8.9
4.2mm	Mean	0.0655	0.4285	0.5205
	%CV	7.2	9.2	9.4

Fig. 4 *Evaluation of Length of Stirrer Bar*

reproducibility, change in absorbance and inside diameter of cuvette, we decided to use the shortest (3.8 mm) among the three lengths tested.

3. Stirring speed

Six stirring speeds, i.e., 700, 752, 800, 900, 1,000, and 1,200 rpm were tested with a stirrer bar of diameter 1.2 mm and length 3.8 mm. The dOD/min at different VWF:RCo activity levels tended to become larger with increase in stirring speed. There was, however, a tendency at high rpm for the slope of the calibration curve to be reduced at high VWF:RCo activity. Besides this, when we checked the relationship between stirring speed and dOD/min, we found that the difference in dOD/min between 94% and 188% VWF:RCo activity was small at 700 and 1,200 rpm, suggesting inferior resolution. Good resolution between different activity levels was obtained with 800, 900, and 1,000 rpm. Among these, 900 rpm gave the best reproducibility (CV 4.9 - 6.8%). The stirring speed was thus set at 900 rpm (***Fig. 5***).

4. Stirring in the low activity range

In order to improve the detection sensitivity in the low activity range of 20% and less, we examined the effect of stirring speed during the lag phase (0 - 15 seconds) from the addition of the reagent up to the photometric measurement. The low activity sample such as 0, 8.7, and 17.4% were examined with three stirring speeds, 900, 400, and 150 rpm.

Three stirring speeds, 900, 400, and 150 rpm, were examined with low activity sample such as 0, 8.7 and 17.4%. The dOD/min at different VWF:RCo activity levels showed that at 900 rpm, chosen in Section 4 - 3, there was little difference between VWF:RCo activity 0% and 17.4%, and that 400 rpm gave the highest sensitivity. Based on this result, we selected 400 rpm as the stirring speed in the lag phase (***Fig. 6***).

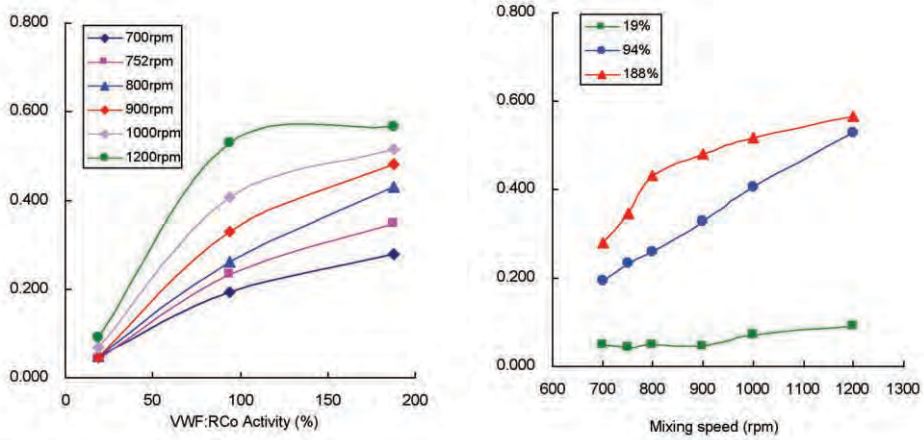
5. Setting of assay conditions

Based on the above examination, we set the conditions for measuring VWF:RCo using CS-2000*i* (***Table 1***). Two choices were provided for the assay, the "low mode" that covered the 10 - 50% measurement range and the "medium mode" in which the sample is pre-diluted 4-fold, and the 40 - 200% range is covered.

BASIC PERFORMANCE AT THE ASSAY CONDITIONS SET

1. Within-run reproducibility

Normal plasma and pathological plasma were measured 20 times continuously to test within-run reproducibility. The normal range with Control Plasma N (CPN) had a CV of 4.4% and the pathological range with Control Plasma P (CPP) had a CV of 3.3% (***Table 2***).



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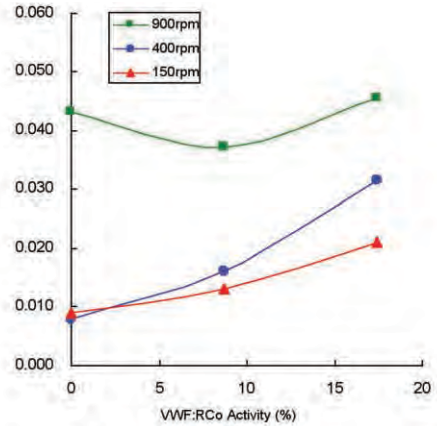
Mixing Speed	dOD/min			
	VWF: RCo	18.8%	94%	188%
700rpm	Mean	0.048	0.193	0.280
	%CV	5.8%	6.0%	13.7%
752rpm	Mean	0.043	0.234	0.346
	%CV	25.7%	6.6%	6.7%
800rpm	Mean	0.048	0.261	0.431
	%CV	15.0%	3.5%	8.0%
900rpm	Mean	0.045	0.329	0.480
	%CV	6.8%	4.9%	5.1%
1000rpm	Mean	0.070	0.406	0.516
	%CV	26.5%	7.1%	2.4%
1200rpm	Mean	0.092	0.530	0.567
	%CV	7.3%	5.0%	7.7%

Fig. 5 *Evaluation of Mixing Speed of Stirrer Bar*



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Lag Phase Mixing Speed	dOD/min				Sensitivity
	VWF: RCo	0%	8.70%	17.40%	17.4% - 0%
900rpm	Mean	0.043	0.037	0.046	0.003
400rpm	Mean	0.006	0.016	0.032	0.024
150rpm	Mean	0.009	0.013	0.021	0.012

Fig. 6 *Evaluation of Mixing Speed of Stirring Bar in Lag Phase*

Table 1 *Measurement Settings of VWF:Rco*

Parameter		Setting Value
Sample Volume		18 µL
Diluent Volume		54 µL
Incubation Time		190 seconds
Detection Time		100 seconds
Stirring Speed	0 - 15 seconds (Lag phase)	400 rpm
	15 - 100 seconds (Reaction phase)	900 rpm
Stirrer Bar Size	Diameter	1.8 mm
	Length	3.8 mm

Note: 4-fold diluted sample is used in the medium mode

Table 2 *Within-run Reproducibility of VWF:Rco*

	VWF: RCo	
	CPN %	CPP %
1	96.4	24.1
2	90.8	24.6
3	80.8	23.4
4	92.8	24.2
5	92.4	23.8
6	96.0	23.4
7	85.6	22.9
8	88.8	24.7
9	92.8	22.5
10	88.4	23.5
11	91.2	23.5
12	90.4	24.0
13	90.0	23.4
14	90.4	24.4
15	88.0	25.1
16	85.2	22.2
17	91.6	22.3
18	83.2	23.4
19	91.2	23.3
20	92.8	23.6
Mean	89.94	23.62
SD	3.93	0.78
%CV	4.4%	3.3%

2. Linearity

A sample with high VWF:RCo activity was diluted stepwise with Owren Veronal buffer and each sample assayed. The dilution ration was changed for the measurement of samples with activity 200% or more and 10% or less. There was linearity in the range 5 - 330% (***Fig. 7***).

3. Detection limit

A sample with low VWF:RCo activity was diluted stepwise with Owren Veronal buffer and each diluted sample assayed. The lowest activity level at which the mean values for each concentration - 2SD and the mean value at 0 concentration + 2SD did not overlap was taken as the detection limit. With the analyzer tested here the detection limit was 5% activity (***Fig. 8***).

4. Correlation with manual method

Correlation of the assay results obtained using CS-2000*i* and the manual method was studied using 40 patient plasma samples. The results for n = 40 were r = 0.925, y = 0.933x - 0.354 (***Fig. 9***).

DISCUSSION

To make it capable of VWF:RCo measurements, we provided a built-in mixing function in the CS-2000*i*, comprising a reaction cuvette in which a magnetic stirrer bar is inserted. The mixing function in the cuvette is necessary to promote the aggregation of platelets during the reaction, as the VWF:RCo assay reagent has stabilized human platelets as one of its components. The shape (diameter and length) of the stirrer bar and the stirring speed have major impact on data in this measurement. We conducted various investigations for optimizing the assay conditions to obtain good quality data. The thicker the stirrer bar and faster the stirring, the larger the dOD/min tended to become. However, the stirrer bar of the diameter of 1.2mm or more was not evaluated because there is a possibility of blocking the light from light source in the cuvette. As for the stirrer bar length, there was no major difference among the lengths examined. After comprehensive evaluation including reproducibility, the slope of the calibration curve and inside diameter of cuvette, we set the stirrer bar diameter at 1.2 mm and length at 3.8 mm and stirring speed at 900 rpm as the assay conditions. The selected

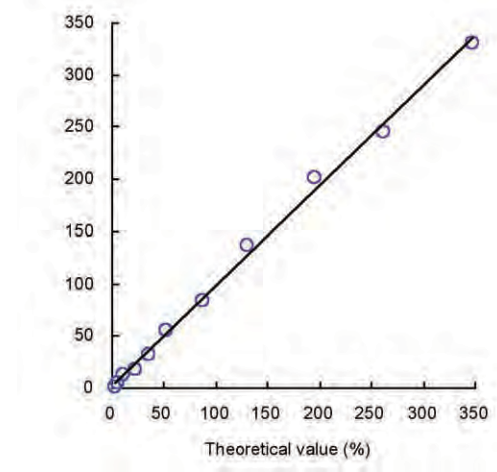

Fig. 7 *Linearity*

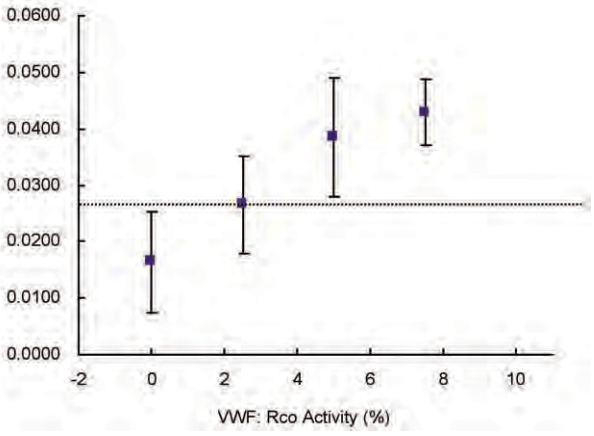

Fig. 8 *Detection Limit*

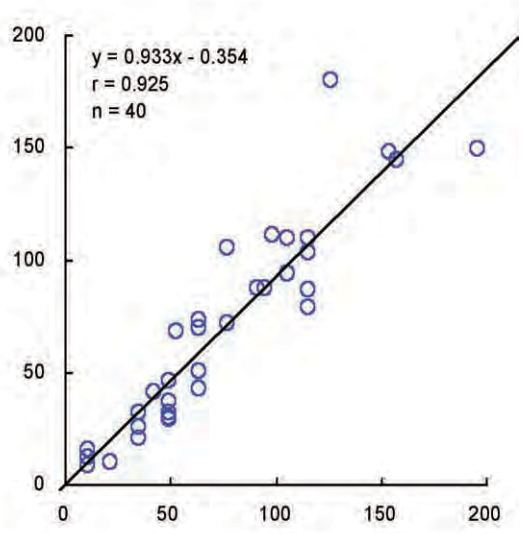

Fig. 9 *Correlation Between CS-2000i and Manual Method*

rpm was within the 800 - 1,200 rpm range, which is used by most of the commercially available platelet aggregometers[7]. It however became clear that when stirring was done at 900 rpm immediately after addition of the reagent, partial aggregation occurred before the reaction phase (16 - 100 seconds from the start to the completion of the photometric measurement). And, this caused poor sensitivity in the low activity range. Therefore, we used a different stirring speed for the lag phase (0 - 15 seconds from the addition of the reagent up to the photometric measurement of turbidity) and the reaction phase. It was confirmed that a stirring speed of 400 rpm in the lag phase, which was a milder speed than used in the reaction phase, permitted thorough mixing of the reagent and the sample during the lag phase without causing aggregation, and thus improved sensitivity in the low activity range.

Assay results obtained with two levels of control plasma showed good within-run reproducibility, the CV being 4.4% for normal plasma and 3.3% for pathological plasma. When the sample dilution was changed before measurement in the very low and very high activity ranges, dilution linearity was shown in the 5 - 330% VWF:RCo activity range. The detection limit determined with low VWF:RCo activity samples was found to be 5% activity. This was slightly inferior to the detection limit of 2% by the manual method. However, as the normal reference range of the activity is 50 - 150%[8,9], this detection limit provides sufficient sensitivity in the detection of VWD. The results obtained with the CS-2000*i* were correlated with those of the manual method. The two were especially close in the low activity range. The manual method relies on visual assessment by the person carrying out the analysis. Therefore, the results vary depending on the person. Automated analysis has the merit of providing stable data independent of the person doing the analysis.

Among the tests for VWD diagnosis, VWF:RCo is a highly sensitive secondary screening test[10,11]. It can be used for disease type classification also when combined with VWF:Ag or FVIII:C, and a few diagnostic flow charts have been proposed[12-14]. We believe that the CS-2000*i*, capable of measuring VWF:Ag, FVIII:C and VWF:RCo, can provide useful information for VWD diagnosis. It has also been recently shown that VWF:Ag and VWF:RCo are high in certain conditions including cerebral infarction and myocardial infarction, and thus are risk factors in vascular diseases[15,16]. VWF analysis would therefore be useful not only for detecting hemorrhagic diseases but also for detecting thrombotic microangiopathies and other conditions.

CONCLUSION

The investigations reported here have made the VWF:RCo assay possible with the Sysmex® CS-2000*i*, a general purpose blood coagulation analyzer, with sufficiently high assay accuracy for routine testing. The CS-2000*i* has the capability to measure VWF:Ag, FVIII:C, etc as well. By combining these data, we can obtain useful information for disease type classification which would be helpful in VWD diagnosis.

A part of the work reported here was presented at the 38th conference of The Japan Society for Clinical Laboratory Automation held in Kobe in September 2006.

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