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The rivaroxaban-adjusted normalized ratio: use of the prothrombin time to monitor the therapeutic effect of rivaroxaban

B Kim[a], S Jang[b,c], YJ Lee[c], N Park[c], YU Cho[b] and CJ Park[b]

[a]Department of Laboratory Medicine, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, Cheonan, Republic of Korea (South Korea); [b]Department of Laboratory Medicine, University of Ulsan College of Medicine and Asan Medical Center, Seoul, Republic of Korea (South Korea); [c]Asan Clinical Research Center, Seoul, Republic of Korea (South Korea)

ABSTRACT

Background: The prothrombin time may be used to monitor the plasma concentration of rivaroxaban. However, there is variability in the responsiveness of rivaroxaban to different thromboplastins. We aimed to develop a rivaroxaban-monitoring method using the prothrombin time to reduce the differences in the sensitivity among reagents.

Methods: Rivaroxaban-spiked pooled normal plasma at a 0–1000 ng/ml concentration was used to generate a rivaroxaban-adjusted sensitivity index (SI) values, and was tested with three thromboplastins. The warfarin-adjusted international sensitivity index (ISI-warfarin), rivaroxaban-adjusted sensitivity index (SI-rivaroxaban), international normalized ratio (INR) calculated with ISI-warfarin, normalized ratio (NR) calculated with SI-rivaroxaban, and their coefficient of variances (CVs) were compared. The NR-rivaroxaban value was compared with the results of an anti-Xa assay.

Results: The ISI-warfarin and SI-rivaroxaban using different thromboplastins were 1.02 and 1.88, respectively, with Thromborel S, 0.90 and 1.00 using Recombiplastin 2G, and 1.30 and 1.15 using Neoplastin CI-plus. Between-thromboplastin variability expressed as CV were 6.3%–25.1% when expressed as INR-warfarin and 1.7%–4.7% when expressed as NR-rivaroxaban. CVs for the NR-rivaroxaban with another laboratory were significantly lower than those for INR-warfarin. Anti-Xa assay v NR-rivaroxaban correlation coefficients were 0.97–0.99.

Conclusion: Using a rivaroxaban-specific NR effectively minimises inter-thromboplastin variability. By utilizing a NR-rivaroxaban, standardized prothrombin time results could be rapidly obtained, especially useful in standardizing the therapeutic effect of rivaroxaban.

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International normalized ratio (INR); international standardization index (ISI); prothrombin time; rivaroxaban; standardization

Introduction

Anticoagulants such as warfarin and heparin have been widely used to treat venous thromboembolism. However, they require frequent monitoring to ensure the correct plasma therapeutic level [1]. As direct acting oral anticoagulants (DOACs) [2] show predictable pharmacokinetics and pharmacodynamics [3,4], they generally have no need for regular drug monitoring, although this may be needed in certain indications. These include the time before surgery or invasive procedure when a patient has taken DOACs in the previous 24 h, bleeding or recurrent thrombosis upon treatment, suspicious overdose, hepatic or renal impairment [1,5].

Rivaroxaban is a widely used direct inhibitor of activated factor X (Xa), and plasma concentrations can be assessed using prothrombin time, high-performance liquid chromatography (HPLC) and anti-factor Xa assay, although the latter two may not be readily available in many laboratories, especially in an emergency situation [6–8]. The prothrombin time (the most widely used coagulation assay commonly used to monitor warfarin as an INR) is sensitive to rivaroxaban over a range of peak and trough plasma concentrations and shows linear responsiveness to the plasma rivaroxaban concentration [6,9]. The prothrombin time assay using thromboplastin with known sensitivity to rivaroxaban has been recommended as a rapidly accessible laboratory method to estimate the relative degree of the anticoagulation of rivaroxaban [2,10]. Therefore, prothrombin time is a good candidate for a method to measure the plasma concentration of rivaroxaban when drug-specific assays are not available. However, there is significant variability in the responsiveness to various thromboplastins [6,11], resulting in difficulties when comparing the results obtained from different thromboplastins, especially in multicentre clinical trials of rivaroxaban. Several studies have focused on resolving this problem [11–14]. A few studies calculated the rivaroxaban-calibrated sensitivity index (SI) and the normalized ratio (NR) in a single centre, but they require validation through a large, multicentre-based study to confirm the results [12,13]. One multicentre study showed a high inter-laboratory variability when using

CONTACT S Jang ssjang@amc.seoul.kr Department of Laboratory Medicine, University of Ulsan College of Medicine and Asan Medical Center, 88, Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Songpa-gu, Republic of Korea (South Korea)

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local thromboplastin to measure the plasma samples spiked with rivaroxaban [11], and another found that the between-thromboplastin variability was reduced by using an arbitrary reference, such as central thromboplastins or calibrators specific to rivaroxaban [14]. The aim of this study was to identify variations in the prothrombin time results for plasma rivaroxaban concentrations, and to develop rivaroxaban-adjusted SI and NR for standardized monitoring of rivaroxaban treatment with the prothrombin time assay.

Methods

Whole blood was collected via venipuncture in a 3.2% sodium citrate tube (Becton Dickenson, Franklin Lakes, NJ, USA) from 337 healthy individuals (180 males, 157 females, ages 24–63 years (mean 31 years)). From these, 297 citrated blood samples were used to make platelet-poor normal pooled plasma (NPP), which was divided into aliquots and frozen immediately at −70°C. Just before each experiment, frozen NPP aliquots were thawed and heated to 37°C for 10 min. Among the remaining 40 citrated blood samples, 20 were used as normal controls for the prothrombin time assays to determine rivaroxaban-adjusted SI and NR, and the other 20 samples were used to calculate the mean normal prothrombin time (MNPT).

Rivaroxaban was prepared by dissolving 20 mg rivaroxaban (Bayer-Schering-Pharma, Wuppertal, Germany) in 50 mL dimethylsulphoxide (DMSO) to produce a stock solution diluted in phosphate-buffered saline without Ca^{2+} or Mg^{2+} at a final rivaroxaban concentration of 500 µg/mL. Each working solution was obtained by mixing these stock solutions with the NPP. The DMSO concentration in plasma was ≤0.05% (v/v), which does not influence the coagulation [15]. Determination and evaluation of the efficacy of rivaroxaban-adjusted SI and NR were as follows. In preparing rivaroxaban-spiked plasma samples, thawed NPP aliquots were spiked with rivaroxaban to obtain final rivaroxaban concentrations of 0, 50, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900 and 1000 ng/mL, chosen to include the on-therapy ranges for various indications [4,16]. Four aliquots for each concentration were prepared. Thus, a total of 60 rivaroxaban-spiked plasma samples were analysed. In addition, 20 citrated platelet-poor plasma samples were obtained from healthy subjects and analysed.

Three commercial thromboplastins and coagulometers were used, including Thromborel-S (Siemens Healthcare Diagnostics, Marburg, Germany) with CA-7000 coagulometer (Sysmex Corporation, Kobe, Japan), HemosIL RecombiPlasTin 2G with ACL-TOP coagulometer (Instrumentation Laboratory, Milan, Italy) and Neoplastin CI-plus with STA-R coagulometer (Diagnostica Stago, Asnieres, France). In addition, the other clinical laboratory performed the same experiment using four thromboplastins, including Thromborel S, HemosIL RecombiPlasTin 2G and Innovin (Dade Behring, Miami, USA) with a CA-7000 coagulometer.

The rivaroxaban-adjusted SI (SI-RIV) values of the three tested thromboplastins were determined using a modification of the World Health Organization (WHO) reference protocol for warfarin-adjusted ISI (ISI-WAR) [17]. The procedure consists of a set of tests using the thromboplastins and test samples. The assay was repeated for five separate sessions, using fresh thromboplastins in each session. The prothrombin time of the working and arbitrary standard thromboplastins on logarithmic axes was plotted, and the slope of the orthogonal regression line and its coefficient of variation (CV) were calculated to represent the precision of the calibration. The rivaroxaban-adjusted SI of the working thromboplastin was calculated as the slope multiplied by the rivaroxaban-adjusted SI of the arbitrary standard thromboplastin, and the rivaroxaban-adjusted SI of the arbitrary standard thromboplastin was set to 1.00. After determination of rivaroxaban-adjusted SI, rivaroxaban-adjusted NR was calculated using the following equation: Rivaroxaban-adjusted NR = (sample PT/MNPT) $^{\text{Rivaroxaban-adjusted SI}}$

To evaluate sensitivity variations among different thromboplastins, plots were drawn for the spiked concentration of rivaroxaban (horizontal axis) and PT of each sample (vertical axis). The prothrombin time ratio (prothrombin time of rivaroxaban-spiked plasma divided by the MNPT), INR calculated using warfarin-adjusted current ISI (warfarin-adjusted INR, INR-WAR), and NR calculated using rivaroxaban-adjusted new SI (rivaroxaban-adjusted NR, NR-RIV) of each sample were also plotted against the spiked concentration of rivaroxaban. The sensitivities of each thromboplastin to rivaroxaban were determined by the slope of a linear regression analysis.

The Warfarin-adjusted ISI and rivaroxaban-adjusted SI of the tested thromboplastins were compared. To evaluate the between-thromboplastin variability to rivaroxaban and the standardization efficacy of rivaroxaban-adjusted SI, the CVs of clotting time, warfarin-adjusted INR, and rivaroxaban-adjusted NR obtained from our all tested thromboplastin and from our thromboplastins and another laboratory's tested thromboplastin were calculated. Two chromogenic anti-factor Xa assay reagents were used: Biophen DiXal kit (Hyphen Biomed, Neuville-sur-Oise, France), and STA-Liquid Anti-Xa kit (Diagnostica Stago, Asnieres, France). These kits consisted of anti-factor Xa reagents, as well as sets of calibrators and controls: Biophen DiXal kit composed of three lyophilized rivaroxaban calibrators (50, 250 and 500 ng/mL) and two control materials (expected range: 80–120 and

270–330 ng/mL). The STA-Liquid Anti-Xa kit was composed of four lyophilized rivaroxaban calibrators (0, 95, 238 and 464 ng/mL) and two control materials (expected range: 62–100 and 245–339 ng/mL). The specific rivaroxaban anti-factor Xa activity was measured using two different instruments: the Biophen DiXal kit was analysed on a CS-5100 coagulometer (Sysmex Corporation, Kobe, Japan) and the STA-Liquid-Anti-Xa kit was analysed on an STA-R coagulometer. Each anti-factor Xa assay was performed according to the manufacturer's protocol. The results of the anti-Factor Xa assay and rivaroxaban-adjusted NRs of tested thromboplastins were compared.

Statistical analysis was performed using SPSS v 21.0 (SPSS Inc., Chicago, IL) and MedCalc Statistical Software version 14.12.0 (MedCalc, Mariakerke, Belgium). The sensitivity of thromboplastin to rivaroxaban was determined by the slope of the linear regression analysis. The differences between warfarin-adjusted INR and rivaroxaban-adjusted NR of three thromboplastins were estimated using one-way analysis of variance. The CV was calculated as follows: (standard deviation/mean) x 100. Bland–Altman analysis was performed between rivaroxaban-adjusted NR and the rivaroxaban concentration obtained from the anti-factor Xa assay. The statistical significance was established at $P < 0.05$.

Results

Evaluation of sensitivity variations among the different thromboplastins was as follows. The prothrombin time assays showed dose-dependent linear prolongation for plasma rivaroxaban concentration in all three thromboplastins (Figure 1(a)). The prothrombin time and INR also increased with an increasing concentration of rivaroxaban in all three thromboplastins (Figure 1(b,c)). Warfarin-adjusted INR values were different among all three thromboplastins at the same concentration of rivaroxaban. The INR values at 250 ng/mL rivaroxaban concentration were 1.64 with HemoSIL RecombiPlasTin 2G, 1.73 with Neoplastin CI-Plus and 1.17 with Thromborel-S. The sensitivities for rivaroxaban (expressed as the slope of the regression line) of all three tested thromboplastins were different. The highest sensitivity was shown with HemoSIL RecombiPlasTin 2G and Neoplastin CI-plus, which were used as the arbitrary standard thromboplastins to calculate the rivaroxaban-adjusted SI. The rivaroxaban-adjusted SI values of two thromboplastins (Thromborel-S and Neoplastin CI-Plus) relative to the chosen 'standard' thromboplastin (HemoSIL RecombiPlasTin 2G) and their warfarin-adjusted ISI values were ISI-WAR 1.02 and SI-RIV 1.88 for Thromborel-S and ISI-WAR 1.3 and SI-RIV 1.15 for Neoplastin CI-Plus.

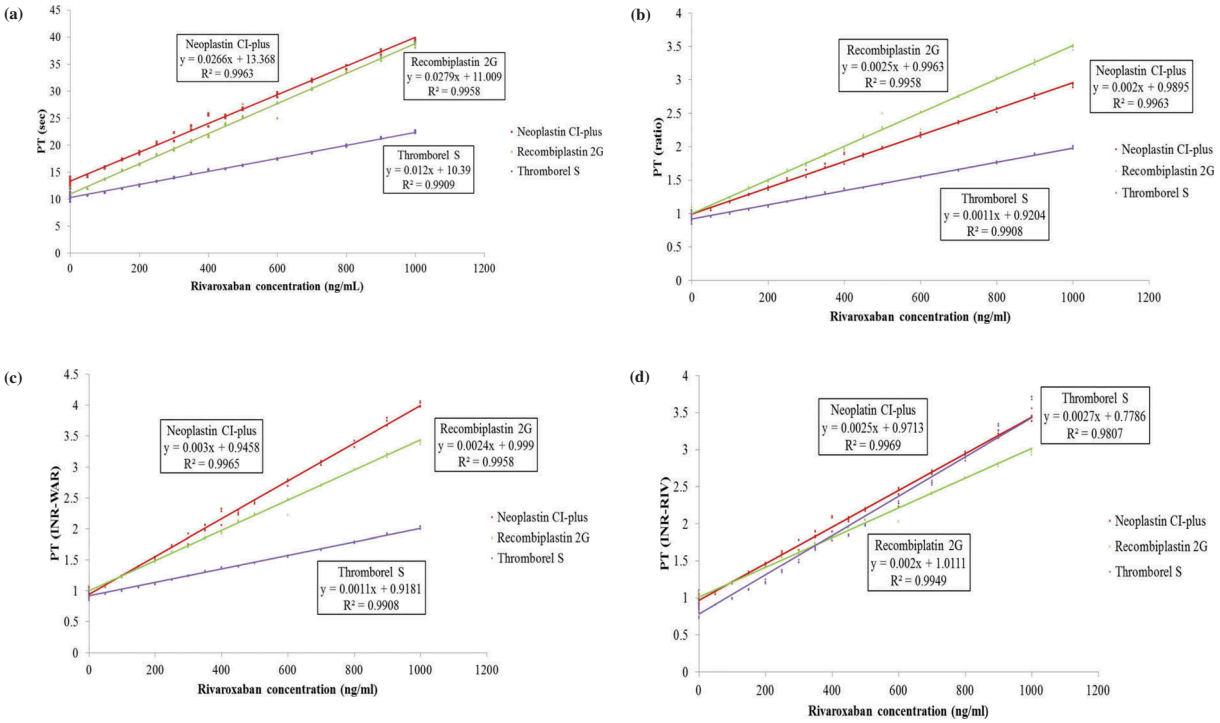

Figure 1. Sensitivities of three thromboplastins for plasma rivaroxaban concentration when the PT results were expressed as (a) PT clotting time, (b) PT ratio and (c) PT warfarin-adjusted INR (INR-WAR). The PT clotting time shows a linear dose-dependent prolongation. (d) The sensitivities of three thromboplastins for plasma rivaroxaban concentration when the PT results are expressed as rivaroxaban-adjusted NR. When PT results are expressed as rivaroxaban-adjusted NR (NR-RIV), the difference in the sensitivity is markedly lower. All three tested thromboplastins had different sensitivities to rivaroxaban. Neoplastine CI-plus and HemoSIL ReCombiPlasTin 2G showed higher sensitivity for rivaroxaban compared to Thromborel S. Redline for Neoplastin CI-plus, green line for HemoSIL ReCombiPlasTin 2G and violet line for Thromborel-S.

The CV of the slope was less than 3% for each thromboplastin, showing an acceptable precision of the SI calibration. The rivaroxaban-adjusted-SI value of Thromborel-S was higher than its warfarin-adjusted ISI. Neoplastin®CI-plus showed rivaroxaban-adjusted SI values lower than the warfarin-adjusted SI. The absolute differences between the warfarin-adjusted ISI and rivaroxaban-adjusted SI were 0.11 to 0.86.

The mean values of warfarin-adjusted INR and rivaroxaban-adjusted NR obtained from three thromboplastins are shown in Table 1. The differences between warfarin-adjusted INR and rivaroxaban-adjusted NR were significant using Thromborel-S and Neoplastin CI-plus and not significant using HemoSIL Recombiplastin 2G. The rivaroxaban-adjusted NR showed similar values among all three thromboplastins at the same concentration of rivaroxaban. The difference in sensitivity to rivaroxaban in all three thromboplastins was considerably lower when expressed as rivaroxaban-adjusted NR (Figure 1(d)).

The inter-thromboplastin variability (expressed as CV %) for warfarin-adjusted INR and rivaroxaban-adjusted NR in single and multicentre analyses are shown in Table 2 and compare with previously reported inter-thromboplastin variability for warfarin INR (7–10%) [18,19]. INR-warfarin CVs were broadly similar between single and multicentre, except at 50 ng/ml, where the multicentre data was superior. However, the NR-rivaroxaban CVs were consistently smaller in the single centre compared to the multicentre analysis.

Validation of suitability of rivaroxaban-adjusted NR was as follows. The rivaroxaban-adjusted NR values of each rivaroxaban-spiked plasma sample were compared to the rivaroxaban concentration obtained from the anti-factor Xa assay. The rivaroxaban concentration measured by the anti-factor Xa assay correlated well with the spiked calculated concentration of rivaroxaban (r^2 = 0.9975). The correlation was strong between rivaroxaban-adjusted NR and rivaroxaban concentration obtained from the anti-factor Xa assay (Figure 2(a)). The mean difference between the spiked concentrations compared to the concentrations obtained from the anti-factor Xa assay was −10.5 ng/mL (95% CI −29.4 to 8.3 ng/mL) (Figure 2(b)).

Table 1. The mean value of warfarin-adjusted INR and rivaroxaban-adjusted NR, and the differences between warfarin-adjusted INR and rivaroxaban-adjusted NR.

	INR-WAR		NR-RIV		
	Mean	SD	Mean	SD	*P* value between mean INR-WAR and NR-RIV
ReCombiPlasTin 2G	1.82	0.77	1.85	0.64	0.741
Neoplastin® CI-plus	1.97	0.80	1.80	0.78	0.047
Thromborel® S	1.29	0.32	1.68	0.97	0.002

*INR-WAR, Warfarin-adjusted INR; NR-RIV, rivaroxaban-adjusted NR

Table 2. The coefficient of variations (CVs) of warfarin-adjusted INR (INR-WAR) and rivaroxaban-adjusted NR (NR-RIV) at specific rivaroxaban concentrations of single- and multi-centre PT results.

		Rivaroxaban concentration (ng/mL)				
		50	100	200	300	700
Single center CV (%)	INR-WAR	7.1%	9.3%	12.3%	14.2%	25.1%
	NR-RIV	1.8%	2.0%	1.7%	3.5%	4.7%
Multicenter CV (%)	INR-WAR	4.7%	8.1%	12.5%	16.7%	26.3%
	NR-RIV	5.1%	6.2%	6.7%	6.3%	6.6%

Discussion

Although rivaroxaban, a direct inhibitor of factor Xa, generally does not need drug monitoring, some studies and guidelines suggest that measuring serum drug concentrations might be useful for specific clinical situations and patient populations [5,11]. The ideal assay to measure the plasma DOAC levels must show excellent accuracy, and it should be sensitive enough to measure the lowest clinically relevant plasma concentration of the DOAC, readily available at all times, yield results quickly (which is essential for emergency situations) and represent agreement over a wide range of drug levels [20,21]. Unfortunately, no assay that satisfies all of the above criteria has been developed [22]. Traditional coagulation tests are speedy, widely available and inexpensive, but limited in terms of the accuracy in determining plasma DOAC levels [23]. In contrast, DOAC-specific assays are not widely available, have a relatively long turnaround time, high cost and require a skilled scientist [2,8,23,24]. Rivaroxaban prolongs the prothrombin time in a dose-dependent, linear manner [24,25], but there is a known variation in the sensitivity of different thromboplastins to rivaroxaban, and the conventional warfarin-adjusted INR values cannot correct this variability [12,13].

We found that the rivaroxaban-adjusted SI values were different from the current warfarin-adjusted ISI in all tested thromboplastins, with the absolute difference range being the largest in Thromborel-S and the smallest in HemoSIL Recombiplastin 2G. A previous study suggested that a rivaroxaban-adjusted SI would vary depending on the prothrombin time measurement methods due to different thromboplastin composition and coagulometers [13]. Our study was conducted based on a thromboplastin- and coagulometer-specific prothrombin time analysis. Therefore, more accurate results could be obtained using a rivaroxaban-specific prothrombin time with rivaroxaban-adjusted SI. We found that use of a rivaroxaban-adjusted NR effectively minimized the inter-thromboplastin variability, as have others [12,13,26]. Some external quality programs report that the inter-thromboplastin variability in the prothrombin time INR for warfarin is about 7–10% [18,19]. The inter-thromboplastin variability of rivaroxaban-adjusted NR showed smaller values at all rivaroxaban doses. Therefore, the use

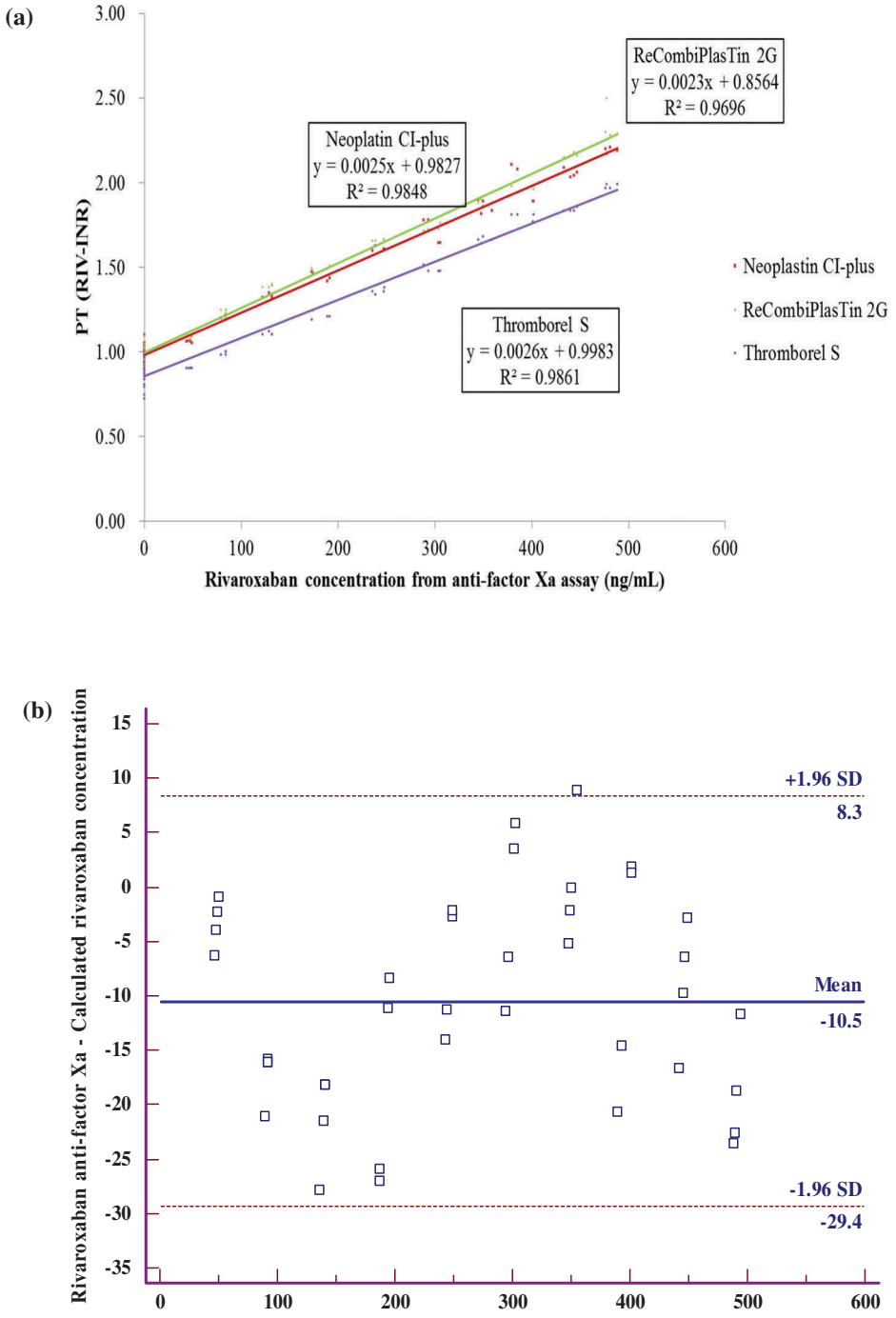

Figure 2. Correlation between rivaroxaban-adjusted NR and anti-Xa assay. (a) Correlation between spiked concentrations of rivaroxaban. (b) Correlation between rivaroxaban-adjusted NR of all tested thromboplastins and the concentration of rivaroxaban obtained from the anti-Xa assay. (c) Bland-Atman plot for the differences between rivaroxaban concentrations obtained from anti-factor Xa assay and spiked concentration. Redline for Neoplastin® CI-plus, green line for HemoSIL® ReCombiPlasTin 2G and violet line for Thromborel® S.

of rivaroxaban-adjusted SI and NR could overcome the variability related to different thromboplastins and coagulometers, and it could be reliably used, especially in an urgent situation.

Several studies have revealed a good correlation between the anti-factor Xa assay and mass spectrometric measurement of rivaroxaban in normal plasma samples spiked with known amounts of rivaroxaban [27–29]. The chromogenic anti-factor Xa assays are suitable methods to measure the concentration of rivaroxaban using rivaroxaban-specific calibrators and controls [5,16,24,30]. In this study, the rivaroxaban-adjusted NR showed a high degree of correlation with the rivaroxaban concentrations obtained from an anti-factor Xa assay using rivaroxaban-spiked NPP samples. Since the chromogenic anti-factor Xa assays are well correlated with the results from mass spectrometry, the gold-standard method to measure the plasma rivaroxaban concentration, rivaroxaban-adjusted NR is considered to have good accuracy.

Therefore, rivaroxaban-adjusted NR using a PT assay system may be effective in determining the plasma concentration of rivaroxaban, especially in an emergent situation.

Our study has some limitations. First, in common with others [11–13] the rivaroxaban-spiked NPP sample was obtained from healthy subjects, instead of patient samples treated with rivaroxaban, spiked samples may not reflect the potential variations of sensitivity between thromboplastins and between *in vivo* samples with similar levels of rivaroxaban [31]. Using samples obtained from rivaroxaban-treated patients undergoing elective surgery may produce especially meaningful results, including a comparative analysis of postoperative bleeding complications. Second, the IRP was not used to calculate the rivaroxaban-adjusted SI. Instead, arbitrary standard thromboplastins were chosen which have been known to show good sensitivity for rivaroxaban [26,32]. Technically, an accurate SI for rivaroxaban in the arbitrary standard thromboplastins has not been determined. Therefore, the SI of the arbitrary standard thromboplastin was designated as 1.00. Further experiments are required to identify the accurate rivaroxaban SI of these thromboplastins using IRP. In addition, HemoSIL Recombiplastin 2G originates from a human source, so this thromboplastin represents the WHO calibration method. Third, the sensitivity of thromboplastin at a lower concentration of rivaroxaban was not validated. Current thromboplastins cannot discriminate the presence of rivaroxaban in a patient's plasma sample due to the low sensitivity to rivaroxaban. However, further investigations are possible using a dilute PT assay with increased sensitivity to rivaroxaban to quickly and accurately determine whether or not rivaroxaban was used. Fourth, mass spectrometry was not used to measure the plasma rivaroxaban concentration, which is the current validated standard. Instead, a defined amount of rivaroxaban was added to the plasma, and the anti-factor Xa assay was used to validate the calculated rivaroxaban concentration. Finally, the correlation of rivaroxaban-adjusted NR with clinical efficacy or safety outcomes should also be evaluated.

In conclusion, rivaroxaban-specific NR reduces the differences between the PT results from different thromboplastins and coagulometers. The rivaroxaban-specific NR correlates with the rivaroxaban concentration determined by the anti-factor Xa assay. By utilizing a rivaroxaban-specific NR, accurate, standard PT results could be obtained simply and rapidly. Based on this finding, the study demonstrates the feasibility of accurate standardization of a PT result to estimate the plasma rivaroxaban concentration using rivaroxaban-adjusted SI and NR. Especially, the rivaroxaban-specific NR has the potential to standardize the therapeutic effect of rivaroxaban in a multicenter study.

This work represents an advance in biomedical science because it shows that the prothrombin time-based rivaroxaban-adjusted normalized ratio (NR) could be an appropriate assay to measure the concentration of plasma rivaroxaban.

Summary Table

What is known about this subject:

- In certain circumstances, monitoring of plasma rivaroxaban concentration might be needed.
- The prothrombin time could be an appropriate method to measure the plasma rivaroxaban concentration.
- However, variability in the responsiveness to rivaroxaban was observed among thromboplastins.

What this paper adds:

- Development of a rivaroxaban monitoring method using prothrombin time.
- A rivaroxaban-specific normalized ratio minimizes inter-thromboplastin variability.
- With a rivaroxaban-specific normalized ratio, standardized prothrombin time results could be rapidly and simply obtained.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

B Kim http://orcid.org/0000-0003-4456-5612
S Jang http://orcid.org/0000-0002-0045-1747
CJ Park http://orcid.org/0000-0003-4396-8348

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