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Laboratory monitoring of rivaroxaban and assessment of its bleeding risk

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ABSTRACT

Background: The aims of this study were to investigate the effects of rivaroxaban on routine coagulation assays using our local, widely available, reagents and to study the relationship between sensitive coagulation assays and bleeding risk caused by rivaroxaban.

Methods: Prothrombin time (PT), activated partial thromboplastin time (APTT) and anti-factor Xa (FXa) chromogenic assays (Biophen DiXaI) and inhibition of FXa activity were performed in normal pooled plasma (NPP) spiked with rivaroxaban and plasma samples from patients treated with rivaroxaban.

Results: *In vitro*, the linear correlation coefficient of measured concentrations of rivaroxaban, by Biophen DiXaI, and spiked concentrations of rivaroxaban was 0.99. PT and APTT showed good linear correlation with rivaroxaban concentrations, while other assays showed poor correlation. *In vivo*, PT showed a moderate linear correlation with rivaroxaban concentrations while APTT had a weak correlation with rivaroxaban concentrations. In vitro and *in vivo*, the rivaroxaban concentrations, measured by Biophen DiXaI, always showed good correlation with the inhibition of FXa activity, and PT values showed moderate correlation with the inhibition of FXa activity.

Conclusions: Biophen DiXaI can be considered as a quantitative method to monitor the anticoagulation activity of rivaroxaban, and could be used to evaluate bleeding risk caused by rivaroxaban. The PT reagent (Thrombosis S) could be considered as a rough method to monitor the anticoagulation activity of rivaroxaban and evaluate bleeding risk caused by rivaroxaban.

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Rivaroxaban; monitoring; prothrombin time; inhibition of factor Xa activity; anti-factor Xa chromogenic assay; bleeding risk

Introduction

Rivaroxaban is an oral, direct factor Xa (FXa) inhibitor that has been approved in more than 100 countries worldwide, for the prevention of venous thromboembolism in adult patients after elective hip or knee replacement surgery. The absorption of rivaroxaban after oral intake is near 80%; C_{max} is reached within 2–4 h, and the half-life is between 7 and 11 h in patients with normal renal and hepatic functions. After administration of 10 mg rivaroxaban, the peak plasma concentrations are in the range of 91–195 ng/ml (mean: 125 ng/ml).[1–4] Due to predictable pharmacokinetics and pharmacodynamics, it is postulated that rivaroxaban can be administered at a fixed dose without routine coagulation monitoring. However, clinical surveillance is recommended in several clinical situations such as in patients with bleeding, deteriorating renal or hepatic functions, before urgent surgery or invasive procedure and overdose.[5] Currently, there is no specific antidote available for rivaroxaban in the clinical setting. So it is important that clinical laboratories rapidly measure the degree of anticoagulation and assess the risk of bleeding due to rivaroxaban.

To date, most studies and guidelines have recommended that the anti-FXa chromogenic assays could be used as a quantitative method and prothrombin time (PT) could be used as a qualitative method to assess the anticoagulation activity of rivaroxaban.[5–11] However, the sensitivity of PT varies with different thromboplastin reagents and instruments used for assessment.[12–14] Consequently, it is suggested that laboratories should be aware of the sensitivity of their own reagents in regard to rivaroxaban. In addition, the relationship between both assays and bleeding risk due to rivaroxaban has not been known well. As rivaroxaban is a direct FXa inhibitor, the measured inhibition of FXa activity may have a direct correlation with risk of bleeding, although the strength of this correlation is currently unknown.[1,2,15]

The primary focus of the present study is to investigate the effects of rivaroxaban on routine coagulation assays using our local, widely available, reagents. The secondary focus of the study is to investigate the relationship between sensitive coagulation assays and inhibition of FXa activity to assess which assays have a stronger correlation with bleeding risk.

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Materials and methods

This study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethnic Committee of the Fifth People's Hospital of Shanghai, Fudan University, China. Written informed consent was obtained from each study participant.

In vitro

Ten healthy individuals (5 males and 5 females, between the ages of 23–45) of our laboratory were included in this study. These subjects all had normal coagulation tests including PT, activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen, no history of thromboembolic or haemorrhagic diseases, and no other disease that required the use of medications that could have a direct effect on the functions of platelet and/or coagulation factors during a two-week period prior to sampling. Blood was obtained via venepuncture, and collected into 0.109-M sodium citrate (9:1 v/v) tubes (BD, Plymouth, UK). Platelet-poor plasma (PPP) was obtained by centrifuging blood samples for 10 min at 3,000 rmp at room temperature. PPPs from the 10 individuals were mixed to obtain the normal pooled plasma (NPP) immediately, which was frozen at –80 °C without delay. Frozen NPP aliquots were thawed and heated at 37 °C for 5 min just before the experiment.

The stock solution of rivaroxaban was obtained from Selleck (S3002) at a concentration of 4.36 mg/ml in 100% dimethyl sulfoxide (DMSO). This stock solution was diluted in phosphate-buffered saline without Mg^{2+} and Ca^{2+} in order to obtain intermediate solutions at 5.45, 2.18, 1.09 and 0.545 µg/ml.[8] Working solutions of 545, 218, 109 and 54.5 ng/ml were obtained by mixing intermediate solutions with NPP. The DMSO concentration in plasma was ≤0.05% (v/v), which did not influence the coagulation.[16]

In clinical patients

Twenty-five patients were included in this study (9 males, 16 females; mean age, 77 ± 11 years; median age, 79.5 years). Each patient was treated with 10 mg once daily for the prevention of deep vein thromboembolism after elective hip or knee replacement surgery. Exclusion criteria were severe liver or renal dysfunction, coagulation disorders, malignancy or receiving other anticoagulation drugs.

After receiving rivaroxaban for ≥5 days, plasma samples were collected. PPP was obtained as described above for the healthy individuals. Each plasma sample was aliquot into tubes and PT, APTT, TT and fibrinogen were performed immediately. All other aliquots were immediately frozen at –80 °C and heated to 37 °C for 5 min prior to testing for other assays.

Coagulation assays

PT, APTT, TT and fibrinogen were performed in NPP samples spiked with rivaroxaban (rivaroxaban concentrations of 545, 218, 109 and 54.5 ng/ml) and PPP samples from patients treated with rivaroxaban on a CS-5100 automatic haemostasis analyser (Sysmex Corporation, Kobe, Japan). These assays were performed using the Calibrator and Control (Dade Behring) according to the manufacturer's instructions. Results of PT and APTT were reported in seconds and as ratios (vs. NPP).

Biophen DiXaI (Hyphen BioMed, Neuville-Sur-Oise, France) kit can be used for quantitative measurement of the concentration of rivaroxaban on human-citrated blood plasma. This assay is based on the inhibitory action of rivaroxaban on exogenous FXa which specifically cleaves para-nitroaniline (p-NA) linked to a chromogenic substrate. Then, pNA is released from the substrate and measured at 405 nm. The amount of pNA released is directly proportional to residual FXa activity. There is an inverse relationship between the concentration of rivaroxaban in the tested sample and colour development, measured at 405 nm. Due to a high ionic strength pH 7.9 buffer, this test is specific for FXa direct inhibitors (DiXaIs), without interference of indirect polysaccharide inhibitors.[6] This assay was performed in citrated NPP samples spiked with rivaroxaban (rivaroxaban concentrations of 545, 218, 109 and 54.5 ng/ml) and PPP samples from patients treated with rivaroxaban on an ACL-TOP 700 coagulometer (Beckman Coulter, USA) using the Biophen Rivaroxaban Calibrator and Control (Hyphen BioMed, Neuville-Sur-Oise, France).

The inhibition of FXa activity was determined using the Biophen Factor X assay (Hyphen BioMed, Neuville-Sur-Oise, France) according to kit instructions with minor adjustments.[17] This assay is based on activation of endogenous FX by Russell's viper venom (RVV), and then the specific chromogenic FXa substrate is cleaved by the residual FXa, releasing para-nitroaniline (pNA), whose colour is measured at 405 nm. There is a direct relationship between colour development and FX activity in the tested plasma. In this study, the system was calibrated in a range from 0 to 200% using NPP, and was used to calculate FXa activities in percentage for each sample. In each sample, the inhibition of FXa activity was calculated as the percentage difference of the FXa activity in undiluted NPP samples (100% FXa activity) and the FXa activity measured for samples. The test was performed in citrated NPP samples spiked with rivaroxaban (rivaroxaban concentrations of 545, 218, 109 and 54.5 ng/ml) and PPP samples from patients treated with rivaroxaban using manual protocols as follow. The plasma samples were diluted 1:10 in Tris-NaCl buffer. Fifty microlitres of these mixtures were incubated for 2 min at 37 °C. After the addition of 50 µl of a solution of the chromogenic

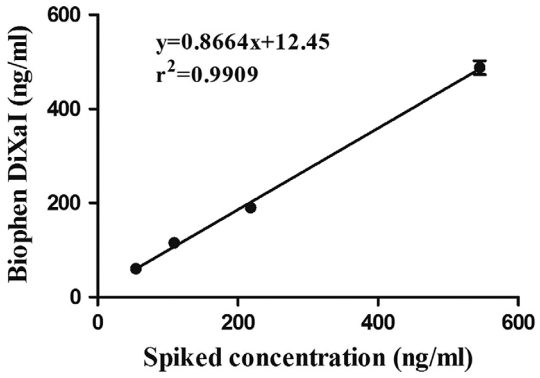

Figure 1. *In vitro*, the linear regression model fitted using the least square approach showed the relationship between measured concentrations by Biophen DiXaI and spiked concentrations. Biophen DiXaI, Biophen Direct Factor Xa Inhibitor.

FXa substrate, the mixtures were incubated for an additional 2 min at 37 °C. Then, 50 µl of a RVV solution was added and the reaction was terminated by introduction of 50 µl of acetic acid (20%) after 3 min. Finally, the UV absorptions were measured at 405 nm.

Statistical analysis

Statistical and graphing analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com) for Windows. Regression analyses were fitted using the least squares method and reported with the correlation coefficient (r^2). Recovery was calculated as the (measured value/expected value) *100%. The D'Agostino–Pearson Omnibus K2 test was used to assess for normality. For all statistical tests, *P*-values <0.05 were considered to be statistically significant and degrees of freedom (DF) were reported when appropriate.

Results

Sensitive assays

In vitro, the spiked plasma samples were tested four times over four days at each of the four concentrations (545, 218, 109 and 54.5 ng/ml). The linear correlation coefficient (r^2) of measured concentrations of rivaroxaban, by Biophen DiXaI, and spiked concentrations of rivaroxaban was 0.99 (Figure 1). The measured concentrations of rivaroxaban were reliable and within 20% (recovery range 83–119%) of the spiked concentrations. PT showed a high linear correlation with measured concentrations (Figure 2(a)), and the correlation coefficient (r^2) was 0.95. PT ratio showed a higher linear correlation coefficient (r^2) of 0.98 (Figure 2(b)). APTT showed a good linear correlation with measured concentrations (Figure 2(c)), and the correlation coefficient (r^2) was 0.87. APTT ratio also showed a higher linear correlation coefficient (r^2) of 0.97(Figure 2(d)). TT and fibrinogen values were not affected by rivaroxaban Figure 2 (e) and (f).

In vivo, patient samples were obtained between 1 April 2015 and 30 November 2015.From each patient, two plasma samples at two different time points were collected after receiving rivaroxaban. A total of 50 clinical samples, obtained from orthopaedic patients under prophylactic treatment with rivaroxaban 10 mg once daily, were tested. Eighteen samples were obtained at the time of peak drug activity after drug administration (mean, 2.6 ± 0.6 h), 18 samples were collected directly at 0–2 h before rivaroxaban dosing and 14 samples were obtained at unfixed time points after drug administration. No thrombotic event or bleeding occurred during the study. PT and PT ratio showed a moderate linear correlation with rivaroxaban concentrations (Figure 3(a) and (b)) and the correlation coefficient (r^2) was 0.68 and 0.73, respectively, while APTT and APTT ratio showed a poor correlation with rivaroxaban concentrations as shown in Figure 3(c) and (d). When rivaroxaban concentrations reached 101.7 ng/ml, the PT value was above the upper limit of the normal range (normal range: 10.0–13.0 s) (Figure 3(a)).

Bleeding risk

Inhibition of FXa activity showed a curvilinear correlation with rivaroxaban concentrations measured by Biophen DiXaI *in vitro* (Figure 4(a)) with $r^2 = 0.99$. PT showed a curvilinear correlation with inhibition of FXa activity (Figure 4(b)) with $r^2 = 0.77$. *In vivo*, the inhibition of FXa activity also had a curvilinear correlation with rivaroxaban concentrations measured by Biophen DiXaI (Figure 5(a)) with $r^2 = 0.78$. PT also showed a curvilinear correlation with inhibition of FXa activity (Figure 5(b)) with $r^2 = 0.61$.

Discussion

The gold standard for measuring the concentration of rivaroxaban is liquid chromatograph (LC)–mass spectrometry (MS).[18] However, many clinical laboratories do not have access to LC–MS analysis, which makes this technique unsuitable for routine measurement of rivaroxaban. A number of studies have indicated that anti-FXa chromogenic assays are specific and sensitive measurement methods for direct FXa inhibitors.[6–9] Recently, *in vitro* and *in vivo*, most studies have investigated the suitability of commercially available chromogenic anti-FXa assay Biophen DiXaI with rivaroxaban calibrators and controls for determination of rivaroxaban plasma concentrations.[6,8] The availability and methodology of this assay can be easily implemented in clinical laboratories, and it is in good agreement with LC–MS/MS measurements. Our study showed that rivaroxaban concentrations, measured by Biophen DiXaI, had a high linear correlation with spiked concentrations and the recovery was acceptable. Therefore, we suggest that Biophen DiXaI could be used to measure the plasma concentration of rivaroxaban.

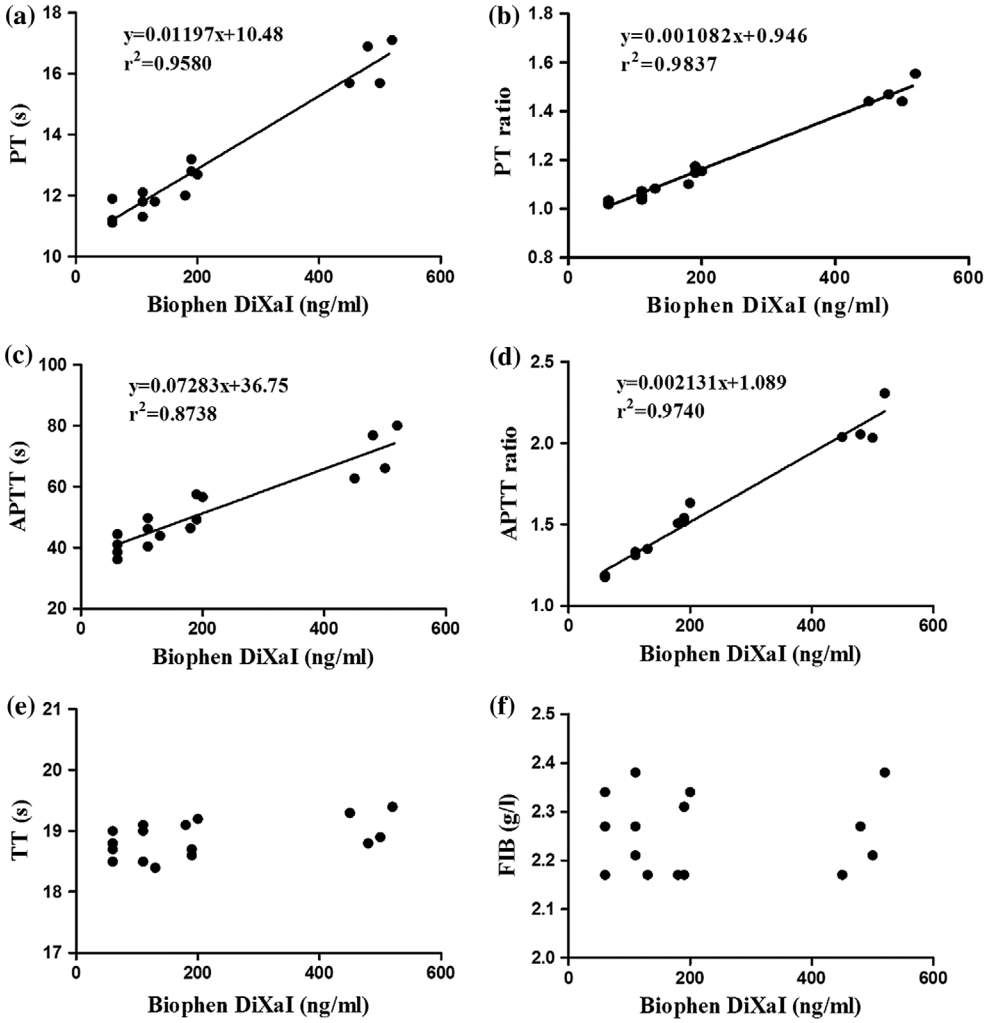

Figure 2. *In vitro*, the linear regression model fitted using the least square approach showed the relationship between rivaroxaban concentrations measured by Biophen DiXaI and PT (a), PT ratio(b), APTT(c) and APTT ratio(d), respectively. Scatter plot of TT (e) and FIB (f) vs. rivaroxaban concentrations measured by Biophen DiXaI. Biophen DiXaI, Biophen Direct Factor Xa Inhibitor; PT, prothrombin time; PT ratios, prothrombin time vs. the NPP; APTT, activated partial thromboplastin time; APTT ratios, activated partial thromboplastin time vs. NPP; TT, thrombin time; FIB, fibrinogen.

The FX deficiency is defined such that the FX plasma activity levels are as low as 6–10% of the normal range to be considered mild deficiency, and below 1% are considered to be severe deficiency.[19,20] The FX plasma activity levels above 20% are rarely associated with bleeding, while patients with the FX plasma activity levels <10% present with mucocutaneous bleeding and patients with levels <5% may have symptoms including hemarthrosis, intracranial haemorrhage and gastrointestinal bleeding. Sae Heum Song et al. [15] reported that the duration of time that the intrinsic FXa activity was suppressed to 15% (the inhibition of FXa activity = 85%) of normal or lower by edoxaban (another FXa inhibitor) was the most significant predictor for the observed bleeding risk. This implied there was a threshold for the inhibition of FXa relative to bleeding risk. Therefore, the inhibition of FXa activity above a limiting value may result in excessive bleeding. So in this study, the inhibition of FXa activity could be considered as a predictor for risk of bleeding.

In vitro, PT and APTT showed good correlation with rivaroxaban concentrations. However, *in vivo*, PT showed moderate linear correlation with rivaroxaban concentrations, while APTT showed a poor correlation with rivaroxaban concentrations. *In vitro* and *in vivo*, PT showed a moderate correlation with the inhibition of FXa activity. When the inhibition of FXa activity reached 85%, the PT value was 15.6s (90% confidence intervals = 13.01s to 18.82s) approximately *in vivo*. Consequently, the PT reagent (Thrombosis S) may also provide relevant information about cases of bleeding in clinical, and could be considered as a rough method to monitor the anticoagulation activity of rivaroxaban and evaluate bleeding risk caused by rivaroxaban.

In spiked plasma and patient's plasma, the rivaroxaban concentrations measured by Biophen DiXaI always showed a good correlation with the inhibition of FXa activity. As no formal therapeutic drug levels or safety intervals have been established, the close relation between the inhibition of FXa activity and rivaroxaban concentrations measured by Biophen DiXaI may be used to propose an approximate threshold associated with bleeding risk.

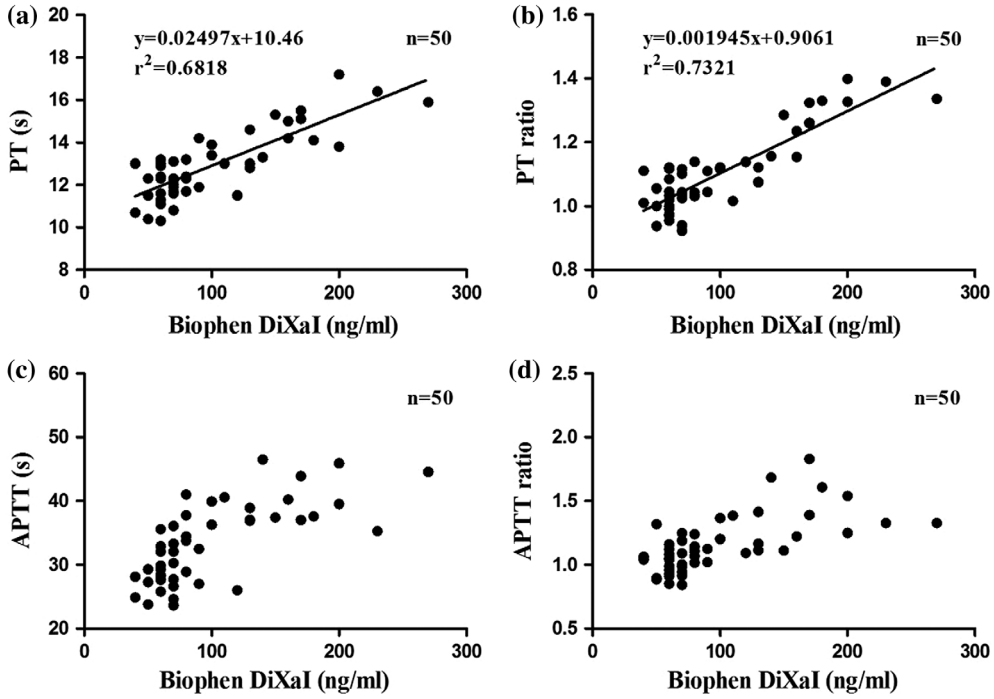

Figure 3. *In vivo*, scatter plot of PT (a), PT ratio(b), APTT(c) and APTT ratio(d) vs. rivaroxaban concentrations measured by Biophen DiXaI. Biophen DiXaI, Biophen Direct Factor Xa Inhibitor; PT, prothrombin time; PT ratios, prothrombin time vs. the NPP; APTT, activated partial thromboplastin time; APTT ratios, activated partial thromboplastin time vs. NPP.

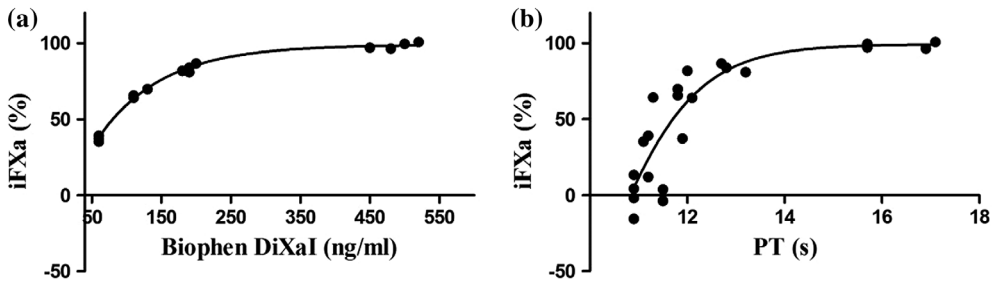

Figure 4. *In vitro*, the inhibition of FXa activity correlation with rivaroxaban concentrations measured by Biophen DiXaI (a) and PT (b). FXa, factor Xa; Biophen DiXaI, Biophen Direct Factor Xa Inhibitor; PT, prothrombin time.

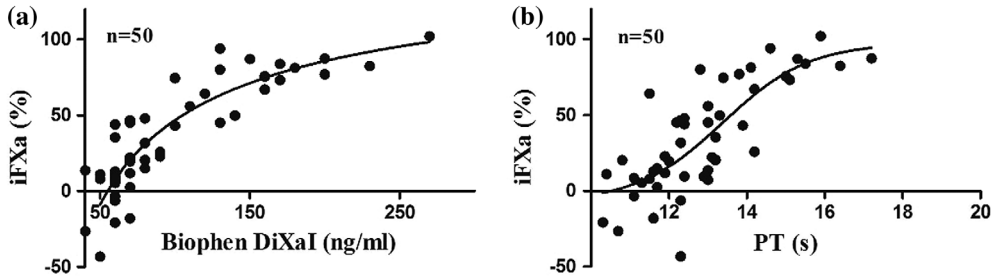

Figure 5. *In vivo*, the inhibition of FXa activity correlation with rivaroxaban concentrations measured by Biophen DiXaI (a) and PT (b). FXa, factor Xa; Biophen DiXaI, Biophen Direct Factor Xa Inhibitor; PT, prothrombin time.

In vitro, the values of PT showed slightly shorter and the values of APTT showed slightly longer than those values *in vivo*. Due to freezing and thawing before testing *in vitro*, the values of shortened PT and prolonged APTT may have been affected.[21] The limitation of this study is that only the inhibition of FXa activity was measured as the predictor for bleeding risk.

We conclude that Biophen DiXaI could be used as a quantitative method to monitor the anticoagulation activity of rivaroxaban. The rivaroxaban concentrations measured by Biophen DiXaI showed a close correlation with the inhibition of FXa activity, which could be used to propose an approximate threshold associated with bleeding risk. The PT reagent (Thrombosis S) could be

used as a rough method to monitor the anticoagulation activity of rivaroxaban and evaluate bleeding risk due to rivaroxaban. It is possible that the present results in patients treated with rivaroxaban can be applied to other oral direct FX inhibitors (apixaban and edoxaban). It is the first time that this type of data has been reported in association with the Chinese ethnic group. This work represents an advance in biomedical science because it shows that Biophen DiXaI could not only be used as a quantitative method to assess the anticoagulation activity of rivaroxaban, but also could be used to evaluate the risk of bleeding caused by rivaroxaban (Table 1).

Table 1. Summary.

What is known about this subject
- Rivaroxaban can be used without routine coagulation monitoring, but clinical surveillance is recommended in several situations
- The sensitivity of PT to assess the anticoagulation activity of rivaroxaban varies depending on reagents and instruments used
- Anti-factor Xa (FXa) chromogenic assays and PT assays associated with bleeding risk are currently unknown.

What this paper adds
- The anti-FXa chromogenic assay (Biophen DiXaI) has a close correlation with the inhibition of FXa activity
- The close relation between the inhibition of FXa activity and rivaroxaban concentrations measured by Biophen DiXaI could be used to propose an approximate threshold associated with bleeding risk
- The PT reagent (Thrombosis S) could be used as a rough method to monitor the rivaroxaban activity and evaluate the risk of bleeding

Conflicts of interest

There are no conflicts of interest.

Disclosure statement

No potential conflict of interest was reported by the authors.

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