Comparison of calibrated chromogenic anti-Xa assay and PT tests with LC-MS/MS for the therapeutic monitoring of patients treated with rivaroxaban

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Summary

Possibilities to monitor rivaroxaban therapy could be useful in certain circumstances. Prothrombin time (PT) or chromogenic anti-Xa assays such as the Biophen Direct Factor Xa Inhibitor® (DiXaI) have been proposed to estimate rivaroxaban concentrations but are mainly based on *in vitro* studies. The study aim was to compare PT and Biophen DiXaI® measurements with liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements in plasma samples from patients treated with Xarelto®. Fifty-two plasma samples were included. PT was performed using Innovin® and Triniclot PT Excel S®. Biophen DiXaI® was performed according to instructions from the manufacturer. The rivaroxaban plasma concentration ranged between 0 and 485 ng/ml as measured by LC-MS/MS. The limits of quantification were 30 ng/ml and 5 ng/ml for Biophen DiXaI® and LC-MS/MS, respectively. The linear correlation between Biophen DiXaI® and LC-MS/MS analyses was high for all rivaroxaban concentrations (r^2 = 0.95). For concentrations ≤100 ng/ml, r^2-value was 0.83. The Bland-Altman analysis showed a mean difference of –16 ng/ml (SD: 25 ng/ml). The PT methods did not correlate well with plasma concentrations measured by LC-MS/MS ($r^2 \approx 0.60$). In conclusion, the important inter-individual variability and the poor correlation with LC-MS/MS preclude the use of PT to estimate rivaroxaban concentrations. Thanks to its small inter-individual variability and good agreement with LC-MS/MS measurements, we recommend the use of Biophen DiXaI® assays to estimate concentrations of rivaroxaban >30 ng/ml. Quantification of low rivaroxaban levels (<30 ng/ml) requires the LC-MS/MS method.

Keywords

Rivaroxaban, monitoring, prothrombin time, chromogenic anti-Xa assay, LC-MS/MS

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hourIntroduction

Rivaroxaban (Xarelto®) is approved by the European Commission and the Food and Drug Administration (FDA) for several indications. These include the primary prevention of venous thromboembolic events in adult patients who have undergone elective total hip or total knee replacement, the prevention of stroke in adult patients with non-valvular atrial fibrillation (NVAF), the treatment of deep-vein thrombosis (DVT) and the prevention of recurrent DVT and pulmonary embolism (PE) following an acute DVT in adults (1-4). Rivaroxaban has also been tested for long-term treatment of venous thromboembolism (3). Recently, rivaroxaban also received a positive opinion by the European Medicines Agency (EMA) for the prevention of thrombotic events in adult patients after an acute coronary syndrome with elevated cardiac biomarkers when co-administered with acetylsalicylic acid (ASA) alone or with ASA plus clopidogrel or ticlopidine (5). The European Society of Cardiology guidelines for the management of AF, the American College of Chest Physicians Guidelines and the Canadian Cardiovascular Society Guidelines recommend the "new oral anticoagulants" (NOACs) as broadly preferable to vitamin K antagonists (VKAs) in the vast majority of patients with NVAF, suggesting a wider use of these compounds in the near future (6-8). Although the new anticoagulants may replace some of

the traditional agents, certain changes in hospital routine and patient management strategies are required when introducing these new agents to clinical practice (9). Therefore, the knowing of the pharmacology and the impact of these new compounds on a series of routinely used coagulation assays is of great importance to achieve optimal patient outcome. The pharmacokinetic properties of rivaroxaban include renal and hepatic elimination (10, 11). Thanks to its predictable pharmacokinetic and pharmacodynamic profiles, monitoring is not recommended in the large majority of patients (12). However, it is anticipated that a non-negligible proportion of patients will achieve either insufficient or supra-therapeutic level when given at fixed dose. Moreover, the European summary of product characteristics (EU-SmPC) states that clinical surveillance is recommended throughout the treatment period in several subgroups of patients (1). Therefore, biological monitoring would be valuable in acute situations such as recurrence of thrombosis or bleeding, before urgent surgery or before fibrinolytic therapy of acute ischaemic stroke, in bridging and in patients with risk factors for rivaroxaban accumulation or too low levels, i.e. several drug-drug interactions, patients with extreme body weight, or presenting hepatic or renal impairment. Although robust data on drug levels associated with therapeutic or harmful ranges are currently lacking, estimation of plasma drug concentrations can be interesting to identify poor or high responders (13).

The recent recommendation from the Subcommittee on control of anticoagulation of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (ISTH) proposed that the plasma levels of rivaroxaban may be estimated by different coagulation assays such as specific prothrombin time (PT) assays or chromogenic anti-Xa assays using rivaroxaban plasma calibrators (14). However, these findings are only based on *in vitro* analysis and further studies in patients treated with rivaroxaban are required to determine their usefulness (14). The accuracy of these estimates compared to the true concentrations from patients treated with rivaroxaban has not been established.

The aim of this study is to assess the correlation between chromogenic anti-Xa assay and PT with the reference method for the measurement of rivaroxaban in plasma, i.e. liquid chromatography with tandem mass spectrometry (LC-MS/MS), in patients treated with rivaroxaban.

Materials and methods

The study was performed in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the "Centre Hospitalier Universitaire UCL Mont-Godinne – Dinant (Yvoir, Belgium)". Written informed consent was obtained from each donor.

Normal pooled plasma and home-made calibrators

Twenty-seven healthy individuals were included. The exclusion criteria were thrombotic and/or haemorrhagic events, antiplatelet and/or anticoagulant medication, hormonal therapy, pregnancy, and use of drugs potentially affecting platelet and/or coagulation factor functions during two weeks prior to sampling. Blood was taken by antecubital venipuncture and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Venosafe®, Terumo, Belgium) using a 21-gauge needle (Terumo, Belgium). Platelet-poor plasma (PPP) was obtained from the supernatant fraction after double centrifugation for 15 minutes (min) at 2000 g at room temperature. Immediately after centrifugation, PPPs from the 27 donors were mixed to obtain the normal pooled plasma (NPP) which was frozen at –80°C without delay. Frozen NPP aliquots were thawed and heated to 37°C for min 5 min just before experiments.

Powder of rivaroxaban for analyses was a generous gift of Bayer A.G. (Bayer A.G, Leverkusen, Germany).

Rivaroxaban for coagulation analysis was prepared from a stock solution at 2.2 mg/ml in 100% DMSO to obtain an intermediate solution at 10.9 µg/ml, 5.5 µg/ml, 2.2 µg/ml, 1.1 µg/ml, 0.5 µg/ml, 0.2 µg/ml and 0.1 µg/ml diluted in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

Working solutions of 1090 ng/ml, 545 ng/ml, 218 ng/ml, 109 ng/ml, 55 ng/ml and 11 ng/ml of rivaroxaban were obtained by mixing these stock solutions with NPP. The DMSO concentration in plasma was ≤ 0.05% (v/v) which does not influenced the coagulation (15).

Linezolid (internal standard) for LC-MS/MS analyses was purchased from Sigma-Aldrich (Sigma-Aldrich, BVBA, Diegem, Belgium). The use of linezolid as internal standard can be considered due to structural similarities with rivaroxaban (►Figure 1). None of the patients included in this study was taken linezolid making possible the use of this compound as internal standard.

Clinical samples

Fifty-two plasma samples from real-life patients treated with rivaroxaban were included in the study for retrospective analysis. Exclusions criteria were concomitant treatment with linezolid or other anticoagulant(s) within the last two weeks. To be included in the study, patients must be on rivaroxaban since at least two weeks, without any bridging with other anticoagulant(s). All patients on rivaroxaban were treated for stroke prevention in non-valvular AF or for the prevention of recurrent DVT and PE following an acute DVT. Blood was taken by venipuncture and PPP was obtained as described above for the healthy volunteers. Plasma samples were frozen at –80°C without any delay and heated to 37°C for 5 min immediately before coagulation testing. For LC-MS/MS drug measurements (see below) heating of the sample is not needed.

Coagulation assays

Chronometric assay: Prothrombin time (PT)

Prothrombin time was measured using two different reagents, Triniclot PT Excel S® (TrinityBiotech, Bray, Ireland) and Innovin® (Siemens Healthcare Diagnostics, Deerfield, IL, USA) on a STA-R Evolution® coagulometer. The same batch was used for each

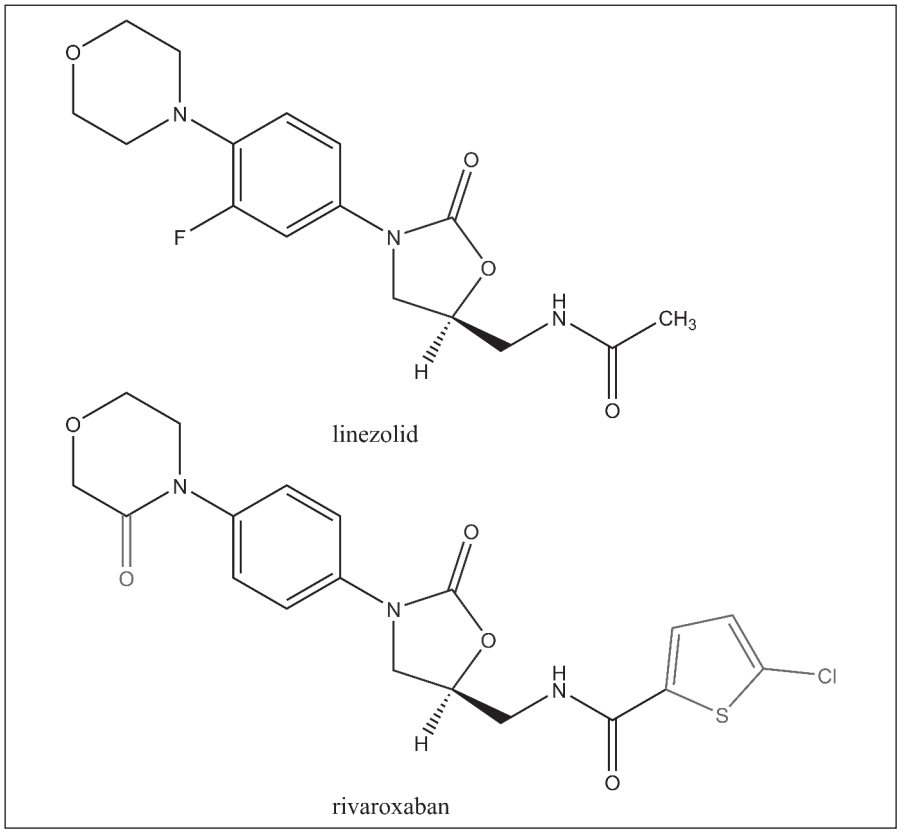


Figure 1: Structural comparison between rivaroxaban and linezolid. Similarities in the structure allow the use of linezolid as internal standard for LC-MS/MS.

reagent. Triniclot PT Excel S® is derived from rabbit brain while Innovin® is a recombinant human tissue factor. The two PT tests were performed as previously described (16, 17). Results are given in seconds, as ratios (vs NPP), and in ng/ml according to calibration with home-made calibrators (ranging from 0 to 1090 ng/ml). We have chosen these reagents to confront the results obtained with the more sensitive reagent to our knowledge (Triniclot PT Excel S®) and the less sensitive (Innovin®), as mentioned previously (17).

Chromogenic assay: Biophen Direct Factor Xa Inhibitor (DiXaI)

Biophen DiXaI® (HYPHEN BioMed, Neuville-Sur-Oise, France) is a chromogenic assay for *in vitro* quantitative measurement of direct factor Xa (FXa) inhibitors on human citrated blood plasma. It is based on the inhibition of a constant amount of exogenous FXa and the hydrolysis of a FXa specific chromogenic substrate (CS-11(65) consisting of -D-Arg-Gly-Arg-pNA, 2HCl) by the residual FXa. Para-nitroaniline (pNA) is then released from the substrate and measured at 405 nm. The amount of pNA released is a direct relationship of the residual FXa activity. Thanks to its high ionic strength pH 7.90 buffer, this test is specific for direct FXa inhibitors, without interference of indirect polysaccharide inhibitors (18). The test was performed as previously described on a STA-R Evolution® coagulometer (17). Calibration is performed using a reference, lyophilised preparation of rivaroxaban (from 0 to 480 ng/ml in the initial sample after reconstitution) (HYPHEN BioMed).

Liquid chromatography coupled with tandem mass spectrometry

This method represents an adaptation of the procedure described by Rohde et al. (19). Plasma concentrations of rivaroxaban were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) after sample preparation by protein precipitation of 200 µl citrated plasma with methanol containing linezolid as internal standard. Rivaroxaban for LC-MS/MS analyses was a generous gift from Bayer A.G. (Bayer A.G.) and linezolid was purchased from Sigma Aldrich (Sigma-Aldrich). Sample preparation consisted of mixing 500 µl of methanol containing the internal standard and 200 µl of plasma sample. The mix was gently shaken and centrifuged. An aliquot (20 µl) of the final extract was injected into the LC-MS/MS system. Separation of the analytes was achieved on an HPLC Kinetex column (Phenomenex© C18, 2.6 µm, 3.0 mm x 150 mm), using a gradient run with mobile phase A (10 mM ammonium formate) and mobile phase B (methanol). The analytes were detected using a Waters© Quattro Micro mass spectrometer operating in positive electrospray ionisation (ESI) mode utilising multiple reaction monitoring (MRM) for the transitions 436.01 ± 144.9 m/z (rivaroxaban, quantitative ion product), 436.01 ± 231.0 (rivaroxaban, qualitative ion product), 338.11 ± 296,0 (internal standard, quantitative ion product), 338.11 ± 195,1 (internal

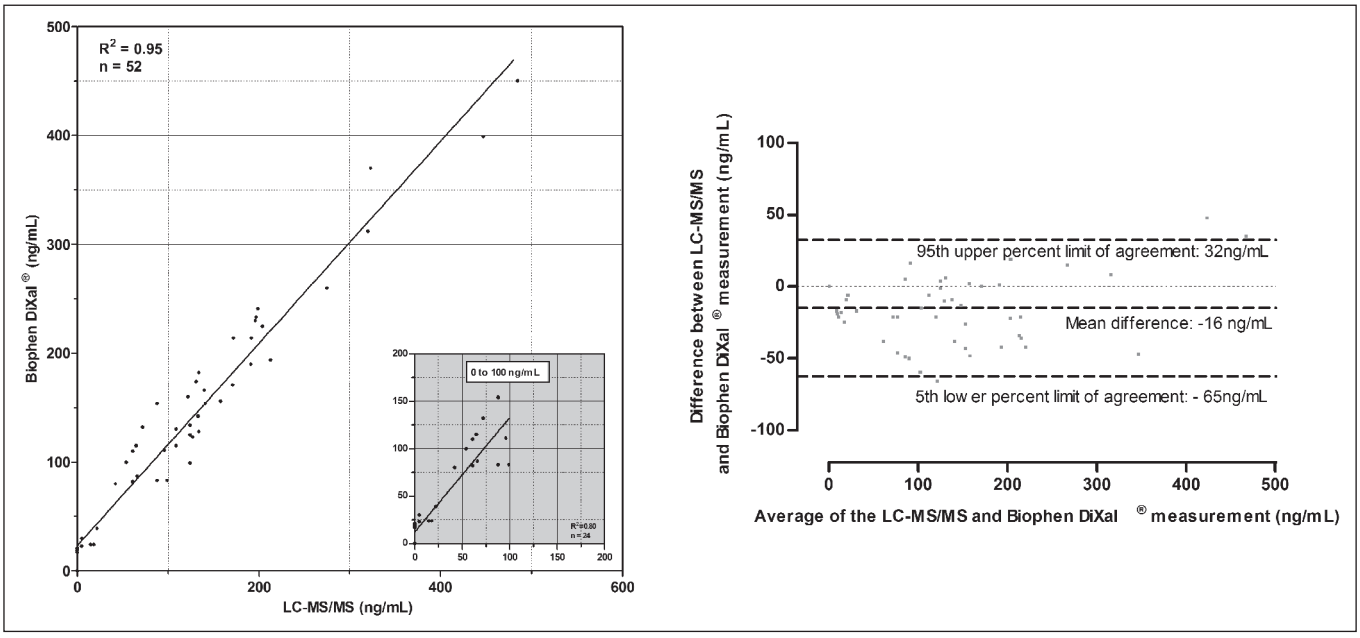


Figure 2: Correlation and Bland-Altman analysis between liquid chromatography-tandem mass spectrometry (LC-MS/MS) and the Biophen Direct Factor Xa Inhibitor® (DiXaI) assay for the measurement of rivaroxaban concentrations in patient plasma samples. The insert shows the relationship at rivaroxaban concentrations ≤100 ng/ml as determined by LC-MS/MS. For the Bland-Altman analysis the difference is calculated as follow: [difference (A-B) vs average] where A is the result of the LC-MS/MS and B the result of Biophen DiXaI®.

standard, qualitative ion product). No interfering peaks were observed in 15 blank plasmas. The calibration curve for rivaroxaban in plasma was linear over the range 5-500 ng/ml, and the lower limit of detection (LLOD) was estimated to 3 ng/ml. Validation experiments with three levels of control samples (15, 60 and 125 ng/ml) on three different occasions (15 determinations per concentration), showed an inter-assay precision between 5.79 % and 8.11 % and an inter-assay accuracy within the confidence range (± 20%). The method was validated according to FDA Guidelines for Industry for Bioanalytical Method Validation.

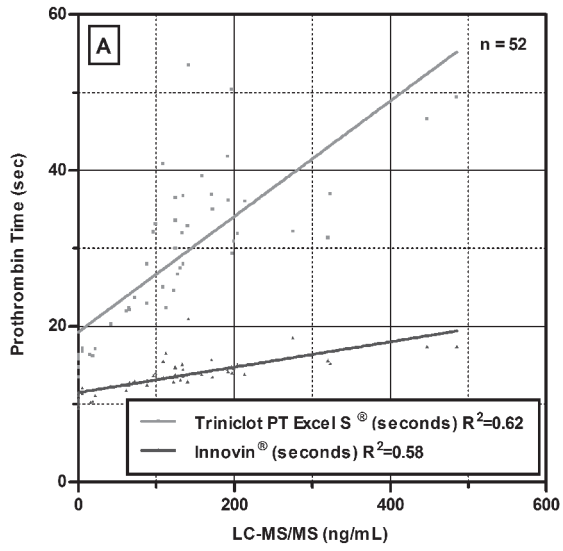
Statistical analysis

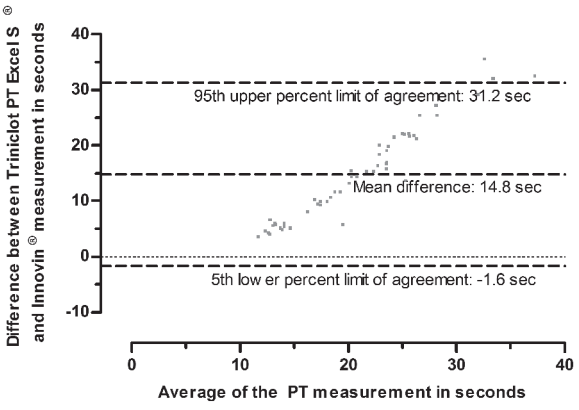
Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results for the Biophen DiXaI® and PT vs the LC-MS/MS methods were compared by Spearman correlation analysis as well as by linear regression. Bland-Altman analyses were also proposed. The 95th limits of agreement of the Bland-Altman analyses are calculated as follows: Mean difference – or + 1.96*standard deviation for the 5th and the 95th limit of agreement, respectively. For the Biophen DiXaI®, the lower limit of detection (LOD) and the lower limit of quantitation (LOQ) were calculated as follow: LOD = [(3*standard deviation of Y0)/ slope] and LOQ = [(10*standard deviation of Y0/ slope]. For the LC-MS/MS method, the LOD and lower limit of quantitation (LLOQ) were calculated following preparation of sample at very low concentrations (2-5 ng/ml). Each sample was prepared in 10 copies. The LOD was defined as the concentration showing a three-fold superior signal compared to the background noise. The LLOQ is defined as the lowest concentration providing these two criteria: the variation coefficient must be lower than 20% and the deviation from the target must be less than 20%.

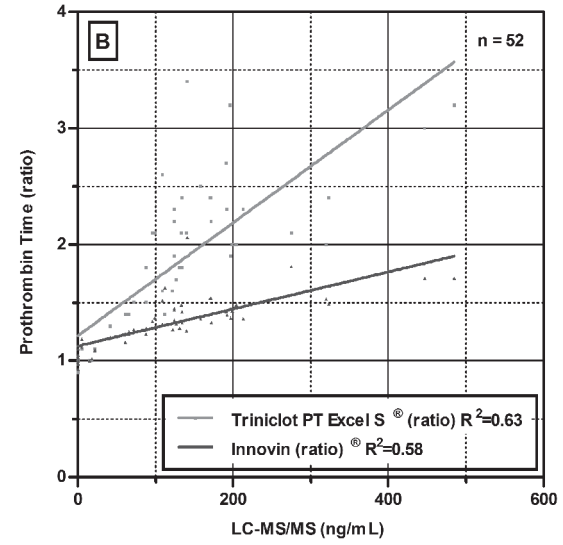
Results

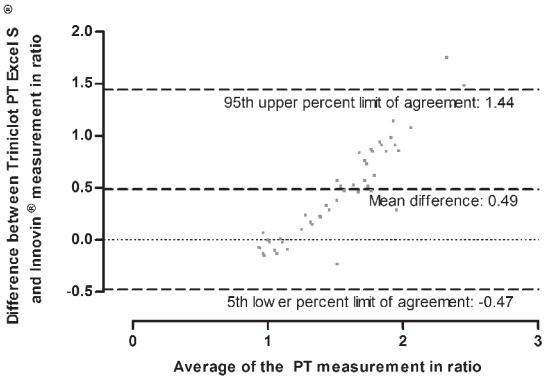
Fifty-two samples were analysed by LC-MS/MS, Biophen DiXaI® and PT. The correlation between Biophen DiXaI® and LC-MS/MS method as well as the Bland-Altman analysis are provided in ►Figure 2. ►Figure 3 and ►Figure 4 summarise the results obtained with the two PT reagents (Triniclot PT Excel S® and Innovin®) in comparison with LC-MS/MS. Commercial calibrators at concentrations of 0 ng/ml (confidence interval [CI]: 0 ng/ml – 2 ng/ml) and 96 ng/ml (CI: 76.8 – 115.2 ng/ml) were tested at five recoveries by LC-MS/MS to verify the accuracy of our method. For the zero concentration, all samples were below the LLOD (i.e. 3 ng/ml). For the calibrator at 96 ng/ml, four measurements were within the confidence interval and one measure was just at the lower limit of the confidence interval (i.e. 76.8 ng/ml). Nevertheless, it would have been interesting to test more "external controls"

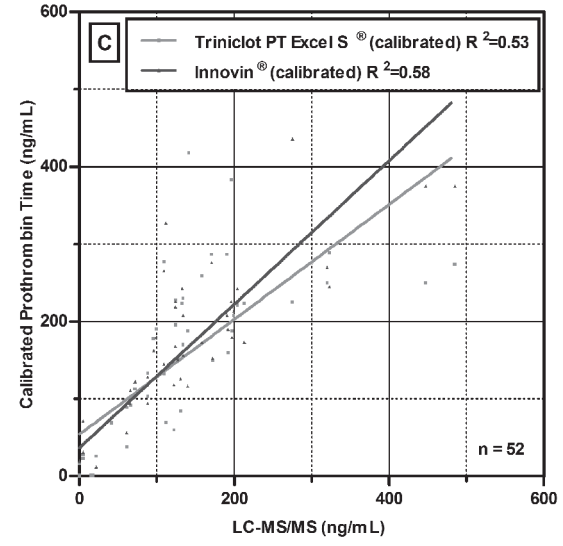
Figure 3: Correlations between PT and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results are expressed in seconds (A), ratios (B) or ng/ml (C). Graphics of the Bland-Altman analysis comparing the two reagents when results are given in seconds, ratio or ng/ml are also provided. For the Bland Altman analysis the difference is calculated as follow: [difference (A-B) vs average] where A is the result of Triniclot PT Excel S® and B the result of Innovin®.

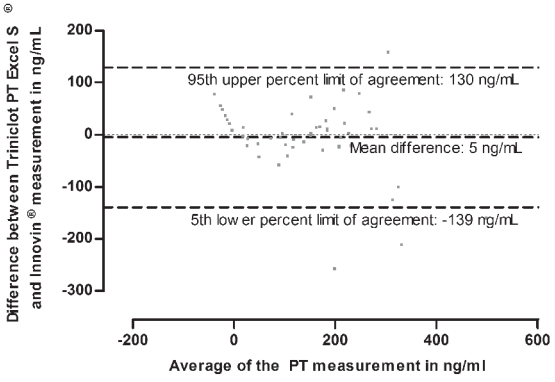












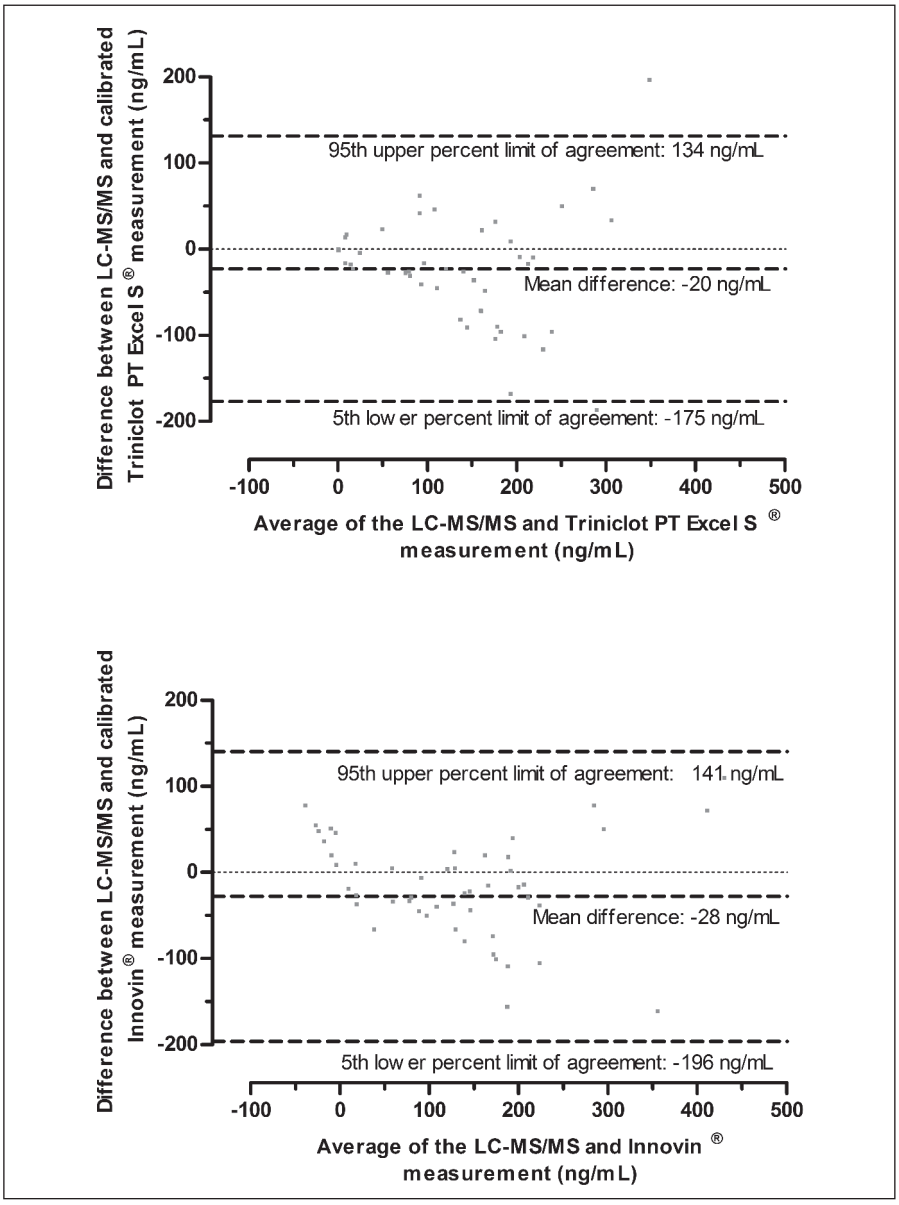


Figure 4: Bland-Altman analyses comparing LC-MS/MS and calibrated PT reagent. The difference is calculated as follow: [difference (A-B) vs average] where A is the result of LC-MS/MS and B the result of Triniclot PT Excel S® and Innovin®, respectively.

on our LC-MS/MS method. "Home-made controls" were always within the 20% of the expected values either with the LC-MS/MS method or with the Biophen DiXaI® showing the accuracy and the precision of our methods.

Correlation between the Biophen DiXaI® test and LC-MS/MS

Fifty-two samples were tested for comparison. The Spearman correlation between Biophen DiXaI® and LC-MS/MS had a coefficient (rs) of 0.96 (95% CI: 0.94 to 0.98; p<0.0001; r^2 for linear regression = 0.95) (►Figure 2). Results from the Bland-Altman analysis are provided in ►Figure 2. For plasma concentrations ≤100 ng/ml (n=24) the Spearman correlation coefficient was 0.92 (95% CI: 0.81 to 0.96; p< 0.0001; r^2 for linear regression = 0.83) (►Figure 2; insert). The Bland-Altman analysis for samples with ≤100 ng/ml rivaroxaban showed a mean difference of -20 ng/ml for plasma concentrations estimated by the Biophen DiXaI® in comparison with the LC-MS/MS (SD: 22 ng/ml; 95% limits of agreement: -63 to 23 ng/ml).

For Biophen DiXaI®, the LOD in plasma given by the manufacturer is 20 ng/ml. None information is given by the manufacturer regarding the LOQ. However, a previous study has calculated the LOQ at 20 ng/ml and the LOD at 10 ng/ml (20). These values are quite similar than those obtained with our own fresh calibrator (30 and 10 ng/ml, respectively).

Correlations between the PT assays and LC-MS/MS

Fifty-two samples were tested for comparison. The PT tests showed concentration-dependent prolongations of clotting time. The Spearman correlation coefficient between PT and LC-MS/MS

was 0.86 (95% CI: 0.77 to 0.92; p< 0.0001; r^2 for linear regression = 0.62) and 0.87 (95% CI: 0.77 to 0.92; p< 0.0001; r^2 for linear regression = 0.58) for Triniclot PT Excel S® and Innovin®, respectively (►Figure 3A). When expressing results as ratios, the Spearman correlation coefficient was 0.87 (95% CI: 0.78 to 0.92; p< 0.0001; r^2 for linear regression = 0.63) and 0.87 (95% CI: 0.77 to 0.92; p< 0.0001; r^2 for linear regression = 0.58) for Triniclot PT Excel S® and Innovin®, respectively (►Figure 3B). The rivaroxaban concentrations estimated with calibrated methods gave rs-values of 0.86 (95% CI: 0.76 to 0.92; p< 0.0001; r^2 for linear regression = 0.53) and 0.86 (95% CI: 0.76 to 0.92; p< 0.0001; r^2 for linear regression = 0.58) for Triniclot PT Excel S® and Innovin®, respectively (►Figure 3C). ►Figure 4 presents Bland-Altman analyses of the calibrated PT results in comparison with LC-MS/MS. Both PT reagents showed large 5^{th} – 95^{th} limit of agreement (►Figure 4) while the mean difference compared to LC-MS/MS reached -20 and -28 ng/ml for Triniclot PT Excel S® and Innovin®, respectively. When focusing on results >50 ng/ml or >100 ng/ml separately, a weaker linear correlation and a higher standard deviation for the Bland-Altman analysis were obtained. Namely, for concentrations higher than 50 ng/ml as measured by LC-MS/MS, the linear correlation coefficient (r^2) for results expressed in seconds or as ratio was 0.38 and 0.33 for Triniclot PT Excel S® and Innovin®, respectively. When results are expressed in ng/ml, both reagents showed a poor linear correlation coefficient (r^2) of 0.33 and 0.26 for Triniclot PT Excel S® and Innovin®, respectively. For concentrations higher than 100 ng/ml, both reagents showed a very poor correlation (data not shown). The differences between the two PT reagents used in this study had been evaluated by Bland-Altman analyses and are provided in ►Figure 3.

Discussion

Rivaroxaban possess predictable pharmacokinetic and pharmacodynamics profile. However, searching for the optimal dose in the individual patient could be of interest in several situations. Although, harmful and therapeutic range are currently lacking, a similar approach to that proposed for dabigatran (16, 21) would be valuable to identify under- or over-responders. Thus, exceeding the 90^{th} percentile of rivaroxaban trough levels could be considered as an increased risk of bleeding. For patients treated with rivaroxaban 20 mg od for the treatment of acute DVT, it is stated that the 90^{th} percentile of rivaroxaban plasma concentrations measured at trough (24 hours [h] after the previous dose), was about 239 ng/ml (1). The FDA - Clinical Pharmacology Biopharmaceutics Review(s) of Xarelto® supports this approach, showing that a two-fold increase in exposure due to intrinsic and extrinsic factors will increase the risk of major bleeding by 50%. This report also shows poor protection against the composite outcome of any DVT, non-fatal PE and death from all causes when plasma drug concentrations were above the 10^{th} percentile of rivaroxaban plasma concentration at trough (22).

In this study, we report on the correlation between rivaroxaban concentrations in patients treated with Xarelto® as determined by the calibrated Biophen DiXaI® and a LC-MS/MS method. We also compared the LC-MS/MS method with two PT assays in order to evaluate the recommendations provided by the literature as well as in the EU-SmPC (1, 14, 23).

Correlation between Biophen DiXaI® and LC-MS/MS

The use of anti-FXa chromogenic assays, more sensitive and specific to the direct inhibition of FXa than PT, could be of interest for the monitoring of direct factor Xa inhibitors (24). The results from a multicentre trial involving 24 laboratories indicate that the anti-FXa chromogenic method, using rivaroxaban calibrators and controls is suitable for the measurement of a wide range (20–660 ng/ml) of rivaroxaban plasma concentration (20). They also present a good repeatability and reproducibility and the estimated concentrations have been comparable with the concentrations measured by LC-MS/MS in a previous study (25). A limitation of the anti-Xa assay is that the standardisation across reagents and methods is not easily achieved but interesting results showed that inter-assay variability can be easily reduced (26).

In our study, the Biophen DiXaI® showed a good correlation with LC-MS/MS and the Bland-Altman analysis revealed an acceptable mean difference (►Figure 1). When focusing on samples ≤100 ng/ml, the mean difference was approximately the same (-19 and -16 ng/ml, for samples ≤100 ng/ml and the entire analysis, respectively). In both cases the 95% limits of agreement was ranging from approximately -65 to 30 ng/ml, suggesting reasonable accuracy of Biophen DiXaI® to estimate rivaroxaban plasma concentration in the low-normal to high concentration range. However, the LOD and LOQ of Biophen DiXaI® (i.e. 10 and 30 ng/ml, respectively, with our own fresh calibrators) are much higher than those obtained with the very sensitive LC-MS/MS method (3 and 5 ng/ml for the LOD and the LLOQ, respectively). This limits the use of Biophen DiXaI® for accurate quantitative estimates of C_{trough} in the orthopaedic indication (median C_{trough} = 9 ng/ml) or in stroke prevention in patients responding poorly to the drug (mean C_{trough} = 32 ng/ml; poor-responders will not reach this value and cannot be accurately assessed).

Biophen DiXaI® can therefore be used to reliably estimate rivaroxaban plasma concentrations higher than 30 ng/ml. However, this precludes its use for the evaluation of sub-therapeutic response at C_{trough}. Moreover, variability of coagulation analysers may further increase the imprecision at such low concentrations. The LC-MS/MS method is more accurate and is useful in the entire range of rivaroxaban concentrations. Consequently, the LC-MS/MS is required for quantification of very low to moderate rivaroxaban concentrations (3 to 30 ng/ml) in clinical samples (e.g. before fibrinolytic therapy of acute ischaemic stroke or before acute surgery associated with an increased bleeding risk) or for pharmacokinetic studies.

Correlation between PT and LC-MS/MS

The Subcommittee of Control of Anticoagulation of the Scientific and Standardisation Committee of the ISTH and the British Com-

What is known about this topic?

- Rivaroxaban is an oral anticoagulant used for primary prevention of venous thromboembolic events in adult patients who have undergone elective total hip replacement surgery or total knee replacement surgery, for long-term use in the prevention of thromboembolic events in patients with atrial fibrillation and in the treatment of deep-vein thrombosis (DVT) and the prevention of recurrent DVT and pulmonary embolism (PE) following an acute DVT in adults.
- Monitoring is generally not required but possibilities to evaluate the intensity of rivaroxaban treatment may be valuable in some situations.
- Prothrombin time can be used for urgent determination of the relative intensity of anticoagulation. Calibrated chromogenic anti-Xa assays can be used to determine the drug concentration.

What does this paper add?

- There is a poor correlation between prothrombin time and the measured plasma rivaroxaban concentration. Prothrombin time must not be used to estimate rivaroxaban concentrations in plasma and poorly reflects the intensity of anticoagulation due to rivaroxaban.
- The calibrated chromogenic anti-Xa assay, Biophen Dixrect Factor Xa Inhibitor®, highly correlates with the reference LC-MS/MS measurement for rivaroxaban concentration higher than 30 ng/ml.
- Detection and quantification of lower levels of rivaroxaban in treated patients requires the more sensitive LC-MS/MS analysis.

mittee for Standards in Haematology mention that the PT (with appropriate reagent) can be used to determine the relative intensity of anticoagulation in emergency situation when required, but should not be used to quantify drug plasma concentration (14, 23). A similar approach is proposed in the EU-SmPC using Neoplastin® as thromboplastin reagent (1). However, no information regarding the reagent (i.e. STA-Neoplastin® CI, CI + or R) or the type of sample (i.e. NPP spiked with rivaroxaban or plasma samples from patients treated with rivaroxaban) is provided. This precludes firm affirmation that PT could be an alternative to accurately estimate plasma drug concentration in real-life patients compared to more specific and sensitive methods. The results of a multi-centre trial on pooled plasma spiked with rivaroxaban involving 21 laboratories, revealed a large inter-laboratory variation with local PT reagents when expressed in seconds. The results were less variable when expressed as rivaroxaban concentrations (ng/ml) or when central PT reagent was used (STA-Neoplastine CI Plus®) (27). These previous findings suggested that it was feasible to measure rivaroxaban plasma concentrations using PT combined with rivaroxaban calibrators and controls (27). However, the authors of this field trial mention that alternative methods that are more specific and more sensitive are needed for the measurement of a wider range of rivaroxaban plasma concentration. This study was performed with apheresis, citrated, pooled plasma originating from transfusion blood banks. This cannot reflect the use of PT in real-life conditions and do not obviously take into account the individual baseline variability.

In this *ex vivo* study, results are expressed in seconds, as ratio or in ng/ml. Namely, INR^{ISI} methodology used for VKAs is not suitable for measurement of rivaroxaban because it dramatically magnifies the between-reagent variability (1, 14). An INR^{ISI} methodology specifically developed for rivaroxaban is feasible but is not currently provided in the insert-kit of the reagents and therefore, has not been tested here (28).

We demonstrate that Innovin® has a poor sensitivity compared to Triniclot PT Excel S® (►Figure 2). This confirms our previous *in vitro* findings regarding the sensitivity of the PT reagents (17) and a recently published case report (29). However and importantly, even if this sensitivity is rather low, the use of Innovin® to determine the clotting factor activity in patients having recently taken rivaroxaban (within 2 h before phlebotomy) revealed a significant decrease of all factor assessed by PT-based assay (30). Therefore, larger variations could be expected with more sensitive reagents but this needs to be confirmed.

Both PT reagents (Innovin® and Triniclot PT Excel S®) show a poor linear correlation coefficient with rivaroxaban concentrations in plasma whatever the results are given in seconds, as ratio or in ng/ml (►Figure 3). Calibration of each reagent with rivaroxaban does not improve the correlation and even gets worse for Triniclot PT Excel S® (►Figure 3C). In addition, the Bland-Altman analyses of the results express in ng/ml show a very large 5^{th} – 95^{th} percent limit of agreement (►Figure 4). For the between reagent comparison, the Bland-Altman analyses reveal high inter-reagent variability for results expressed in seconds and ratio (►Figure 3A and B) precluding standardisation of cut-off expressed in these units. For results expressed in ng/ml, the mean difference between Innovin® and Triniclot PT Excel S® is very low (5 ng/ml) but is associated with a large standard deviation (69 ng/ml). When focusing on results higher than 50 ng/ml or 100 ng/ml, correlations are very poor, and Bland-Altman analyses show higher mean difference with even larger standard deviations.

Limitations of this study

This study was not intended to correlate the rivaroxaban plasma concentrations with clinical efficacy or safety outcomes. We cannot exclude the fact that PT may also give relevant information in connection with cases of bleeding or recurrence of thrombosis. Clinical studies that investigate relationships between either rivaroxaban concentrations in plasma (measured by LC-MS/MS of estimated via Biophen DiXaI®) or PT values and clinical outcomes are still required. Importantly, we only tested Innovin® and Triniclot PT Excel® for the PT-experiments and Biophen DiXaI® for the chromogenic anti-Xa experiments. Therefore, it is important to mention that results discussed in this work could be not extended to other thromboplastin reagents or anti-Xa chromogenic assays. Further studies are required to confirm this hypothesis.

Conclusions

The poor sensitivity (especially for Innovin®), the important variability, and the poor linear correlation with the LC-MS/MS preclude the use of PT to estimate rivaroxaban concentration in plasma. Thanks to its small variability and good agreement with LC-MS/MS measurements, we recommend the use of Biophen DiXaI® to accurately estimate rivaroxaban plasma concentrations >30 ng/ml. Taking into account the lower sensitivity of the Biophen DiXaI® method, detection and quantitation of lower levels in rivaroxaban treated patients (to check for compliance, in case of recurrence of thrombosis, before elective surgery or before fibrinolytic therapy of acute ischaemic stroke) requires LC-MS/MS analyses.

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Conflicts of interest

None declared.

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