Development of bioanalytical assays for variegin, a peptide-based bivalent direct thrombin inhibitor

Aim: Variegin is an anticoagulant peptide that will be tested in porcine models of percutaneous coronary intervention. We developed three bioanalytical assays for variegin quantitation and utilized these methods to evaluate pharmacokinetics of variegin in pigs. **Results & methodology:** The LC–MS/MS, thrombin amidolytic and modified thrombin time assays had a quantitation range of 21.6–5541.7, 10.8–5541.7 and 5.4–5541.7 nM in human plasma, respectively. The elimination half-lives obtained using the LC–MS/MS, modified thrombin time and thrombin amidolytic assays were 52.3 ± 4.4 , 50.4 ± 5.9 and 67.7 ± 6.3 min, respectively. **Conclusion:** We developed three bioanalytical assays for a novel direct thrombin inhibitor, variegin. The thrombin time assay is optimized for variegin quantitation during future porcine studies and clinical trials.

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Injectable anticoagulants are administered to more than a million patients undergoing percutaneous coronary intervention (PCI) and cardiopulmonary bypass surgery each year [1,2]. Thrombin is a central enzyme in the blood coagulation cascade, providing an excellent target for the development of injectable anticoagulants. Heparin, a natural polysaccharide that indirectly inhibits thrombin by binding antithrombin III, is the most commonly used injectable anticoagulant. However, two major limitations of heparin are its variable pharmacokinetics (PK), leading to increased bleeding, and its propensity to cause thrombocytopenia, leading to life-threatening thrombosis (heparin-induced thrombocytopenia) [3]. Furthermore, it is a nonspecific inhibitor that inhibits both thrombin and Factor Xa [4].

Injectable direct thrombin inhibitors (DTIs) have been developed to overcome the inherent pharmacologic variability and off-target effects of heparin [5]. Bivalirudin (Angi-

omax, The Medicines Company, NJ, USA) is an injectable DTI that has been approved as an alternative to heparin for anticoagulation during PCI [1,2]. Large randomized trials have shown that bivalirudin is associated with similar antithrombotic efficacy but less bleeding than a combination of heparin and a glycoprotein IIb–IIIa inhibitor [6–8].

Yet, the clinical community has questioned the true efficacy–safety balance of bivalirudin in light of recent studies showing that bivalirudin use was associated with greater ischemic events but similar bleeding as compared with heparin [9]. The scientific community constantly seeks more efficacious and safer injectable anticoagulants. Previously, we isolated the variegin peptide from salivary gland extracts of the tropical bont tick (*Amblyomma variegatum*). Variegin, a 32-residue peptide, inhibits thrombin more potently than bivalirudin by sevenfold ($K_i = 146.4 \pm 13.6$ pM) [10]. Further-

Norrapat Shih¹, Leonardo Pinto de Carvalho², Yie Hou Lee³, Mauricio Macario Rocha⁴, Adriano Henrique Pereira Barbosa⁴, José Marconi A de Sousa⁴, Antonio Carlos de C Carvalho⁴, R Manjunatha Kini¹ & Mark Y Chan^{*,2}

¹Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Block S2 Level 2, Singapore 117543, Singapore

²Cardiac Department, National University Hospital, Cardiovascular Research Institute (CVRI), National University Heart Centre (NUHCS), National University Health System, 14 Medical Drive, MD6 #08–01, Singapore 117599, Singapore

³KK Research Centre, KK Women's & Children's Hospital, 100 Bukit Timah Road, Singapore 229899, Singapore

⁴Cardiac Department, Federal University of Sao Paulo State (UNIFESP), 715 Napoleao de Barros, Sao Paulo 04024–002, Brazil

*Author for correspondence:
Tel.: +65 677 25538
mark_chan@nuhs.edu.sg

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more, variegin shows high selectivity for thrombin when screened against many serine proteases, and is able to prolong time to occlusion in a zebrafish larvae venous thrombosis model [10,11]. Our plan is to develop this peptide into an injectable anticoagulant drug. To this end, animal studies and human trials will require PK and bioanalytic assays of the peptide.

Despite DTIs having a more predictable PK profile than heparin, it is increasingly recognized that monitoring of DTI treatment with a bioanalytic assay may be useful in situations such as abnormal clearance, severe bleeding, nonadherence and drug overdose [12]. Traditionally, ELISA and radioisotope assays are employed for PK studies of peptides and proteins, as opposed to LC–MS/MS assays [13]. This is mainly because ELISA and radioisotope assays can achieve higher sensitivities and require far lower equipment cost than LC–MS/MS assays. However, both ELISA and radioisotope assays suffer from several disadvantages, such as the risk of cross-reactivity to structurally similar peptides or metabolites for ELISAs, and the inability to distinguish between intact and degraded peptides for radioisotope assays [14]. Furthermore, ELISAs require development of specific, high-affinity antibodies, which is time-consuming and sometimes impossible. More recently, LC–MS/MS is increasingly becoming the platform of choice for bioanalytic assays of peptides. This change is due to increased selectivity of analyte quantitation, a possibility for internal standardization and shorter method development time. As the cost of equipment decreases and sensitivity of instruments increases, mass spectrometers may increasingly be adopted by hospitals for bioanalytical monitoring of therapeutics [15,16].

Apart from LC–MS/MS, activity-based assays such as chromogenic substrate assays and coagulation assays have also been developed for biomonitoring and PK analysis of therapeutics, particularly antithrombotic drugs [17]. Some examples include the Hemoclot® (Aniara, Inc., OH, USA) dilute thrombin time assay for dabigatran and anti-Xa activity assay for heparin, which can be used to determine the plasma concentration of drugs [18,19]. Compared with LC–MS assays, these activity-based assays have the advantage of being suitable for use by nonspecialist laboratories and configuration into point-of-care tests.

In this study, we have developed the LC–MS/MS, thrombin amidolytic (chromogenic substrate) and modified thrombin time assays for variegin quantitation, and successfully implemented these assays for PK studies of the peptide in pigs. These assays can be used for both nonclinical and clinical trials of variegin.

Material & methods

Modified thrombin time assay

Plasma (50 μ l) was mixed with 50 μ l of 50 mM Tris-Cl pH 7.4 in a 96-well microtiter plate and incubated at 37°C for 5 min. Human α -thrombin (diluted in 1 mg/ml of bovine serum albumin [BSA] and 50 mM Tris-Cl, pH 7.4) was preincubated at 37°C for 5 min before 50 μ l of this solution was added to the microtiter plate. The plate was shaken inside a microplate spectrophotometer (Tecan Sunrise™, Tecan, Männedorf, Switzerland) for 10 s to mix the solution before the clotting reaction was monitored at absorbance 650 nm up to 6 h. The formation of a fibrin-based clot will result in increased light absorbance at 650 nm. Thrombin time (time required for reaction to reach 50% maximal absorbance increase) was measured.

Thrombin amidolytic assay

Sample preparation was optimized as described by Jiang *et al.* [20]. Briefly, 50 μ l of plasma was treated with 2.5 μ l of 1 M HCl and incubated at 65°C for 5 min, after which 2.5 μ l of 1 M NaOH was added and centrifuged at 17,000 $\times g$ for 2 min. A specified amount of extracted plasma sample (supernatant) was incubated with 100 μ l of human α -thrombin (diluted in 1 mg/ml of BSA and 100 mM Tris-Cl, pH 8.5) for 5 min, prior to the addition of 100 μ l of S-2238™ thrombin amidolytic substrate (Chromogenix, Milan, Italy). The reaction was monitored using a microplate spectrophotometer (Tecan Sunrise) at absorbance 405 nm for 10 min, and the initial enzyme velocity in each reaction was measured.

LC–MS/MS assay

Porcine plasma (200 μ l) was spiked with 1750 μ l of distilled water, 40 μ l of 10% trifluoroacetic acid and 10 μ l of internal standard (IS) working solution (10 μ g/ml). This solution was then loaded onto a preconditioned Varian Bond Elut C18 SPE cartridge (200 mg; Agilent, CA, USA), washed with 4 ml of 20% acetonitrile/0.2% trifluoroacetic acid and subsequently eluted using 4 ml of 50% acetonitrile/0.2% trifluoroacetic acid. The elution was lyophilized and stored at -80°C. Prior to LC–MS/MS analysis, the lyophilized elution was resuspended in 100 μ l of 15% acetonitrile/0.1% formic acid, and 20 μ l was introduced into the mass spectrometer.

The LC–MS/MS analysis was performed on an Agilent 6490 triple quadrupole mass spectrometer coupled to an Agilent 1290 infinity HPLC pump. Chromatographic separation was performed using an Accucore RP-MS column (2.1 $\times$ 50 mm; 2.6 μ m) with a security guard (2.1 $\times$ 10 mm; 2.6 μ m), and column temperature was maintained at 40°C. MS was conducted on an Agilent 6490 triple quadrupole mass spectrometer with

an ESI interface in positive ion mode. SRM was used for detection of the precursor–product ion transitions of variegin and its stable isotope-labeled IS at m/z 903.1 > 838.5 and 905.2 > 841.1, respectively. A variegin peptide labeled with heavy isotopes $^{13}\text{C}_6$, $^{15}\text{N}_2$ on Lys13 was used as the IS (Figure 1A). Source parameters were as follows: gas temperature, 150°C; gas flow, 14 l/min; nebulizer gas, 40 psi; sheath gas temperature, 400°C; sheath gas flow, 8 l/min; capillary voltage, 4000 V; and nozzle voltage, 2000 V. Parameters for collision-induced dissociation (CID) were as follows: collision energy voltage, 20 V; cell accelerator voltage, 2 V; and fragmentor voltage, 380 V.

Method validation

Intraday and interday accuracy and precision of the method were estimated by analyzing six replicates of samples at different concentrations on the same day and on three consecutive days. Sample sizes were based on regulations stipulated for bioanalytic assays [21]. The assay accuracy was calculated as (observed concentration - spiked concentration)/(spiked concentration) $\times$ 100%. The assay precision for each concentration level was determined as the coefficient of variance of the measured concentrations. Acceptance criteria for accuracy and precision were 85–115 and $\pm$ 15% of the nominal concentrations, respectively. Extraction recovery was evaluated by comparing the peak area responses of variegin in six replicates of samples at three different concentration levels with peak area of variegin added to postextracted blank plasma at equivalent concentrations. The matrix effect was evaluated by comparing the peak area responses of variegin in processed blank plasma extract with variegin present in 15% acetonitrile and 0.1% formic acid at equivalent concentrations.

Analyte stability

The stability of variegin in plasma and whole blood anticoagulated with 3.2% sodium citrate was evaluated by analyzing replicates ($n = 6$) of spiked plasma or whole blood samples that were exposed to different conditions over time. Stability in whole blood was investigated up to 8 h. Stability in plasma with or without a protease inhibitor was investigated up to 24 h. Stability in plasma with or without lyophilization was investigated up to 1 month. Stability in plasma at -80°C was investigated up to 2 months. At different time points during the investigation period, samples were processed for LC–MS/MS.

Porcine PK studies

Five male pigs (*Sus domesticus*; 23–27 kg) were supplied by UNIFESP (Sao Paulo, Brazil). PK stud-

ies were approved by the Federal University of Sao Paulo IACUC committee (Protocol Number: N6001300114). Pigs were selected due to the availability of larger volume blood draws and its previous use in preclinical PK/pharmacodynamic studies [22,23]. Variegin (ChinaPeptides Co., Ltd, Shanghai, China) was dissolved in saline (0.9% sodium chloride) prior to administration to animals. Pigs were anesthetized and placed under mechanical ventilation. A longitudinal incision was made over the right neck region, and a segment of the jugular vein was exposed via blunt dissection. A 6F triple lumen catheter was introduced and secured inside the jugular vein for drug administration and blood collection. Baseline blood sample (10 ml) was collected prior to drug administration. Variegin (1.11 mg/kg) was administered to pigs by intravenous injection. Subsequently, 10 ml of blood samples was collected through the jugular vein catheter at the following time points after drug administration: 1, 3, 7, 10, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min. Blood samples were centrifuged at $3000 \times g$ for 15 min at room temperature for plasma isolation. Plasma samples (supernatant) were lyophilized and stored at -80°C. Prior to performing the LC–MS/MS, thrombin amidolytic and modified thrombin time assays, the lyophilized plasma samples were reconstituted with distilled water.

For evaluation of PK parameters, the plasma concentration–time profiles were subjected to a noncompartmental analysis using the linear-trapezoidal rule on the WinNonlin Professional software (WinNonlin, Version 4.0.1, Pharsight Corporation, CA, USA). The linear regression fit of the terminal data points on the plasma concentration (log scale) versus time profile was used to calculate the slope or elimination rate constant (k). The elimination half-life ($t_{1/2}$) was calculated as $0.693/k$. Volume of distribution (V_d) was calculated as dose/C_0 , where C_0 is the initial plasma concentration. Mean residence time (MRT) was calculated as the area under the first moment time curve ($\text{AUMC}_{0-\infty}$) divided by $\text{AUC}_{0-\infty}$. Clearance (Cl) was calculated as $\text{dose}/\text{AUC}_{0-\infty}$. The linear trapezoidal rule was used to calculate AUC up to the last measurable time point ($\text{AUC}_{0-4\text{ h}}$) or to infinity ($\text{AUC}_{0-\infty}$) by $\text{AUC}_{0-t} + C_t/k$, where C_t is the last measurable drug concentration.

Data analysis

Results are presented as the mean $\pm$ standard deviation. For analyte stability and comparison of PK parameters, Student's t -tests were used to decide if the differences among the means were statistically significant and $p < 0.05$ constituted the threshold for significance.

Figure 1. Development of an LC-MS/MS method for variegain quantitation. (A) Sequence of variegain peptide. IS variegain peptide was labeled with heavy isotopes $^{13}\text{C}_6$, $^{15}\text{N}_2$ on Lys13 (emboldened and underlined). (B) The MS spectrum of variegain in positive ion mode. (C) MS/MS spectra of variegain in positive ion mode with CID at 903.1 m/z. (D) The MS spectrum of variegain-IS in positive ion mode. (E) MS/MS spectra of variegain-IS in positive ion mode with CID at 905.2 m/z. (F) SRM chromatogram of variegain and variegain-IS in plasma. (G) Calibration curve of variegain in plasma ($n = 6$) with internal standardization. Each data point represents mean $\pm$ SD. CID: Collision-induced dissociation; IS: Internal standard; SD: Standard deviation; Variegain-IS: Internal standard variegain peptide labeled with heavy isotopes $^{13}\text{C}_6$, $^{15}\text{N}_2$ on Lys13.

Materials

Human α -thrombin (95% purity) was from Haematologic Technologies, Inc. (VT, USA). Tris, HCl, NaOH, LC-MS grade formic acid and BSA were from Sigma-Aldrich. Chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNA.2 HCl) was from Chromogenix. Varian bond elut C18 SPE cartridge (200 mg) was from Agilent Technologies (CA, USA). LC-MS grade acetonitrile and Accucore RP-MS column was from Thermo Fisher Scientific (MA, USA). Trifluoro-

acetic acid was from Tokyo Chemical Industries (Tokyo, Japan).

Results

LC-MS/MS assay: direct quantitation of variegain by SRM

The $[M + 4H]^{4+}$ ions at m/z 903.1 and 905.2 were used as the precursor ions for variegain and variegain-IS (internal standard variegain peptide labeled with heavy isotopes $^{13}\text{C}_6$, $^{15}\text{N}_2$ on Lys13), respectively

(Figure 1A, B & D). CID of variegin at m/z 903.1 yielded noticeable product ions at m/z 800.6, 838.5, 1096.5 and 1256.8 (Figure 1C). The highest intensity product ion at m/z 838.5 [b_{16} -NH₃²⁺] was selected for variegin quantitation, since it displayed the highest sensitivity for peptide quantitation in plasma. Therefore, the transitions used were m/z 903.1 > 838.5 and 905.2 > 841.1 for variegin and variegin-IS, respectively.

The chromatographic separation was achieved using a short (~8 min) and shallow separation gradient on an Accucore RP-MS (2.1 × 50 mm; 2.6 μm) with a security guard (2.1 × 10 mm; 2.6 μm). The standard curve obtained using the optimized SRM method has an LLOD of 2.7 nM, and is linear in the range of 21.6–5541.7 nM in plasma with a correlation coefficient of $r^2 = 0.9998$ (Figure 1G).

For extraction of variegin from plasma, a Varian C18 SPE cartridge was used. Although recovery was more than 85% using this extraction method, there was a relatively high matrix effect from co-extracted plasma proteins (Supplementary Table 1). Despite this, Supplementary Table 1 shows that the method was able to quantify variegin in plasma with an interday and intraday accuracy between -11.9 and 1.4% and -3.4 and 4.7%, respectively, with the corresponding precisions in the ranges of 5.6–8.2% and 1.6–5.0%, respectively (Supplementary Table 1). These accuracy and precision values were well within regulatory limits stipulated for bioanalytic assays [21].

Modified thrombin time assay: functional assay based on coagulation time

This method is based on clotting of citrate-anticoagulated plasma induced by addition of excess thrombin, without the need for sample extraction. This reaction can be monitored at absorbance 650 nm using a microplate spectrophotometer. Thrombin time is the time taken following addition of excess thrombin until half of the maximal absorbance increase is reached (Figure 2A). Plasma variegin concentration can be inferred from the thrombin time of the plasma clotting reaction. Due to the limited linear range of each enzymatic assay, four assays were optimized with varying concentrations of thrombin to achieve the desired quantitation range. The first assay containing 7.2 NIH U/ml thrombin had a linear range of 692.7–5541.7 nM of variegin in plasma (Figure 2B). The second assay containing 1.0 NIH U/ml thrombin had a linear range of 173.2–1385.4 nM of variegin in plasma (Figure 2C). The third assay containing 0.15 NIH U/ml thrombin had a linear range of 21.6–173.2 nM of variegin in plasma (Figure 2D). Last, the fourth assay containing 0.037 NIH U/ml thrombin had a linear range of 5.4–43.3 nM of variegin in plasma (Figure 2E).

No sample extraction was required, and the collected plasma can be directly analyzed using this assay. Furthermore, the method was able to quantify variegin in plasma with an interday and intraday accuracy and precision within accepted limits (Supplementary Table 2) [21]. For the assay containing 7.2 NIH U/ml thrombin, the interday and intraday accuracy was between -0.42 and 4.12% and -1.61 to 5.13%, respectively, with the corresponding precision in the ranges of 6.05–11.2 and 5.89–11.44%, respectively. For the assay containing 1.0 NIH U/ml thrombin, the interday and intraday accuracy was between -11.07 to 4.34% and -4.75 to 14.11%, respectively, with the corresponding precision in the ranges of 2.15–5.71 and 1.63–4.17%, respectively. For the assay containing 0.15 NIH U/ml thrombin, the interday and intraday accuracy was between -8.37 to 13.46% and -4.57 to 5.56%, respectively, with the corresponding precision in the ranges of 2.45–9.87 and 1.25–5.68, respectively. Last, for the assay containing 0.037 NIH U/ml thrombin, the interday and intraday accuracy was between -5.60 to 15.63% and -12.58 to -2.99%, respectively, with the corresponding precision in the ranges of 2.20–5.15 and 3.63–6.68%, respectively.

Thrombin amidolytic assay: functional assay using chromogenic substrate

The thrombin amidolytic assay is reliant on the S2238 chromogenic thrombin substrate that releases a product detectable at 405 nm upon thrombin cleavage. Variegin in the extracted plasma will inhibit thrombin in the assay such that the plasma variegin concentration can be deduced from the enzymatic velocity (Figure 3A). Due to the limited linear range of each enzymatic assay, several assays were optimized with varying concentrations of thrombin, substrate and extracted-plasma sample to achieve the desired quantitation range. More specifically, the assay containing 3.6 NIH U/ml thrombin, 800 μM S2238 and 1 μl extracted-plasma sample had a linear range of 692.7–5541.7 nM of variegin in plasma (Figure 3B). Another assay containing 0.45 NIH U/ml thrombin, 150 μM S2238 and 2 μl extracted-plasma sample had a linear range of 86.6–346.4 nM of variegin in plasma (Figure 3C). The final assay containing 0.45 NIH U/ml thrombin, 150 μM S2238 and 16 μl extracted-plasma sample had a linear range of 10.8–43.3 of variegin in plasma (Figure 3D).

Using an acid-based extraction method, the plasma recovery rate was between 74.9 and 96.3% from the LLOQ to ULOQ, respectively (Supplementary Table 3). For the assay containing 3.6 NIH U/ml thrombin, 800 μM S2238 and 1 μl extracted-plasma sample, the interday and intraday

accuracy was between -3.52 to 9.34% and -13.43 to 7.16%, respectively, with the corresponding precision in the ranges of 1.03–6.85 and 1.09–7.10%, respectively. For another assay containing 0.45 NIH U/ml thrombin, 150 μ M S2238 and 2 μ l extracted-plasma sample, the interday and intraday accuracy was between 7.85–42.43% and -0.85 to 11.17%, respectively, with the corresponding precision in the ranges of 3.85–12.27 and 5.72–17.93%, respectively. As for the final assay containing 0.45 NIH U/ml thrombin, 150 μ M S2238 and 16 μ l extracted-plasma sample, the interday and intraday accuracy was between 6.15–28.97 and -7.03 to 7.54%, respectively, with the corresponding precision in the ranges 0.061–9.12 and 0.43–9.51%, respectively. Although the intraday accuracy, intraday and interday precision were within the acceptable limits, this assay had relatively high variability in the interday accuracy, especially when measuring the lower variegins concentrations of the assay. To this end, a standard curve was run alongside every assay for accurate quantitation.

Analyte stability

Variegins stability was investigated in both whole blood and plasma anticoagulated with 3.2% sodium citrate under different storage conditions over time and quan-

tified using the developed LC–MS/MS assay. Incubation of variegins in citrate-anticoagulated whole blood at room temperature resulted in a significant decrease in the amount of peptide over the 8-h incubation period (Figure 4A). However, variegins was stable in citrate-anticoagulated plasma with or without a protease inhibitor for up to 24 h (Figure 4B). Thereafter, a significant decrease in the amount of peptide was apparent. By incubating the peptide in lyophilized citrate-anticoagulated plasma, the stability can be prolonged for up to 2 weeks (Figure 4C). To further prolong the stability of peptide in the citrate-anticoagulated plasma samples, the peptide was incubated in citrate-anticoagulated plasma and stored at -80°C for up to 2 months, where the variegins peptide was stable throughout this period (Figure 4D).

Porcine PK studies

The plasma concentration–time curve of variegins, obtained using the LC–MS/MS, modified thrombin time and thrombin amidolytic assays, all showed a biexponential profile with a rapid initial decrease in plasma concentration followed by a slower elimination phase (Figure 5). The elimination half-lives obtained using the LC–MS/MS, modified thrombin time and thrombin amidolytic assays were 52.3 ± 4.4 , 50.4 ± 5.9 and 67.7 ± 6.3 min, respectively (mean $\pm$ standard deviation; Table 1).

Figure 2. Modified thrombin time assay for variegins quantitation in plasma. (A) Representative absorbance curve of plasma clotting reaction following excess thrombin addition. Thrombin time was taken as the time required to reach half of the maximal absorbance increase from baseline. Calibration curves for variegins plasma concentration using assays containing (B) 7.2, (C) 1.0, (D) 0.15 and (E) 0.037 NIH U/ml thrombin in assay ($n = 6$). Each data point represents mean $\pm$ SD. SD: Standard deviation.

Figure 3. Thrombin amidolytic quantitation assay for variegin in plasma. (A) Representative absorbance curve for extracted-plasma sample with low versus high variegin concentration. Enzyme reaction was monitored at absorbance 405 nm. Calibration curves for variegin plasma concentration ($n = 6$): (B) enzyme assay with 3.6 NIH U/ml thrombin, 800 μ M S2238 and 1 μ l extracted-plasma sample; (C) enzyme assay with 0.45 NIH U/ml thrombin, 150 μ M S2238 and 2 μ l extracted-plasma sample; and (D) enzyme assay with 0.45 NIH U/ml thrombin, 150 μ M S2238 and 16 μ l extracted-plasma sample. Each data point represents mean $\pm$ SD. SD: Standard deviation.

For comparison of the three bioanalytical assays, the variegin plasma concentrations obtained from each of the methods were plotted against each other (Supplementary Figure 1). The comparison graph between variegin plasma concentrations obtained from the LC–MS/MS and modified thrombin time assays resulted in a slope gradient of 1.0694 (Supplementary Figure 1A). In contrast, the comparison graphs between variegin plasma concentrations obtained from the LC–MS/MS and thrombin amidolytic assays, and from the modified thrombin time and thrombin amidolytic assays, resulted in slope gradients of 0.7689 and 0.6978, respectively (Supplementary Figure 1B & C).

Apart from this, the PK parameters from the porcine PK studies obtained using the three bioanalytical assays were compared using a t-test, and p-values are listed in Table 2. All the PK parameters obtained using the both the LC–MS/MS and modified thrombin tie assays showed no significant difference, with calculated p-values above 0.05. In contrast, the comparison between the LC–MS/MS assay and thrombin amidolytic assay, and between the modified thrombin time assay and thrombin amidolytic assay, resulted in PK parameters that were significantly different from each other, with p-values below 0.05.

Discussion

In comparison with the thrombin amidolytic and modified thrombin time assays, the LC–MS/MS assay has a wider linear quantitation range and more favorable intraday and interday accuracy and precision, which is mostly due to the internal standardization using the heavy-isotope-labeled variegin peptide.

Recent studies suggest that, compared with heparin, bivalirudin is associated with a slightly increased risk of stent thrombosis in the first few hours after PCI [9]. The greater use of glycoprotein IIb–IIIa inhibitors in the heparin comparator arm has also been raised as possible reason for the lower bleeding rates observed with bivalirudin in earlier clinical trials [24]. Moreover, as compared with heparin, which is administered as a single bolus injection, bivalirudin must be continuously infused, a logistic disadvantage during emergency procedures.

LC–MS/MS is widely used for bioanalysis of small molecule therapeutics, and is becoming increasingly used for peptide quantitation for PK studies. The SPE method used for sample preparation was, however, relatively time-consuming and resulted in a high matrix ion suppression effect, due to the similar stationary-phase chemistry for both the SPE cartridge and LC column. Further development of this method may include using

Figure 4. Sample processing stability of variegins in citrated plasma and whole blood. Samples were processed after specified incubation time and variegins quantified by LC-MS/MS ($n = 6$). (A) Variegins stability in citrated whole blood at room temperature up to 8 h. (B) Variegins stability in citrated plasma at room temperature with or without a protease inhibitor up to 24 h. (C) Variegins stability in lyophilized and nonlyophilized plasma at room temperature up to 1 month. (D) Variegins stability in citrated plasma at -80°C up to 2 months. Each bar represents mean $\pm$ SD. Data were tested using the Student's t -test, where $* p < 0.05$ in comparison with 0 day or 0 h. SD: Standard deviation.

Figure 5. Plasma concentration–time profiles of variegin. Variegin (1.11 mg/kg) was administered intravenously to pigs ($n = 5$) and variegin plasma concentration was quantified using (A) LC–MS/MS, (B) modified thrombin time and (C) thrombin amidolytic assays from pig plasma collected postadministration. Each data point represents mean $\pm$ SD. SD: Standard deviation.

an ion-exchange SPE cartridge and reverse-phase LC column which will allow for orthogonal separation of the analyte from the matrix [25,26].

By using LC–MS/MS to study the analyte stability, it was discovered that the amount of variegin rapidly decreased in the presence of whole blood. This could potentially be due to absorption or degradation by blood cells. We therefore immediately isolated plasma by centrifugation following blood collection from animals. When incubated in citrate-anticoagulated plasma, the peptide was stable at room temperature for up to a day. This stability is extended up to a week if the plasma is lyophilized, and up to at least 2 months if kept at -80°C .

The thrombin amidolytic assay is reliant on the S2238 chromogenic thrombin substrate, which releases a *p*-nitroaniline product detectable at 405 nm upon thrombin cleavage. Plasma variegin concentration can be deduced from the enzymatic velocity. Initially, we attempted to use neat plasma in the assay. However, this resulted in not only coagulation when adding thrombin but also a high background absorbance

signal. Therefore, we decided to use an acid-based extraction method following the protocol described by Jiang *et al.* [20], which provided a relatively fast (<20 min) extraction method with good recovery and a significantly reduced background absorbance and higher sensitivity. Despite this, the assay had an inter-assay accuracy value outside the accepted limits (>15% accuracy), which necessitated the use of an intra-assay standard curve on each assay run.

The modified thrombin time assay is based on the commonly used thrombin clotting time test. The advantage of this assay as compared with the LC–MS/MS and thrombin amidolytic assays is the ability to use neat plasma, avoiding the need for any sample extraction procedure. However, to extend the linear quantitation range, the assay is measured for up to 6 h after thrombin addition. For future use as a point-of-care coagulation time assay, less time, and thus smaller linear range, can be used when the therapeutic plasma concentration has been established.

Both the modified thrombin time and thrombin amidolytic assays have the advantage of determining

Table 1. Pharmacokinetic parameters of variegins determined using LC–MS/MS, modified thrombin time and thrombin amidolytic assays.

Pharmacokinetic parameters	LC–MS/MS assay	Modified thrombin time assay	Thrombin amidolytic assay
Elimination rate constant, k (min^{-1})	0.0133 ± 0.012	0.0139 ± 0.0017	0.0103 ± 0.00098
Elimination half-life, $t_{1/2}$ (min)	52.3 ± 4.4	50.4 ± 5.9	67.7 ± 6.3
Volume of distribution, V_d (ml/kg)	725.2 ± 53.8	741.7 ± 100.3	471.0 ± 61.9
Mean residence time, MRT (min)	43.2 ± 4.5	42.8 ± 3.7	56.3 ± 5.5
Clearance, Cl (ml/min/kg)	9.6 ± 0.5	10.2 ± 1.1	4.9 ± 0.8
Area under curve, $\text{AUC}_{0-4\text{h}}$ (ng/ml min)	$111,536.1 \pm 5255.7$	$106,819.2 \pm 12,196.7$	$214,057.8 \pm 34,678.7$
Area under curve, $\text{AUC}_{0-\infty}$ (ng/ml min)	$115,527.3 \pm 5800.5$	$109,333.7 \pm 12,193.4$	$234,215.5 \pm 46,627.3$

the anticoagulant activity of variegins. Furthermore, the activity results in the form of thrombin time (coagulation time), and initial velocity has also been calibrated to variegins concentration in plasma samples. Therefore, the modified thrombin time and thrombin amidolytic assays are able to estimate both PK and pharmacodynamic activities.

For porcine PK studies, the dose of 1 mg/kg variegins was chosen based on preliminary studies showing that this dose of variegins can effectively prevent stent thrombosis (data not shown). From the PK analysis, the peptide had a relatively large apparent distribution volume suggesting distribution and accumulation in peripheral tissues. This may be explained by previous observation showing accumulation of radiolabeled variegins in kidneys of rats [NUTTALL PA, UNPUBLISHED DATA]. The parameters obtained using the LC–MS/MS and modified thrombin time assays were similar. In comparison, the PK parameters obtained using the thrombin amidolytic assay had a longer elimination half-life, larger AUC value and a lower volume of distribution.

From the comparison graphs among the three bioanalytical assays, the comparison graph of

LC–MS/MS assay and modified thrombin time assay resulted in a slope gradient closest to 1. This suggests that there is a high correlation between variegins plasma concentrations obtained from both these assays. Furthermore, the LC–MS/MS assay and modified thrombin time assay resulted in the most congruent PK parameters. In contrast, comparison of PK parameters obtained using the LC–MS/MS assay or modified thrombin time assay to the thrombin amidolytic assay resulted in significantly different parameters. Using the LC–MS/MS assay as a reference standard, the modified thrombin time assay can give more accurate results in PK studies compared with the thrombin amidolytic assay.

In comparison with the LC–MS/MS and modified thrombin time assays, the difference in PK parameters and variegins plasma concentrations obtained using the thrombin amidolytic assay could be explained by the presence of partially degraded variegins peptide from the plasma samples. These partially degraded fragments of variegins, not detectable by the LC–MS/MS assay, can be co-extracted from the plasma sample and still inhibit thrombin in the amidolytic assay. These fragments could also be present in the modified thrombin time assay, but

Table 2. p-values from comparisons of pharmacokinetic parameters obtained using LC–MS/MS, modified thrombin time and thrombin amidolytic assays.

Pharmacokinetic parameters	LC–MS/MS assay vs modified thrombin time assay	LC–MS/MS assay vs thrombin amidolytic assay	Modified thrombin time assay vs thrombin amidolytic assay
Elimination rate constant, k (min^{-1})	0.5940	0.0023	0.0101
Elimination half-life, $t_{1/2}$ (min)	0.6127	0.0010	0.0059
Volume of distribution, V_d (ml/kg)	0.7660	0.0013	0.0192
Mean residence time, MRT (min)	0.8932	0.0016	0.0036
Clearance, Cl (ml/min/kg)	0.3170	0.0001	0.0005
Area under curve, $\text{AUC}_{0-4\text{h}}$ (ng/ml min)	0.4633	0.0001	0.0006
Area under curve, $\text{AUC}_{0-\infty}$ (ng/ml min)	0.3561	0.0001	0.0005

Pharmacokinetic parameters were compared using the Student's t-test where $p < 0.05$ constituted the threshold for significance.

cannot compete with the fibrinogen substrate and other plasma components for binding to thrombin. However, in the thrombin amidolytic assay, with a lower concentration of co-extracted plasma proteins, these fragments could compete more easily for binding to thrombin.

Plasma samples from the porcine PK study was lyophilized and stored at -80°C . Although plasma samples were lyophilized, when resuspended in distilled water to the original volume prior to measurement, the pH was 7.4. Furthermore, in early preliminary experiments, plasma that was resuspended in distilled water resulted in similar coagulation times to fresh nonlyophilized plasma in the activated partial thromboplastin time, prothrombin time and thrombin clotting time assays (data not shown). Even though variegin showed responsiveness in these standard coagulation assays, we did not use these assays for the porcine PK study. This is because the focus of this study was to develop specific bioanalytical assays for variegin quantitation, and not to perform basic coagulation profile screening for variegin activity.

A limitation of this study is the lack of clinical data to support the utility of these bioanalytical assays for guiding variegin usage in the clinic. Although these assays have been validated using the porcine PK studies, they will need to be further optimized and validated in a clinical setting when therapeutic and toxic levels of the drug are known.

Conclusion

In this comparison of bioanalytical assays for variegin, peptide-based anticoagulant, thrombin time

was superior to the thrombin amidolytic assay, when compared with LC–MS/MS assay as the reference method. Following calibration of the thrombin time assay to LC–MS/MS-measured PK concentrations, the thrombin time assay is well suited to the development of clotting time assays already in clinical use. However, with increasing adoption of MS-based PK measurements in clinical laboratories, already and reality in highly specialized clinical chemistry laboratories, the LC–MS/MS assay may eventually become the preferred bioanalytical assay for peptide-based anticoagulants [27].

Future perspective

With the increasing development of new anticoagulants, new bioanalytical assays must be developed for drug testing and point-of-care monitoring. Current standard coagulation assays (e.g., prothrombin time, thrombin clotting time and activated partial thromboplastin time) have a limited quantitation range and do not directly measure drug levels. Although enzyme-based bioanalytical assays will continue to be used in clinics due to their ease of use by nonspecialist laboratories, as the cost, reliability and physical footprint of mass spectrometers are decreasing. We expect increased use of MS for accurate bioanalytical monitoring of therapeutics, presently only a reality in highly specialized clinical chemistry laboratories. Future work to assess the efficacy and safety of variegin in preclinical models of thrombosis and bleeding is necessary before consideration for human clinical trials.

Executive summary

Background

- To develop bioanalytical point-of-care assays for monitoring of the novel anticoagulant peptide, variegin.
- Implementation of these bioanalytical assays for pharmacokinetic (PK) studies of variegin.

Experimentation

- Development and validation of an LC–MS/MS, modified thrombin time and thrombin amidolytic assays for variegin quantitation.
- Utilization of the bioanalytical assays for PK studies of variegin in pigs.

Results & discussion

- The LC–MS/MS, thrombin amidolytic and modified thrombin time assays had a quantitation range of 21.6–5541.7, 10.8–5541.7 and 5.4–5541.7 nM in human plasma, respectively.
- Method validation shows that the LC–MS/MS and modified thrombin time assays had an intraday and interday precision and accuracy within regulatory limits stipulated by the US FDA.
- The concentration–time profile of variegin can be fitted to a two-compartment model. The elimination half-lives obtained using the LC–MS/MS, modified thrombin time and thrombin amidolytic assays were 52.3 ± 4.4 , 50.4 ± 5.9 and 67.7 ± 6.3 min, respectively.
- Comparisons of PK parameters and variegin plasma concentrations obtained using the LC–MS/MS and modified thrombin time assays showed high correlation.

Conclusion

- The modified thrombin time assays can be used for accurate quantitation of variegin plasma concentrations following calibration with known plasma concentrations of variegin measured by LC–MS/MS in a large animal PK model.

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Ethical conduct of research

Pharmacokinetics studies on pigs were approved by the Federal University of Sao Paulo IACUC committee (Protocol Number: N6001300114).

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