

# A performance evaluation of a novel human recombinant tissue factor prothrombin time reagent (Revohem™ PT)

Chris Gardiner<sup>1</sup>, Kiyoko Kohama<sup>2</sup>, Irfan Patel<sup>1,3</sup>, Philip Lane<sup>1</sup>, Steve Dwyer<sup>4</sup>, Sam J Machin<sup>1</sup>, Ian J Mackie<sup>1</sup>

<sup>1</sup> University College London, London, UK. <sup>2</sup> Sysmex Corporation, Kobe, Japan, <sup>3</sup> Sysmex UK, Milton Keynes, UK. Sidra Medical and Research Center, Doha, Qatar

## Corresponding author

Chris Gardiner. Haemostasis Research Unit, Research Department of Haematology, Cancer Institute, University College London, 1<sup>st</sup> Floor, 51 Chenies Mews, London, United Kingdom, WC1E 6HX

Email: [c.gardiner@ucl.ac.uk](mailto:c.gardiner@ucl.ac.uk)

## **Abstract**

### **Introduction**

A new prothrombin time reagent (Revohem™ PT) based on recombinant human tissue factor produced by the silkworm-baculovirus expression system was tested. The aim of this study was to compare the performance of the new PT reagent with two widely used routine PT reagents.

### **Methods**

All testing was performed on a Sysmex CS-5100 coagulometer. Revohem™ PT was tested for imprecision and stability using normal and abnormal lyophilised commercial control plasmas. Comparability was assessed with two widely used reagents; one containing recombinant human tissue factor (Reagent A) and the other a human placental thromboplastin (Reagent B) using a wide range of normal and abnormal plasmas and analyser specific ISI values.

### **Results**

Excellent between-day imprecision was obtained for Revohem™ PT (CV < 1.0%) and acceptable open vial on-board stability over 7-days. There was good agreement between methods in samples from patients with liver disease and patients receiving warfarin and no significant differences between methods with increasing INR values. Both recombinant reagents suffered less interference from lupus anticoagulant than the placental thromboplastin. Revohem™ PT had similar sensitivity to reagents A and B for FII, V, VII and X deficiency; and demonstrated dose responsiveness to Dabigatran, Apixaban and Rivaroxaban with steeper response curves than the comparison reagents

### **Conclusions**

Revohem™ PT showed comparable or improved performance relative to two widely used reagents and is suitable for use in warfarin control, detection of inherited factor II, V, VII and X deficiency and assessment of liver disease coagulopathy.

## **Introduction**

The prothrombin time (PT) is widely used for monitoring vitamin K antagonist anticoagulant therapy. It is also used and as a screening test for single or combined deficiencies of the extrinsic coagulation pathway due to inherited defects or acquired defects due to liver disease . Currently there is considerable interest in the sensitivity of PT reagents to direct oral anticoagulants (DOAC) because DOAC might interfere in screening for coagulopathies in patients bleeding where the anticoagulation status of the patient is unknown, and in determining whether a patient is still anticoagulated (e.g. prior to surgery). Recombinant thromboplastins, using recombinant tissue factor (generated in bacteria) and synthetic phospholipids are sensitive PT reagent which overcome much of the lot to lot availability associated with thromboplastins prepared from traditional biological sources . Insect systems are widely used to produce proteins from higher eukaryotes because they have a similar pattern of glycosylation, phosphorylation, and protein processing. We report on the performance of a novel prothrombin time reagent (Revohem™ PT, Sysmex Corporation, Kobe, Japan) which utilises human recombinant tissue factor produced by the silkworm-baculovirus expression system and synthetic phospholipids. Revohem™ PT was tested for imprecision, stability and comparability to two widely used PT reagents (Reagent A and Reagent B) in a wide range of sample types.

## **Materials and methods**

Blood samples taken into 0.105M sodium citrate (Vacutainer, Becton Dickinson, Oxford, UK) from 100 patients receiving warfarin anticoagulation, 26 with known lupus anticoagulant and 45 with liver disease were obtained from residual, anonymised samples collected after all routine testing had been completed, in compliance with local ethical committee rules and the Human Tissue Act. All testing was performed on a Sysmex CS-5100 coagulometer (Sysmex Corporation). Revohem™ PT is a lyophilised reagent containing recombinant human tissue factor, synthetic phospholipids, calcium ions, a heparin neutralizing compound, amino acids, HEPES and preservatives. The comparison reagents were a recombinant human thromboplastin (Reagent A) and a lyophilized human placental thromboplastin (Reagent B). ISI assignment and INR calibration were performed using certified reference plasmas (AK calibrant, Technoclone, Vienna, Austria) on 5 different days as previously described . Imprecision and reagent stability were assessed by testing normal and abnormal control plasmas (Dade® Ci-Trol® levels 1 and 2, Siemens Healthcare, Marburg, Germany).

Normal reference ranges were derived using citrated plasma collected locally from 90 apparently normal healthy volunteers and 50 commercially sourced normal plasmas (CRYOcheck™ Normal Donor Set, Precision BioLogic Inc. Dartmouth, Canada). Informed consent was obtained from normal donors (approved by the UCL Research Ethics Committee. Project ID Number: 7029/001).

Sensitivity to factor deficiencies was assessed by testing immunodepleted plasmas deficient in factor II, factor V, factor VII and factor X (CRYOcheck, Precision BioLogic or Hyphen Biomed immune-depleted deficient plasma) supplemented with normal reference plasma (CRYOcheck, Precision BioLogic or locally prepared pooled normal plasma) to achieve concentrations of approximately 10%, 20%, 30%, 40%, 50%, 60%, 70% and 80% of normal. In addition, plasma from eleven patients with congenital factor V deficiencies (6 FV, 2 FVII and 3 FX) were also tested. The factor II, V, VII and X levels were verified by one-stage PT-based assays using multiple dilution analysis and fibrinogen levels by Clauss assay, using reagents from Siemens Healthcare. Heparin sensitivity was assessed by spiking normal plasma with unfractionated and low molecular weight heparin at 0.1, 0.2, 0.4, 1.0 and 1.5 IU/mL. Sensitivity to direct oral anticoagulants was assessed by testing plasma from patients receiving Rivaroxaban, Apixaban and Dabigatran where available, in addition to normal plasma samples spiked with drug, plasma controls and calibrators (Hyphen Biomed, Neuville-sur-Oise, France) to achieve a range of concentrations.

Statistical analysis was performed on Excel (Microsoft, WA, USA) and Minitab 17 (State College, PA, USA). Parametric statistics were used unless otherwise stated. Correlation plots were made using Pearson's coefficient of correlation. A probability value of  $p < 0.05$  was considered statistically significant. Normal reference ranges were defined by the mean  $\pm 2$  standard deviations of log transformed data.

## Results

Locally assigned ISI values were 1.00 for Revohem™ PT, 1.02 for Reagent A and 1.07 for Reagent B. Revohem™ PT demonstrated < 1% imprecision with both normal and abnormal control plasmas over 5 days (Table 1). To assess reagent stability, freshly reconstituted normal and abnormal controls were tested at day 0, day 4, day 7 and day 8 using an open 8 mL vial of Revohem™ PT which was stored on-board the analyser. After 7 days, the PT measurements for the normal and abnormal control plasmas had decreased by 4.1% and 5.4% relative to day 0. Normal reference ranges and geometric mean PTs were established in 140 normal plasmas (Table 2).

Comparability was assessed in 100 plasma samples from patients receiving warfarin using the local INR calibration. Revohem™ PT demonstrated good correlation with Reagent A (Figure 1a) with no significant differences between methods with increasing INR values for samples within the therapeutic range, although Reagent A appeared to give higher INR values above the therapeutic range (Figure 1b). A difference of more than 0.5 INR units was observed in 10 samples (6 higher by

Reagent A and 4 higher by Revohem™ PT), but 7 of these samples were from patients with an INR >4.5 using Reagent A. Revohem™ PT also showed good correlation with Reagent B (Figure 1c), again with no significant differences between methods and no discernible trend with increasing INR values for samples within the therapeutic range (Figure 1d). The two comparison reagents demonstrated good correlation (Figure 1e) although Reagent A appeared to give slightly higher INRs above the therapeutic range (Figure 1f).

Figure 1a

Figure 1b

Figure 1c

Figure 1d.

Figure 1e

Figure 1f

Sensitivity to lupus anticoagulant was assessed in 26 patients with lupus anticoagulant tested according to the most recent ISTH criteria. All had positive dilute Russell viper venom ratios and/or Taipan/Ecarin ratios. As all 26 patients were also receiving warfarin, equal volume mixes of these plasmas with pooled normal plasma were also tested. Excellent correlation was obtained between Reagent A and Revohem™ PT (Figure 2a), with an average difference of -0.14. Only two samples showed a difference of more than 0.5 INR units. Reagent B and Revohem™ PT also showed excellent correlation (Figure 2c) but Reagent B gave significantly higher INR values (difference 0.34,  $p < 0.0001$ ) with 14 samples showing a difference of more than 0.5 INR units. The two comparison reagents demonstrated excellent correlation (Figure 2c) but Reagent B again gave significantly higher INR values (difference 0.32,  $p < 0.0001$ ).

The effect of haemostatic abnormalities secondary to liver disease was studied in 45 patients with a median MELD score of 15 (range 9 – 32). Good correlation was observed between reagents (Figures 3d-e). A small but statistically significant difference between Revohem™ PT and Reagent A (Wilcoxon signed rank  $p = 0.008$ ) was observed but this difference was not clinically significant (all <0.5 PT ratio).

Figure 2a

Figure 2b

Figure 2c

Figure 2d

Figure 2e

Figure 2f

The sensitivity for specific factor deficiencies was determined by plotting the factor level against PT (Figures 3a –d) to determine the highest concentration of a given factor to produce prolongation of the PT above the upper limit of the normal reference range. Revohem™ PT demonstrated similar sensitivity to factors II, V, VII and X as the two comparison reagents (Table 3). All deficient plasmas had normal levels of the non-depleted extrinsic factors (0.80 – 1.20 IU/mL for II, V, VII and X) and fibrinogen (2.5 -3.0 g/L). Five plasmas from patients deficient in factor V (<0.01, 0.08, 0.19, 0.18 and 0.33 IU/dL), two plasmas from patients deficient in factor VII (0.12 and 0.24 IU/dL) and three plasmas from patients deficient in factor X (0.03, 0.04 and 0.21 IU/dL) all had prolonged PT with all three reagents. Both Revohem™ PT and Reagent A were insensitive to unfractionated and low molecular weight heparin levels of up to 1.5 IU/mL (i.e. < 5% increase above the PT of untreated plasma, whereas Reagent B suffered interference with unfractionated heparin at 1 IU/mL (data not shown). Revohem™ PT was more sensitive to Dabigatran, Rivaroxaban and Apixaban than Reagent A or Reagent B (figure 3) which demonstrated similar sensitivities to those previously reported .

Figure 3a

Figure 3b

Figure 3c

Figure 3d

Figure 3e

Figure 3f

Figure 3g

## Discussion

We report on the performance of the first PT reagent to use human recombinant tissue factor generated by the silkworm-baculovirus expression system Revohem™ PT was compared to two

widely used PT reagents. Revohem™ PT demonstrated acceptable levels of imprecision and open vial stability. Excellent comparability of INR values was achieved within the therapeutic range in plasma from patients receiving warfarin and of PT ratios in plasma from patients with liver disease, and there were no significant trends with increasing INR. Since it has been suggested that some PT reagents give unreliable INR values in the presence of lupus anticoagulant, 26 plasma samples with lupus anticoagulant and equal volume mixes of the same plasmas with pooled normal plasma were tested by all three methods. Revohem™ PT suffered less interference from lupus anticoagulant than the placental thromboplastin (Reagent B) and had similar sensitivity to reagent A.

The comparison reagents were less sensitive to reduction in FII than to reductions in FV, FVII and FX as previously reported. The sensitivity of Revohem™ PT to FV, FVII and FX was similar to that of the comparison reagents. Reagent B was slightly more sensitive to FII than Revohem™ PT or Reagent A. The prothrombin time is known to have limited utility for the detection of mild clotting factor deficiencies in the extrinsic pathway. It should be emphasised that the prothrombin time is a global test, which is influenced by the levels of all clotting factors within a spiked deficient plasma, not just the clotting factor that has been adjusted. Although guidance is available on the assessment of coagulation factor sensitivity of the APTT no clear information has been provided for PT, but it is generally assumed that similar mixing studies may be informative. The factor sensitivity data and ISI determination both indicate that Revohem PT is highly sensitive to factors II, V, VII and X. The apparent oversensitivity to factors V, VII and X may be explained by the inherent problems with error associated with mixing tests, including problems with mixing plasmas from different sources, the impact of any buffers and stabilisers that a manufacturer may have added (in both PT reagents and plasmas), as well as differences in citrate concentration. In the case of factor V, due to its thermal lability, it is also possible that there is some loss of FV activity during the course of the experiment, so that actual FV levels were lower than those predicted. It has been suggested that the use of well characterised patient samples with a single deficiency of each extrinsic factor (covering the range of desired sensitivity; 30-50%) might offer a better approach than mixing (spiking) experiments, but such samples are difficult to obtain in suitable numbers.

The sensitivity of PT reagents to DOACs is highly variable, so plasma samples from patients receiving Rivaroxaban and Dabigatran, standards and controls and normal plasma spiked with Dabigatran, Apixaban and Rivaroxaban at a range of concentrations were tested by all three methods. The comparison reagents were relatively insensitive to Rivaroxaban and Apixaban as previously reported. Revohem™ PT demonstrated dose responsiveness to all three DOACs with steeper response curves than the comparison reagents. It has been reported that the use of drug calibrators may over-

estimate the sensitivity of PT reagents to DOACs and our data from patients receiving Rivaroxaban (Figure 3) support this. Unfortunately, it was not possible to obtain sufficient samples from patients receiving Dabigatran or Apixaban to compare with drug spiked plasmas. Although the PT should not be used to determine the plasma concentration of DOACs, it is important that laboratories know the sensitivity of its PT reagent to them .

In conclusion, Revohem™ PT showed comparable or improved performance relative to two widely used PT reagents and is suitable for use in the control of warfarin, detection of inherited factor II, V, VII and X deficiency and assessment of coagulopathy in liver disease.

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Table 1. 5-day imprecision. Ten measurements were performed on each control plasma each day.

	Normal plasma			Abnormal plasma		
	Revohem™ PT	Reagent A	Reagent B	Revohem™ PT	Reagent A	Reagent B
Mean PT (s)	12.4	10.6	11.4	36.1	31.4	39.4
SD	0.10	0.08	0.15	0.30	0.49	0.49
% CV	0.81	0.78	1.30	0.83	1.55	1.24

Table 2. Reference ranges from 140 normal subjects

	Revohem™ PT	Reagent A	Reagent B
Mean PT (s)	11.36	10.86	12.35
mean – 2SD	9.89	9.48	10.46
Mean + 2SD	13.06	12.43	14.57

Table 3. Sensitivity to clotting factor deficiencies as determined by the highest factor concentration at which the PT was prolonged.

	Factor II IU/dL	Factor V IU/dL	Factor VII IU/dL	Factor X IU/dL
Revohem™ PT	24	67	52	59
Reagent A	28	59	47	52
Reagent B	48	67	47	58

## Figure legends

Figure 1. INR values in 100 plasmas from patients receiving warfarin (circles) and 140 plasmas from normal donors (crosses). The regression lines were generated using only plasma from patients receiving warfarin with INR values <4.5 (circles), INR values >4.5 (diamonds). a) Reagent A vs Revohem™ PT, b) Bland Altman, mean difference for +0.04 INR units; c) Reagent B vs Revohem™ PT. d) Bland Altman, mean difference +0.09 INR units (One outlier not shown); e) Reagent A vs Reagent B. f) Bland Altman, mean difference -0.09 INR units (One outlier not shown).

Figure 2. INR values in 26 patients with lupus anticoagulant receiving warfarin (circles) and equal volume mixes of the same plasmas (crosses). a) Reagent A vs Revohem™ PT. b) Reagent B vs Revohem™. c) Reagent A vs Reagent B. PT ratios in 45 patients with liver disease: e) Reagent A vs Revohem™ PT, e) Reagent B vs Revohem™, f) Reagent A vs Reagent B

Figure 3. The effect of single factor deficiency was determined in patient plasma (open symbols) and immunodepleted plasmas spiked with pooled normal plasma (closed symbols): a) factor II, b) factor V, c) factor VII, d) factor X. The effect of DOACs on prothrombin time was assessed using patient plasma (open symbols) and normal plasma spiked with drug including calibrators and controls (closed symbols) e) Dabigatran, f) Apixaban and e) Rivaroxaban