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Validation and implementation of drug-dependent antibody assays in clinical trials for safety monitoring of patients dosed with roxifiban, an orally bioavailable glycoprotein IIb/IIIa antagonist

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Abstract

Thrombocytopenia exposes patients to increased bleeding risk. This serious adverse event was observed with a frequency of approximately 2% in early clinical trials with the potent, orally bioavailable glycoprotein (GP) IIb/IIIa receptor antagonist roxifiban. We previously reported that drug-dependent antibodies (DDAbs) to GP IIb/IIIa are the main cause of thrombocytopenia with roxifiban. Two ELISA assays for detection of free DDAbs (in citrate plasma) and total DDAbs (in EDTA plasma to elute platelet bound DDAbs) were developed and analytically validated. These tests served two purposes during the clinical development program, to pre-screen patients for pre-existing antibodies and monitor patients for increasing antibody titers as a surrogate for eminent thrombocytopenia. The free DDAb assay showed inter and intra-assay precision of 5–12 and 12–14% CV, respectively. The total DDAb assay showed a precision of 5–10 and 4–12% CV, respectively. Three cycles of freeze–thaw did not significantly alter DDAb values in citrate plasma, EDTA plasma or extraction solution. The clinical qualifications of the two assays were conducted in two phase II clinical trials in coronary arterial disease (CAD) patients dosed with roxifiban. Both assays have demonstrated clinical sensitivity of nearly 99–100% and clinical specificity of nearly 95%.

Keywords: Immunogenicity; Drug-dependent antibody assay; Anti-drug antibody assay; Validation; Roxifiban; Differential ELISA

1. Introduction

Roxifiban (DMP 754) is an orally bioavailable ester prodrug that undergoes hydrolysis and produces a metabolite, XV459 that is readily detectable in plasma [1]. XV459, a potent antagonist of the platelet glycoprotein (GP) IIb/IIIa receptor, blocks the binding of fibrinogen to GP IIb/IIIa, thus inhibits the final common pathway of platelet activation [2]. Roxifiban belongs to a class of anti-thrombotic drugs that are commonly used for treatment of unstable angina, specifically during percutaneous coronary interventions, in patients with coronary arterial diseases (CAD). One of the relatively common side effects of this class of agents is thrombocytopenia. Thrombocytopenia is associated with an unfavorable clinical outcome. Patients dosed with Abciximab, an approved humanized monoclonal antibody drug that is specific for GP IIb/IIIa and the vitronectin receptor alpha$_v$ beta$_3$, present with a 1–2% occurrences of thrombocytopenia upon initial administration [2]. Similarly, an approximately 2% thrombocytopenia rate was observed during roxifiban early phase II clinical trial. The cause of the thrombocytopenia associated with roxifiban dosing were drug-dependent antibodies (DDAbs) to GP IIb/IIIa, mediating platelet activation and/or platelet clearance of immunoglobulin loaded platelets [3–4]. XV 459 induces a conformational change in GP IIb/IIIa, and this conformational change is detected by the DDAbs. In the absence of 'offending' drug, no thrombocytopenia is observed even in the presence of high circulating levels of DDAbs, pointing to the exquisite drug dependency of the antibodies [3–5].

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A screening assay to detect XV459-dependent DDAbs was therefore proposed for excluding patients from dosing with roxifiban based on pre-existing antibodies or during the initial dosing period to detect rapidly developing antibody titers. Overall, these strategies significantly reduced the overall frequency of thrombocytopenia [4]. GP IIb/IIIa is a conformational labile molecule and EDTA treatment dissociates platelet bound antibodies when incubated at 37 °C. The DDAbs present in EDTA heat-treated plasma are considered "total" DDAb, which included both platelet bound and circulating antibodies. In contrast, DDAbs in citrate plasma are considered unbound to platelet or "free". Both assays were validated analytically and implemented in clinical trials so that their clinical sensitivity and specificity could be evaluated.

2. Experimental

2.1. Material

Non-fat dry milk (Blotto) was purchased from BioRad (Hercules, CA), Tetramethylbenzadine (TMB) Peroxidase Substrate/Hydrogen Peroxide (H_2O_2) System was from Pierce (Rockford, IL), Tween 20 was from Sigma (St. Louis, MO), H_2SO_4, concentrated (18 M) was from Fisher Chemicals (Fairlawn, NJ), Dulbecco's Phosphate Buffer Saline containing Ca and Mg (PBS) was from Invitrogen (Calsbad, CA), anti-human IgG + HRP (secondary antibody) was from Kirkegaard & Perry (Gathersburg, MD). Normal goat serum (NGS) was from Rockland (Gillbertsville, PA). The 1.5 ml microfuge tubes containing 50 mg C18 material were obtained from Orochem (Lombard, IL). Hirudin (Refludan, 50 mg/vial) was obtained from a local pharmacy.

XP280 (N^3-[2-{3-(4-formamidino-phenyl)-isoxazolin-5(*R*)-yl}-acetyl]-N^2-(1-butyloxycarbonyl)-2,3-(*S*)-diaminopropionate) is an active form of the ester prodrug roxifiban and a different salt form of XV459, the main active metabolite of roxifiban formed in vivo. Dup714 (MW 497) is a proprietary thrombin inhibitor. All chemicals were synthesized at DuPont Pharmaceuticals Company (Wilmington, DE). IgG depleted GP IIb/IIIa receptor was isolated from expired platelets then purified by affinity chromatography at DuPont Pharmaceuticals Company as described [5]. rJK094, developed at DuPont Pharmaceuticals, was an IgG4 recombinant monoclonal antibody with high affinity (kd ~ 0.3 nM) and selectivity for GP IIb/IIIa/XP280 complex as determined by BIAcore [6]. Clones 2 and 12 were modified in the antigen recognition sequences by site-directed mutagenesis, both developed at DuPont Pharmaceuticals Company. Plasma samples from individual donors, IH14, IH15, IH18 were collected at DuPont Pharmaceuticals Company after obtaining written consents. Negative human plasma pools in sodium citrate and EDTA were from Biochemed Pharmacologicals (Winchester, VA).

The 96 well, flat bottom, medium binding microtiter plates were from Costar (Corning Life Science, Acton, MA), ELISA plate readers, spectra max and thermomax, equipped with Softmax® Pro software V. 2.3.1, were from Molecular Devices (Sunnyvale, CA), automated ELISA multi reagent plate washers (MRW) were from Dynatech Laboratories (Chantile, VA).

2.2. Immunoassay reagent preparation

Coating buffer, 2.5 μg/ml GP IIb/IIIa receptor in PBS with or without 100 nM XP280, wash buffer, 0.05% Tween 20 in PBS with or without 100 nM XP280, stock solutions of 2 μM of the thrombin inhibitor Dup714 in deionized water and 20 μM hirudin in PBS were all prepared fresh daily. Assay buffer, 0.1% Blotto + 0.05% Tween 20 in PBS, blocking buffer, assay buffer + 1% NGS were prepared and stored at 4 °C until use. The secondary antibody (HRP conjugated human IgG) solution was prepared at 1/5000 dilution in assay buffer with 100 nM XP280 immediately before use.

The ELISA plates were coated within 20 min of preparation of the coating buffer. Half of a plate (six columns) was prepared in the absence, the other half in the presence of 100 nM XP280. The plate was incubated at 4 °C for 14–30 h. The half plate that was coated with GP IIb/IIIa in the presence of XP280 was then blocked and washed with buffer containing XP280 and vise versa. All plates were stored at 4 °C and used within 5 days of preparation.

2.3. Assay procedures

2.3.1. Free (citrate) DDAb assay

On the day of assay, plates were washed three times, blotted on paper towels and blocked for 60 min at 37 °C. Samples and quality controls (QCs) were thawed in a 37 °C water batch and then immediately transferred to ice. The final concentrations of 10 nM thrombin inhibitor Dup714 (or 100 nM hirudin) was added to the plasma samples prior to 1/10 dilution in blocking buffer unless otherwise stated. One hundred microlitres of study sample were added in each of the six wells on a plate, three in the half of the plate with and three in the half of the plate without XP280. The plate was then incubated at room temperature on a shaker table for 1 h before the solution was aspirated and the plate was washed three times. The plate was blotted dry as above and 100 μL of diluted secondary antibody solution was added to all wells and the plate was incubated for another 1 h at room temperature on a shaker table. The plate was washed three times again and blotted dry. Immediately after the addition of TMB/H_2O_2 mixture, the plate was read at 650 nm in kinetic mode at 12 s interval for 5 min with maximal OD set at 0.2, and temperature at 23 °C. Final results were reported as the difference in velocity with and without XP280 present (delta, mOD/min).

$$\text{Delta (mOD/min)} = (\text{mOD/min with XP280}) - (\text{mOD/min without XP280})$$

2.3.2. Total (EDTA) DDAb assay

The total DDAb assay in EDTA plasma was the same as described in free DDAb assay in citrate plasma except the following.

The buffer used to dilute samples and QCs was the blocking buffer containing 1.5 mM $CaCl_2$. All QC samples were added with 48 nM XP280 to mimic the patient samples before processing. Study samples were thawed then centrifuged at 1000 × *g* for 2 min before final concentrations of 10 nM Dup714 and 0.3% citrate were added to aliquots of the supernatant to keep consistency with that of the free DDAb assay.

XV459 in EDTA plasma samples was removed using a 1.5-ml microfuge tube containing 50 mg C18 material. The procedure was as followed: C18 material was activated with 500 μL methanol two times before it was equilibrated two times with blocking buffer, followed by the addition of 500 μL of plasma samples. Plasma sample was mixed with C18 material end-over-end for 15 min at room temperature, followed by centrifugation at 2500 × *g* for 5 min. The supernatant was collected and diluted 1/10 with diluting buffer before placed in an ELISA plate.

Both total and free DDAb assays were performed at Covance Laboratories, Vienna, VA.

2.4. Validation/quality control (QC) sample preparation

2.4.1. Recombinant antibody samples

Recombinant antibody rJK094 was prepared using hybridoma technology by immunizing mice with GPIIb/IIIa-XP280 complex [6]. After screening in the differential ELISA, one clone was selected and the cDNAs encoding the antibody were isolated. Subsequently, the constant IgG region was replaced with human IgG4 to enable detection by anti-human Ig antibodies. The antibody was expressed in CHO cells and isolated by protein G affinity chromatography from the conditioned media using standard procedures. In addition, sub-clones with variations in the variable region were prepared to obtain antibodies with different affinities for the XP280-induced conformational changes in GP IIb/IIIa. The antibody stock concentrations were approximately 350 μg/ml. The validation/QC samples were prepared by spiking rRJ094 in 1% Blotto in PBS to a final concentration of 4, 2, 1, 0.5 and 0 ng/ml. The slope of the linear regression was used to measure the reproducibility of the assays over the range of interest.

2.4.2. Human plasma validation/QC samples

Four types samples have been observed previously. (1) *High background negative samples*: Subject samples that have high readings both with and without XP280, and the net difference (delta value) are less than the cut point. (2) *High background positive samples*: Subject samples that have high readings both with and without XP280, and the delta value are greater than the cut point. (3) *Low background negative samples*: Subject samples that have low readings both with and without XP280 and the delta value are less than the cut point. (4) *Low background positive samples*: Subject samples that have low readings without XP280 and high readings with XP280 and the delta value are greater than the cut point. The typical low background plasma samples (without XP280) had kinetic readings of 10–30 mOD/min while the high background plasma samples had readings greater than 50 mOD/min. The exact cause of the high background reading in drug naïve patient is not clearly known, but it is possible that the presence of drug independent antibodies to GP IIb/IIIa played a role. Since drug independent antibody did not appear to have pathophysiological relevance [4], we did not characterize them further.

The validation/QC samples were to mimic the anticipated clinical situation as closely as possible. Thus, the following validation/QC samples were prepared.

2.4.2.1. Negative samples. IH15 is a low background, negative donor plasma sample without additional manipulation. IH18 is a high background, negative donor plasma sample without additional manipulation.

2.4.2.2. Positive samples. IH14 is a low background, positive donor plasma sample without additional manipulation. IH14 was used as a positive QC sample for subsequent clinical trials.

IH14:IH15 was generated by mixing IH14 and IH15 1:1 to produce a low background, moderately positive sample. IH14:IH18 was generated by mixing IH14 and IH18 1:1 to produce a high background, moderate positive sample.

2.4.2.3. Other negative and matrix controls. A commercially available, negative human citrate or EDTA plasma pools was used as part of the plate negative control.

All donors contributed to validation/QC samples were free of roxifiban. The health statuses of these donors were unknown.

2.5. Analytical validation

The analytical validation exercises were conducted using the plasma validation/QC samples as described above. Each sample was measured in three wells with and three wells without XP280 on the same plate, unless otherwise stated. Five samples with rJK094 concentrations of 4, 2, 1, 0.5 and 0 ng/ml in buffer and the pooled negative plasma were included in each assay plate to evaluate the ELISA reagent validity and variability. The information was later used to adjust individual plate cut point. All statistical analyses were performed on the final reported results (delta).

2.5.1. Precision (inter-plate and intra-plate variability)

Inter-plate precision was assessed using 10 individual ELISA plates for the free DDAb assay and 11 individual plates for the total DDAb assay. The plasma validation/QC samples were measured three times per plate in triplicates. Total nine wells with and nine wells without XP280 on the same plate. Due to the space limitation, only two different validation/QC samples were measured on one plate.

The intra-plate precision was assessed using four individual plates for the free DDAb assay and eight individual plates for the total DDAb assay. One of the four plasma validation/QC sample (e.g., IH14) was assayed six different times on a plate in triplicates, with and without XP280. Total 18 wells with and 18 wells without XP280.

2.5.2. Recovery

DDAb recovery from matrix was evaluated by spiking IH14, a positive plasma samples, into two different types of negative donor plasma, IH15 and IH18. IH15 was a low background human plasma and IH18 was a high background plasma.

2.5.3. Freeze–thaw (FT) stability

DDAb freeze–thaw stability was assessed using two EDTA plasma samples, IH14, and IH14:IH15, and three citrate plasma samples, IH14, IH14:IH15 and IH14:IH18. Aliquots of the samples were frozen at <−15 °C then thawed at 37 °C for one, two and three times. Each types of sample (e.g., IH14) were assayed on the same plate. All samples were measured in triplicates, with and without XP280. One set of samples (IH14 and IH14:IH15) that were extracted with C18 in EDTA assay was also tested for extract stability.

2.5.4. Dilution linearity and dilution parallelism

Dilution linearity was performed using rJK094 spiked in IH14, IH15 and IH18 donor plasma. Samples were diluted at 1 part sample + 3 parts buffer (1/4 dilution, 25% plasma), 2 parts sample + 3 parts buffer (1/2.5 dilution, 40% plasma), 1 part sample + 1 part buffer (1/2 dilution, 50% plasma, 2 parts sample + 1 part buffer (1/1.5 dilution, 67% plasma) and 3 part sample + 1 part buffer (1/1.3 dilution, 75% plasma).

Dilution parallelism was assessed using incurred samples obtained from two thrombocytopenia patients who had developed high levels of DDAb. Each plasma sample was diluted with buffer at neat, 1/4, 1/16, and 1/64 and analyzed on the same plate.

2.5.5. Storage stability

The assay was intended to be used as a quick turn-around safety test. A 48-h or less result reporting time was required. No long-term storage stability of samples was therefore assessed.

2.6. Clinical study conduct

2.6.1. QC selection

Since there were no DDAB positive and clinically relevant samples available in large quantity to control a clinical trial that would last 4–12 months, we selected IH14, an endogenous positive sample to serve as the primary QC for assay acceptance. The recombinant monoclonal antibody, rJK094, which had renewable supply, but exhibited somewhat different assay characteristics than human DDAb, such as dilution linearity, were used to help control the assay conditions. Five rJK094 concentrations that yielded delta ranged from negative to near cut point were assayed on each plate. The slope of the linear fit was used as a QC parameter. A near cut point sample was prepared by spiking rJK094 in human plasma. This sample was used to monitor the assay and reagent variations.

2.6.2. Minimal dilution

A minimal dilution of 1/20 was initially used in a free DDAb assay to minimize the matrix interferences in DMP754-010 clinical trial. Subsequently, an attempt was made to improve assay analytical signal read out to capture thrombocytopenia patients' antibody at an earlier time point. A minimal dilution of 1/10 was then instituted in both total DDAb assay and free DDAb assay. Assays were re-validated and cut points were re-determined for the new minimal dilution.

2.6.3. Cut point determination

The initial assay cut point for the free DDAb assay was determined by parametric method in the validation exercise using an upper limit of 95% obtained from 105 healthy volunteers participating in a phase I clinical study [7,8]. The 105 subjects were administered roxifiban and they were free of adverse events at the time of sample collection.

This differential ELISA was used in one phase I, two phase II and one phase III clinical studies with subjects ranging from healthy volunteers, coronary artery disease patients and peripheral artery disease (PAD) patients. For each clinical study, the cut point was re-evaluated and re-established based on prior study experience, assay performances and tolerable risk factors, including false positive and false negative rates.

2.6.4. Cut point normalization

During a phase II clinical study, an individual plate cut point was obtained by calibrating the assay cut point with the delta of a control plasma sample and the slope of rJK094 of that plate as follows: the control plasma delta reading and the slope of rJK094 of a particular plate were compared to the respective mean values obtained from the validation. The two ratios were multiplied with the established assay cut point and the lower of the two cut point was used as that plate's cut point in order to minimize false negatives. For example, the assay cut point for total DDAb assay was set as delta of 73 based on the negative patients results obtained in the validation prior to the clinical trial. The assay slope was set as 12.4 from the mean slope of inter-day measurements of the rJK094 linear fit in the validation. The assay delta for IH14 was set as 106 from the mean inter-day measurement of IH14 in the validation. If a plate in the clinical trial had rJK094 slope of 11, IH14 delta of 91, then the two normalized factors for that plate would be 11/12.4 = 0.89 (slope) and 91/106 = 0.86 (IH14). The plate cut point would be determined by normalization of the assay cut point using smaller of the two normalization factors, which would be $0.86 \times 73 = 63$. In that plate, if a sample had a delta of 70, it would be reported as 70/63 = 111% (positive). If a sample had a delta of 55, it would be reported as 55/63 = 87% (negative).

2.6.5. Patient sample preparation

All clinical studies were approved by local Institutional Review Board (IRB) with written consents obtained from all patients participating in the clinical studies. Free DDAb samples were obtained by collecting human blood in 3.2% citrate tubes. After thorough mixing, plasma were separated by centrifugation at $1500 \times g$ for 15 min and stored at <−15 °C unless otherwise stated. Total DDAb samples were obtained by collecting blood in 15% EDTA tubes followed by incubation at 37 °C for 1 h on an end-over-end rota-

tor. Plasma was then separated and stored at <−15 °C until analysis.

Moderately to severely hemolyzed or lipimic clinical samples were not included in analysis due to possible interferences.

2.6.6. Outlier removal

A custom computer program was written and tested where data calculation was conducted automatically for each clinical sample for outlier removal. The outlier removal principle was as described below.

(1) Establish a critical data range as ±30% of assay cut point of that plate, e.g., if cut point is 73, then the critical data range is 73 × 0.7–73 × 1.3 = 51–95 delta.
(2) Evaluate all delta combinations obtained from three readings with drug − three background readings without drug.
   - If all delta combinations were outside the critical data range, then the final delta was considered not near cut point (either high positive, or negative). No outlier removal was performed.
   - If any of the delta combinations was within the critical data range (i.e., near cut point) and the triplicate wells (with or without drug) had CV >15%, then the one value associated with the largest difference amount the three intra-well difference may be removed.

2.6.7. Report of positives

A patient sample's delta value that was greater than or equal to the normalized cut point was reported as positive. The degree of positivity was calculated using the equation: %Positive = 100 × patient delta/normalized cut point delta. Values that were greater than or equal to 100% were considered as positive. Values that were less than 100% were considered as negative. All positive results were immediately repeated to confirm the finding. All patient results, regardless positive or negative, were faxed to clinical sites immediately after the analysis. Only patient who had "confirmed" positive results was excluded from the study.

3. Results

3.1. Analytical characteristics

3.1.1. Inter and intra-plate precision, recovery and assay sensitivity

3.1.1.1. Free DDAb assay. The intra-plate precision of the positive validation/QC samples, IH14, IH14:IH15 and IH14:H18, with delta values ranging from 60 to 144 mOD/min, were less than 13% CV. The negative sample, IH15, remained below cut point in all measurements. The DDAb analytical recoveries of IH14 in low and high background plasma were 96 and 83%, respectively (Table 1a).

The intra-plate precision of positive plasma validation/QC samples, IH14, IH14:IH15 and IH14:H18, were less than 15% CV and the negative samples, IH15, remained below cut point in all measurements (Table 1b). The rJK094 spiked samples, which have delta values ranging from 9 to 44 mOD/min, closer to the cut point of 35, showed intra-plate variations of 17–24%CV. The slope of the rJK094 curve had a CV of 22% over 23 assay runs (Table 1c).

Table 1
Intra-plate, inter-plate variation and recovery of the free DDAb assay

	Samples; citrate plasma DDAb delta (mOD/min)			
	IH14	IH14:IH15	IH14:IH18	IH15
(a) Intra-plate precision				
Mean	144	69.4	60	−0.8
S.D.	7.2	8.4	5.8	5.1
%CV	5	12	10	NA
%Recovery		96	83	
n	5	6	5	6

	Samples; citrate plasma DDAb delta (mOD/min)				
	IH14	IH14:IH15	IH14:IH18	IH15	Plasma blank
(b) Inter-plate precision					
Mean	138	78.8	80.6	6.2	−3.8
S.D.	20	9.7	9.3	3.3	5.5
%CV	14	12	12	NA	NA
%Recovery		114	117		
n	21	11	17	24	22

	Samples; rJK094 DDAb delta (mOD/min)					rJK094
Concentration	4 ng/ml	2 ng/ml	1 ng/ml	0.5 ng/ml	0 ng/ml	Slope
(c) Inter-plate precision						
Mean	43.9	25.6	14.2	8.7	0.3	10.7
S.D.	9.1	6.0	2.8	1.5	0.7	2.3
%CV	21	23	19	17	NA	22
N	21	16	22	23	23	23

IH14: Low background positive, IH15: low background negative, and IH18: high background negative plasma samples. IH14:IH15 and IH14:IH18 were mixtures of the two donor plasma samples. rJK094: A recombinant antibody to GP IIb/IIIa-Xp280 complex. NA: Not applicable.

The DDAb recoveries of IH14 in low and high background plasma were 114 and 117%, respectively.

3.1.1.2. Total DDAb assay. The intra-plate precision of the positive validation/QC samples, IH14, IH14:IH15 and IH14:IH18, with DDAb ranging from delta 56 to 101 mOD/min, were less than 15% CV. The negative sample, IH15, remained below cut point in all measurements. The DDAb recoveries of IH14 sample in low and high background plasma were 112 and 115%, respectively (Table 2a).

The intra-plate precision of positive plasma validation/QC samples, IH14, IH14:IH15 and IH14:IH18, were less than 13% CV and negative samples, IH15, remained below cut point in all measurements (Table 2b). The rJK094 spiked samples, which have delta values ranged from 8 to 51 mOD/min, lower than the cut point of 70, showed intra-plate variations of 12–20% CV. The slope of the rJK094 curve had a CV of 13% over 21 assay runs (Table 2c). The DDAb recoveries of IH14 sample in low and high background plasma were 114 and 106%, respectively (Table 2b).

Table 2
Intra-plate, inter-plate variation and recovery of the total DDAb assay

	Samples; EDTA plasma DDAb delta (mOD/min)			
	IH14	IH14:IH15	IH14:IH18	IH15
(a) Intra-plate precision and recovery				
Mean	101	56.6	58.2	4.5
S.D.	5.4	5	8	0.7
%CV	5	9	14	NA
%Recovery		112	115	
n	12	18	12	6

	Samples; EDTA plasma DDAb delta (mOD/min)				
	IH14	IH14:IH15	IH14:IH18	IH15	Plasma blank
(b) Inter-plate precision and recovery					
Mean	110	62.7	58.5	3.3	0.8
S.D.	4.1	7.7	6.5	3.4	3.5
%CV	4	12	11	NA	NA
%Recovery		114	106		
n	12	18	17	15	

	Samples; rJK094 DDAb delta (mOD/min)					rJK094
Concentration	4 ng/mL	2 ng/mL	1 ng/mL	0.5 ng/mL	0 ng/mL	Slope
(c) Intra-plate precision						
Mean	50.8	30.4	14.2	7.9	0.5	12.4
S.D.	6.3	4.3	2.7	1.5	0.7	1.6
%CV	12	14	19	19	NA	13
n	27	27	27	27	27	21

IH14: Low background positive, IH15: low background negative, and IH18: high background negative plasma samples. IH14:IH15 and IH14:IH18 were mixtures of the two donor plasma samples. rJK094: A recombinant antibody to GP IIb/IIIa-Xp280 complex. NA: Not applicable.

3.1.1.3. Estimated analytical sensitivity. The concentration of DDAb present in IH14 or other plasma samples could not be quantified due to the heterogeneity nature of the antibodies present in the samples. When tested rJK094 spiked in EDTA plasma, we were able to detect 60 ng/ml DDAb with reasonable precision (similar precision to that of high positive sample) (Table 3).

3.1.2. Freeze–thaw stability

For both citrate and EDTA samples, all replicate measurements of the positive samples remained highly positives with no significant loss in delta responses regardless of the freeze–thaw treatment. An ANOVA based mixed model statistical analysis was conducted on the mean of the technical replicates of each sample using SAS version 9.1 to test the null hypothesis between freeze–thaw cycles of 1–3. A *p*-value of 0.271, 0.987 and 0.091 were obtained for citrate plasma, EDTA plasma and extracted EDTA samples, respectively, suggesting no significant changes in DDAb values after repeated freezing and thawing.

All measurements of negative samples remained below cut point regardless of the freeze–thaw cycles. The overall variation of the IH15 was in agreement with the inter-assay precision. No freeze–thaw related change in delta was observed for negative samples including IH15.

Table 3
Inter-plate variation of the total DDAb assay used in the clinical study, DMP754-017

	Samples; DDAb delta (mOD/min)			
	Buffer blank	rJK094 slope	IH14 EDTA plasma	rJK094 spiked in EDTA plasma
Mean	6.64	13.2	114	43.0
S.D.	1.84	2.93	32.1	11.7
%CV	28.5	22.2	28.1	27.2
No. of plate	279	279	279	252

IH14: Low background positive donor plasma sample. rJK094: A recombinant antibody to GP IIb/IIIa-XP280 complex.

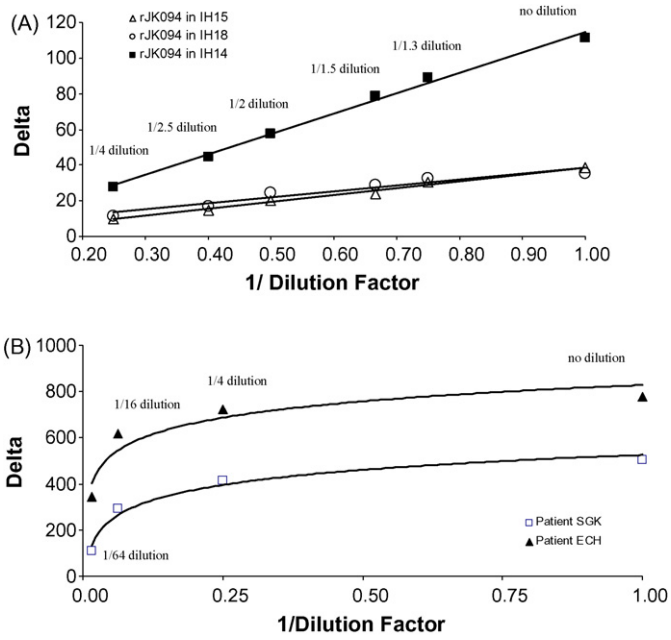

Fig. 1. Dilution parallelism. The dilution factors were the amount of dilutions applied in addition to minimal assay dilution. (A) Validation/QC samples, rJK094 in IH15, rJK094 in IH18 and rJK094 in IH14, were prepared by spiking rJK094 in low background donor IH5, high background donor IH18 and positive donor IH14 plasma samples. (B) Thrombocytopenia patient samples, SGK and ECH.

3.1.3. Dilution linearity and dilution parallelism

When compared to samples with plasma contents ranging from 25 to 75% of that of neat, rJK094 spiked in healthy donor plasma, IH15, IH18 and IH14, showed analytical recovery of 93–105%, 124–130% and 100–107%, respectively. This suggested that DDAb signals are linear in this dilution range (Fig. 1A).

The plasma samples from the two patients who tested DDAb positive and presented with thrombocytopenia, ECH and SGK, were also tested for dilution parallelism (Fig. 1B). A shallow delta signal decrease was observed with increasing dilution factor for both samples. For example, a 16-fold dilution resulted in a mere 42% decrease of DDAb in patient SGK (delta of 504 decreased to 293 mOD/min) and a 21% decrease in patient ECH (delta of 723 decreased to 617 mOD/min). Nevertheless, the shallower than linear dilution curve suggested that the thrombocytopenia patient plasma will likely have elevated DDAb values

even when higher minimal dilutions are used in the assay, a characteristic which may help prevent false negative caused by assay design.

As rJK094 did not appear to have the same dilution parallelism characteristics as that of thrombocytopenia patients, it was determined that the rJK094 samples should be used as quality controls for tracking assay condition variations rather than as standard curve.

3.1.4. Drug interference

EDTA induces a conformational change in GP IIb/IIIa, thereby displacing XP280 (or XV459) from the platelet surface. Consequently, the total roxifiban concentration in ETDA plasma was 8–10 times higher than that in citrate plasma and was sufficient to elicit a change in GP IIb/IIa conformation in wells without XP280 present, thus interfering with the differential assay. Steps of absorption of XV459 via C18 resin were implemented to reduce the drug interference in the differential ELISA. The drug concentrations in patient samples were less than 50 nM, thus the drug interference was evaluated by adding 0, 5, 10, 25, 50 and 75 nM of XP280 to IH14, IH15 and IH18 EDTA plasma samples. The deltas were measured and the negative plasma samples (IH15 and IH18) remained below cut point. IH14 sample showed a delta of 131 mOD/min in control sample (no drug added) and delta values ranged from 123 to 149 mOD/min in aliquots containing divergent amount of XP280, corresponding to 94–114% of that in control. No drug concentrations related changes were observed, suggesting the C18 absorption procedure reduced the XV459 concentration to a clinically non-significant range. It is possible that some antibodies were also lost during the extraction process although it is unlikely that the antibody would be depleted at the same rate as drug since the drug and the antibody were structurally very different.

3.1.5. Robustness

A total of 308 DDAb assays were conducted in DMP754-017 clinical trial by seven analysts (two shifts) over a 4 months period of time (Table 3). Two types of positive controls were evaluated in each assay, an IH14 high positive sample and an rJK094 spiked in plasma with a delta value near the cut point. A buffer blank and a slope generated by rJK094 standards spiked in buffer from this trial were also tabulated for precision evaluation. The critical reagent lots (e.g., TMB, coating GP IIb/IIIa receptor) were changed by necessity multiple times during the trial. Strict criteria were implemented to qualify each critical reagent lot. The end results showed a total assay failure rate of approximately 10% and the variation of the QC samples ranged from 22 to 28% CV. The individual QC (±2S.D.) performances over a 4 months period are presented in a Levey-Jennings chart (Fig. 2).

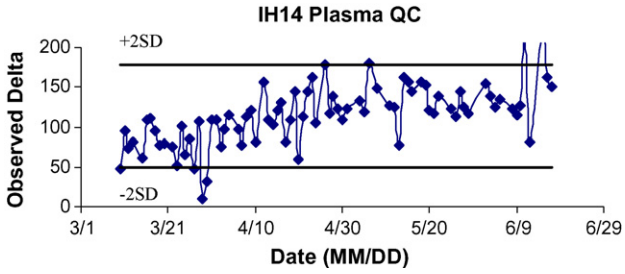

Fig. 2. IH14 QC measured in total DDAb assay during conduct of DMP754-017 clinical trial.

3.2. Cross validation

3.2.1. Dup714 and hirudin

As DDAb assay required a result turn-around time of less than 48 h, all samples from a clinical study were shipped frozen immediately after collection and the DDAb assay was performed upon receipt. After the assay was implemented in the first clinical protocol, we observed that a large number (>20%) of freshly frozen citrate samples (frozen for less than 24 h from collection) coagulated on ELISA plates. We attributed this observation to insufficient anticoagulation activity of citrate present in the samples. Dup714, an in house thrombin inhibitor, was thus added at a final concentration of 10 nM in plasma to reduce sample clotting. Because Dup714 was not commercially available and the supply was limited, we compared a commercially available biological thrombin inhibitor hirudin, with Dup714. A cross validation of hirudin (final concentration of 100 nM) and Dup714 were performed using the free DDAb method. Three positive samples, IH14 with delta ~140, clone 2 with delta ~60, clone 12 with delta ~50, and a negative sample, IH15, with delta of ~8 were added with one of the two anticoagulants. The tests were conducted on 3 different days with three assay runs per day. Duplicated Dup714 and hirudin containing samples were run side by side in each assay run. Hirudin samples showed similar inter-day and intra-day precisions as those treated with Dup714, and both were similar to results observed in the validation. The percentage difference in mean values of the three positive samples ranged from 4.5 to 10%. The negative sample, IH15 remained below the cut point regardless of the thrombin inhibitor added. A two way ANOVA was performed to test the null hypothesis between hirudin and Dup714 and a p-value of 0.4488 was obtained, suggesting that hirudin did not alter the DDAb assay performances established using Dup714.

3.3. Clinical qualification

3.3.1. Cut points

For the free DDAb assay with minimal dilution of 1/10, the cut point was determined as delta of 35. With 1/20 dilution, the cut point was determined as delta of 12.

The total DDAb assay with minimal dilution of 1/10, the cut point was determined as delta = 73.

3.3.2. Repeatability of positives

A total of 28 positive samples from 24 patients (some patients had samples taken on two different dates) were tested two times in two different assays within 24 h to confirm the positive results (Table 4). Twenty-two out of 28 (79%) specimens remained positive after the second assay. The mean percentage difference between the two numeric results was 15%. When compared the tested results obtained from assay 1 to that from assay 2

Table 4
Repeat analysis of positive samples from DMP754-017

Patient ID	%Positive		%Difference
	Result 1	Result 2	
JKL	269	248	8
WAS	348	360	−3
JFD	112	121	−8
RBO	780	539	37
W-B	227	264	−15
F-T	254	258	−2
MAV	248	48	135
M-L	439	409	7
JEB	532	1140	−73
P-L	225	160	34
JJB	114	48.9	80
F-F	238	158	40
I-C	118	150	−24
G-H	320	24.3	172
HSR	152	96	45
F-D	178	130	31
ASD	266	307	−14
ORM	138	130	6
ORM	138	224	−48
JEK	121	118	3
DRH	205	166	21
NRM	199	258	−26
NRM	112	77.6	36
J-K	265	332	−22
J-K	107	87.6	20
EEL	353	393	−11
GSK	148	130	13
GSK	270	326	−19
	Mean		15

Results were from Total DDAb assay. All results were reported as %positive. Result 1: the positive result obtained from initial analysis of a patient sample. Result 2: the repeat analysis for confirmation of positive result. %Difference = 100 × 2 × (Result 1 − Result 2)/(Result 1 + Result 2).

using paired *t*-test, a *p*-value of 0.83 was obtained, suggesting no significant differences between result 1 and result 2.

3.3.3. Clinical utility

Of 1332 patients who were pre-screened using results from citrate DDAb assay as exclusion criteria in a phase II clinical study (DMP754-010), 57 patients (4.3%) tested positive at pre-screen and were therefore excluded from the clinical trial, which is in accord with the upper negative limit of 95% used for cut point determination. Additional patients did not pass other inclusion criteria for the trial and as a consequence, only 832 patients were enrolled. Additional DDAb assays were performed on days 7 and 14 to monitor the developing DDAB titer prior to clinically relevant thrombocytopenia. A total of 22 patients (2.6%) were excluded at the two check points. One patient tested negative at pre-screen and later developed thrombocytopenia during the clinical trial. The free DDAb assay's clinical specificity (ability of a test to correctly identify the absence of disease at a particular decision threshold [9]) was determined as ∼99.9% and the clinical sensitivity (ability of a test to correctly identify disease at a particular decision threshold [9]) was ∼94.1%.

Of 493 patients who were pre-screened using results from EDTA DDAb assay as exclusion criteria in a different phase

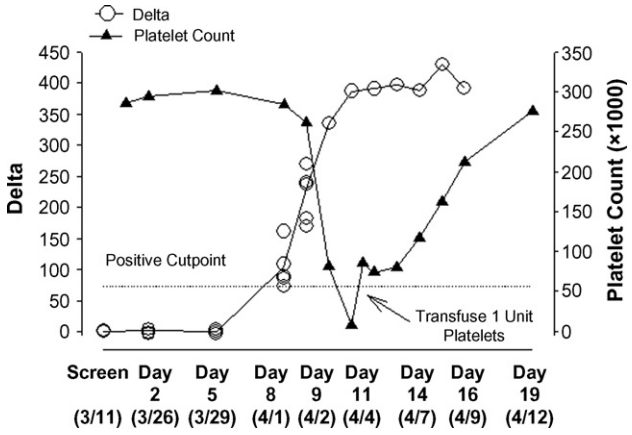

Fig. 3. Total DDAb profile of patient SGK before and after development of thrombocytopenia (redrawn with modifications from Seiffert et al. [5]).

II clinical study (DMP754-017), 15 (3.0%) tested positive at pre-screen, slightly lower than the 5% exclusion rate expected. Additional 8 (2.4%) out of 342 were excluded at the three check points, day 8, 10 and 15, during the study. One patient, SGK who was negative at pre-screen, later tested positive at one study check point and developed thrombocytopenia. The total DDAb assay's clinical specificity was determined as 100% and the clinical sensitivity was 95.5%.

The DDAb profile of patient SGK before and after development of thrombocytopenia is illustrated in Fig. 3. It should be noted that the platelet count results and DDAb titers for patient SGK were previously reported [4] and the key findings are redrawn here with indication of the assay cut point. Also, the platelet count recovered after discontinuation of the study drug despite further increasing DDAb titers, pointing to the exquisite drug dependency of the thrombocytopenia.

4. Discussions

In this paper, we describe the analytical validation of two differential DDAb ELISA assays, as well as our practical experience using these assays as enrollment and dosing termination criteria. The assay performance characteristics are similar to routinely used clinical ELISA's despite the use of a differential ELISA format. Overall, implementation of the DDAb tests during clinical trials of roxifiban reduced the incidence of thrombocytopenia by approximately 10-fold [4,5]. These finding points to the utility of DDAb testing to identify patients at risk for thrombocytopenia.

We also tested the hypothesis that one DDAb assay (either total or free) could be more superior to the other one. More specifically, EDTA treatment of whole blood at 37 °C results in a conformational change in GP IIb/IIIa, and the dissociation of GP IIb/IIIa antagonists as well as DDAbs. Subsequently, after removal of the cellular components of whole blood by centrifugation, EDTA plasma is expected to contain higher concentrations of DDAb than citrate plasma because it included both free and platelet bound DDAb. In initial studies, we validated this concept in vitro using mixtures of plasma from patients that developed DDAbs and whole blood from normal donors

(not shown). We therefore hypothesized that measuring DDAb in EDTA plasma may improve the assay's analytical sensitivity thus improve the assay's clinical sensitivity (reduce false negative rate) as the higher DDAb concentration may allow the identification of DDAb containing specimen at an earlier time point with a sufficient time window to stop dosing prior to clinically significant thrombocytopenia. In DMP754-017 clinical trial, samples in both EDTA and citrate anticoagulant were collected at all time points from all patients. EDTA assay was used to exclude patients from the study, and the citrate assay was tested retrospectively in samples obtained from patient(s) who developed thrombocytopenia. Based on the cut point determination from same patient populations, the patient exclusion rate would have been similar in both assays, at ∼5%. The cut point signal of the EDTA assay was observed to be twice as much as that of the citrate assay. When tested clinically in specimens from the only thrombocytopenic patient, SGK, DDAbs were first detected in both free and total DDAb assays on day 8 and neither assay showed a positive signal before day 8. Retrospective analysis of three other thrombocytopenia patient samples from other trials provided a similar conclusion. These studies have been previously reported [4,5]. This is in accord with the comparable clinical sensitivity and specificity observed for these two assays. Similarly, when a minimal dilution of 1/20 used in the free DDAb assay in DMP754-10 trial was changed to 1/10 in the free DDAb assay in DMP754-017 trial, an improved analytical signal in cut point was observed, with no improvement in clinical sensitivity. These results suggested that an assay's analytical sensitivity might have only limited clinical utility, especially when clinically relevant specimens were not available or difficult to test. The relevant comparisons of two assays are their clinical sensitivity and clinical specificity. When comparing the ease of use of the two procedures, the EDTA assay procedure is much more cumbersome due to the requirement of extracting XV459 from plasma, which incurred additional 25% labor and material cost and lengthened the assay run time by approximately 20%. Furthermore, the EDTA format required EDTA treatment of whole blood at the clinical sites, further increasing the cost of the assay. In addition, the EDTA assay variation was increased due to lack of internal standards to compensate for extraction recovery. These observations resulted in the decision to only implement the citrate format in phase III trials. Whether the decision of using citrate assay was a correct one can only be determined in larger clinical populations at a later phase due to the low event nature of this type of drug.

A patient who developed thrombocytopenia in DMP 754-010 trial tested negative at pre-screen using the free DDAb assay, but developed very high DDAb titer and thrombocytopenia at a later time. When the pre-screen sample was retested without an automated plate washer, the previously negative result became positive, possibly due to the weak affinity of antibody, which can be washed off by the strong force of the automated plate washer. A subsequent phase III clinical study (DMP754-302) was conducted using a complete manual plate wash format instead of automated plate wash; the assay clinical sensitivity was approximately 99.6%, with one patient having negative DDAb at pre-screen developed thrombocytopenia after administration of roxifiban. Clearly, there are different reasons why a DDAb assay could miss to identify a patient who later develop thrombocytopenia. Although encouraged by how well a purified transmembrane protein coated on microtiter wells could recapitulate the conformational changes induced by GP IIb/IIIa antagonist and subsequent DDAB binding to the platelet surface. However, compared to platelet surface, the possibility remains that certain conformational changes and antibody binding events are not mirrored by immobilized GP IIb/IIIa. Alternatively, this patient might have an idiosyncratic, non-immune thrombocytopenia. Nonetheless, a significant improvement in clinical safety has been achieved with thrombocytopenia rate decreased from ∼2% to <0.2% when either format of DDAb assay was implemented.

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