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Development and validation of a new assay for assessing clot integrity

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ABSTRACT

Introduction: Research and routine laboratory assessment of clot integrity can be time consuming, expensive, and cannot be batched as it is generally performed in real time. To address these issues, we developed and validated a micro-titre based assay to quantify thrombogenesis and fibrinolysis, the purpose being to assess patients at risk of cardiovascular events by virtue of hypercoagulability. In further validation, thrombogenesis results were compared to similar indices from the thrombelastograph (TEG).
Methods: Our assay determines three indices of thrombogenesis (lag time to the start of thrombus formation (LT), rate of clot formation (RCF), and maximum clot density (MCD)) and two of fibrinolysis (rate of clot dissolution (RCD) and time for 50% of the clot to lyse (T50)). Plasma was tested fresh and again after being frozen at −70 °C. Some samples were tested immediately, others after being left at room temperature for up to 24 h.
Results: The intra-assay coefficients of variation (CVs) of the three thrombogenesis measures (LT, RCF, MCD) and two fibrinolysis measures (RCD, T50) varied between 2.7 and 12.0% in fresh plasma and between 1.3% and 10.8% in frozen plasma respectively. Similarly, the inter-assay coefficients of variation of the thrombogenesis and fibrinolysis measures were 4.9–10.8% in fresh plasma and 2.2–6.5% in frozen plasma respectively. TEG assays intra- and inter assay CVs were around 25%. There were no significant differences in all plate assay indices up to 6 h at room temperature. Certain plate assay thrombogenesis data were comparable to TEG indices after analysis by Pearson's correlation. The reagent processing cost per sample is £15 for TEG and £2 for the plate assays.
Conclusion: Our micro-titre based assay assessing plasma thrombogenesis and fibrinolysis has good intra- and inter-assay CVs, can assess plasma up to 6 h after venepuncture, is more efficient (in terms of throughput) and is more economical than that of the TEG.

1. Introduction

Correct haemostasis functioning (the balance between thrombosis and fibrinolysis) is an essential physiological process. Increased thrombosis and/or impaired fibrinolysis leads to life-threatening conditions such as ischaemic stroke, myocardial infarction and pulmonary embolism. Conversely, failure of thrombosis (perhaps due to over-anticoagulation) and/or excessive fibrinolysis leads to life threatening haemorrhage, such as of the gastro-intestinal tract and in causing haemorrhagic stroke [1,2]. The need for information regarding the potential occurrence of these events, their treatment with anti-coagulants, in stable thrombotic disease such as coronary artery disease and atrial fibrillation, and in critical conditions such as disseminated intravascular coagulopathy and trauma, and in investigating cardiovascular pharmacology, such as the effects of anti-coagulants, calls for reliable laboratory tests of haemostasis [3,4].

The thrombelastograph (TEG) is an established laboratory tool for the investigation and management of haemostasis, simultaneously delivering numerous indices on various aspects of clot formation and fibrinolysis, a selection of which are shown in Table 1 [5,6]. Despite its flexibility in being able to assess haemostasis in trauma, haemorrhage and the effects of anticoagulation and fibrinolysis [7–9], it has several disadvantages. These include the requirement of whole blood to be assessed in real time, a maximum of two samples to be assessed per analyser at the same time, relatively high coefficients of variation, poorly standardised methodologies, quality control/assurance issues, and limitations on the stability of whole blood samples [10–12]. These issues also mean that it is a poor choice of assay in clinical research and drug development. Although used most commonly with whole blood, some of these problems may be addressed by using plasma, and the use of frozen plasma allows non-urgent assessments to be batched, possibly to be processed out of hours. However, slow and limited throughput of samples, and other problems leave room for alternative technologies.

The insoluble nature of the fibrin clot, and so the interruption of beam of light whilst it forms, is an established feature of the laboratory assessment of thrombogenesis [13–16]. Based on these principles, we set out to develop and validate a micro-method for a high-throughput assay that can assess thrombogenesis and fibrinolysis in citrated plasma, and that may be useful in clinical practice and research. We determined that the most convenient and efficient platform for this would be a

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Table 1
Major TEG indices.

Index	Function in ex-vivo haemostasis
R-time	The time from when the sample is put on the TEG until the first sign of clot formation (amplitude of 2 mm) is reached.
K-time	The time from the R or beginning of clot formation to a fixed level of clot firmness (amplitude of 20 mm) is reached.
Angle (α)	The rate of clot growth.
MA (maximum amplitude)	Maximum strength or stiffness (maximum shear modulus) of the developed clot. MA measures the strength or elasticity of the clot in mm.
LY30	Measures percent lysis at 30 min after MA is reached.

standard 96-well microtitre plate, and we compared our method with parallel data obtained from an established haemostasis device, i.e. the TEG.

2. Subjects, materials and methods

2.1. Subjects

Following local research ethics committee approval and written informed consent, whole blood was obtained from 19 healthy (i.e. not taking prescription medications) volunteers (mean age 35, standard deviation 8, range 26–52 years old, 12 males) into citrated vacutainers. A portion of whole blood was processed on the TEG. Plasma was obtained following centrifugation of the latter for 20 min at 3000 rpm: an aliquot of the fresh plasma was processed promptly whilst some plasma was frozen immediately and stored at −70 °C. Prior to assay, frozen plasma was thawed in a 37 °C water bath.

2.2. General principles of the assays

When plasma is exposed to thrombin, the polymerisation of fibrin can be monitored by measuring the amount of light passing through the solution. As fibrinogen is converted to protofibril monomers and fibrin, this solution will become more turbid and thus cause more scattering and absorption of light [16]. With subsequent addition of exogenous tissue plasminogen activator (tPA), the solution becomes less turbid due to dissolution of fibrin threads by plasmin, so that light passage is restored. We adopted and amended this process, developing a technique for concurrently assessing both thrombogenesis and fibrinolysis, as these share common features.

2.3. The plate assay for thrombogenesis

The method for the thrombogenesis assay calls for 25 μL of plasma to be added to the well of a standard ELISA-quality 96-well microtitre plate (R&D Systems Europe Ltd, Abingdon. UK), followed by 75 μL of a TRIS-NaCl buffer (1.51 g Tris–HCl [CAS 1185-53-1], 1.75 g NaCl [CAS 7647-14-5], 200 mL distilled water). Coagulation is initiated by the addition of 50 μL of a thrombin/calcium solution. For a batch of 20 patient samples in triplicate (total of sixty samples), this reagent will consist of 20 μL of a solution of 1000 IU thrombin [Sigma catalogue number T9549] reconstituted in 4 mL phosphate buffered saline [Sigma P4417] /0.1% bovine serum albumin (BSA [Sigma P5368]), 500 μL of 500 mM calcium chloride [CAS 10043-52-4] in the Tris/NaCl buffer, and 3 mL of the Tris/NaCl buffer (all reagents Sigma-Aldrich, Gillingham, Kent). Bulk addition of buffers to multiple wells of the micro-titre plate is facilitated by an 8-channel micro-pipette. The thrombin/calcium solution can be bulk reconstituted and stored frozen at −70 °C in aliquots.

The plate is immediately loaded into a Tecan Sunrise (Tecan Group Ltd, Männedorf, Switzerland) plate reader at 37 °C programmed to measure the optical density (OD) at 340 nm every 6 s (with an intermediate 2-second shaking period) for 30 min. The raw OD data at each time point can be printed out as an excel file and as a graphic. A typical graphical print-out is presented in Fig. 1, and shows change in OD over time as the fibrin clot forms. From these, three key indices can be obtained: (a) the lag time (LT), which is the time in seconds from the initial measurement (addition of the calcium/thrombin) to the start of the exponential part of the thrombogenesis curve, (b) the rate of clot formation (RCF), calculated as the change in OD unit/second, being [The OD at the end of the exponential phase minus the OD at the start of the exponential phase] / [The time in seconds between these two points], and (c) the maximum increase in OD taken at 30 min, which give us the maximum clot density (MCD).

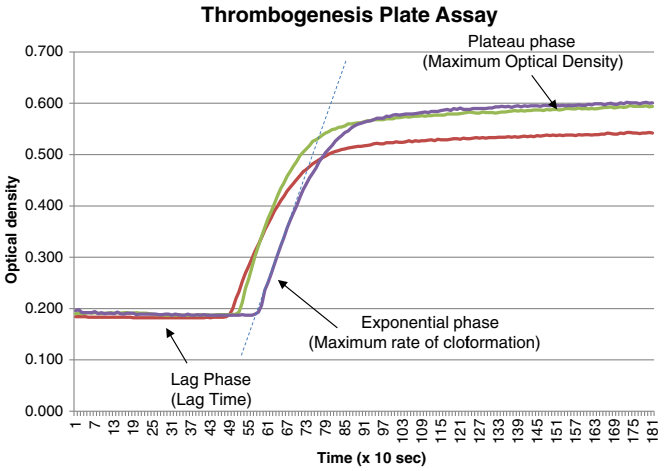

Fig. 1. The thrombogenesis assay.

2.4. The plate assay for fibrinolysis

A strength of our method is that many of the features of the thrombogenesis assay are part of the fibrinolysis assay. In the fibrinolysis assay, 75 μL of plasma is added to the well of a microtitre plate. To this is added 75 μL of a Tris/NaCl/calcium buffer supplemented with thrombin and tPA, to be made up fresh per assay run. For a batch of 20 patient samples in triplicate (therefore sixty wells), this buffer will consist of 9 mL Tris/NaCl buffer, 400 μL calcium chloride solution, 20 μL thrombin (as above) and 800 μL tPA (Technoclone TC41072): stock solution of 100 μg tPA reconstituted in 400 μL of 1 molar potassium bicarbonate [CAS 298-14-6] /1% BSA (both Sigma). The tPA solution can be bulk reconstituted and stored frozen at −70 °C in aliquots.

The plate is immediately loaded into a Tecan Sunrise plate reader as for the thrombogenesis assay, and data was collected for 30 min. Again, the raw data of the OD at each time point can be printed out as an excel file and as a graphic. A typical graphical print-out is presented in Fig. 2, and shows change in OD over time as the fibrin clot is initially formed and then lysed. The data is post-processed to plot into line charts, and from these the rate of clot dissolution (RCD), being the slope of the right hand portion of the graph, and the time for 50% clot lysis (T50) can be determined (as demonstrated in Fig. 2).

As the parameters and settings for the plate reader are identical for each assay, they can be performed in parallel on the same plate. However, should it be necessary, each assay can be run independently. The duration of time required for both plate assays is dependent on the thrombogenesis and fibrinolysis potential of the samples, and thus processing time may be variable. However, for most samples, results may be obtained within 20 min, at which point the assay can be terminated. Our data shows results at 30 min, as this is one of the TEG endpoints.

2.5. The TEG

Manufacturer's instructions were followed. Briefly, 340 μL of citrated whole blood or plasma is added to a reaction cuvette, to which is added