New approaches for measuring coagulation


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Summary. Although specific assays of coagulation factors are essential for diagnostic purposes they only give partial information about an individual’s haemostatic state. This can be better assessed by various global tests, and recent developments and evaluations of five such tests are described in this symposium: the PFA-100; waveform analysis; thrombin generation; overall haemostasis potential; thrombelastography. Each test has advantages in various applications, but the thrombin generation test and waveform analysis have been found most useful in haemophilia, whilst the PFA-100 is helpful in von Willebrand’s disease.

Keywords: coagulation, global tests, bleeding disorders

Introduction

As knowledge of the coagulation system developed in the 1950s onwards, specific assays were devised for most of the main clotting factors, and these largely replaced most of the non-specific global tests such as thrombin generation and thrombelastography. However, in recent years there has been a recognition that specific assays may not be sufficiently informative to assess the patient’s overall haemostatic state and that more global tests may contribute useful additional information. Another reason for the revival of global assays is the development of technical innovations and more sophisticated methods of analysis, which have improved their reliability and reproducibility and led to more widespread use.

In this symposium, the clinical utility of three such global assays is described. Dr Marco Cattaneo assesses the PFA-100 as an in vitro substitute for the bleeding time; Dr H. Coenraad Hemker describes the use of thrombin potential, as determined by the thrombin generation test, in haemophilia; Dr Cheng Hock Toh discusses the application of waveform analysis in bleeding disorders. Finally, Dr Jorgen Ingerslev compares the use of waveform, thrombin generation and traditional bioassays in haemophilia patients.

The PFA-100® versus the bleeding time in the diagnosis of bleeding disorders

We compared the performance of PFA-100® closure time (CT) and skin bleeding time (BT) in the diagnostic work-up of 128 consecutive patients referred to our centre to be screened for bleeding disorders. The correlation with the severity of the bleeding symptoms and the sensitivity of BT and PFA-100 CT for known defects of primary haemostasis were evaluated. All patients underwent (i) a careful medical interview and were assigned a ‘bleeding score’, based on the number, type, frequency and severity of bleeding symptoms; (ii) first-line screening [prothrombin time, activated partial thromboplastin time (APTT), PFA-100 CT both the collagen-ADP (C-ADP) and the collagen-epinephrine (C-EPI), cartridges and BT]. The search for von Willebrand’s disease (VWD), platelet function disorders (PFD), clotting factor defects and abnormalities of fibrinolysis was performed according to the results of the first-line screening tests and the severity and type of bleeding history. Seven (6%) patients had type 1 VWD, 12 (9%) PFD, 29 (23%) defects of clotting factors, 18 (14%) prolongations of the APTT as a result of the abnormalities that are not associated with bleeding (factor XII deficiency and...
The application of waveform analysis in coagulation

Waveform analysis is the optical profile of clot formation on simple assays of coagulation, such as the activated partial thromboplastin time (aPTT) [1]. Optical profiles can be characterized using a set of parameters describing onset and completion of coagulation, magnitude of signal change, rate of coagulation and other properties. Work from our group has shown that this can be atypical in patients with haemostatic dysfunction [2]. The biphasic waveform (BPW) was initially described when a decrease in plasma light transmittance prior to clot formation on the MDA 180® automated coagulation analyzer (bioMérieux, Marcy, France) was shown to correlate with disseminated intravascular coagulation (DIC) in critically ill patients [3]. In contrast to the normal sigmoidal waveform pattern that is characterized by an initial 100% light transmittance phase prior to clot formation, patients with a biphasic pattern had an immediate, progressive fall in light transmittance that occurred even in the preclotting phase [4]. The magnitude of this BPW often varied with sequential samples taken from individual patients and appeared early in samples from patients who were later diagnosed with DIC by more conventional criteria [5]. The utility of this for the forewarning of DIC, the diagnosis of sepsis and the risk of mortality have been validated by others both in the intensive care and in the emergency room setting [6–8].

In contrast to the significance of the initial slope (slope 1), work by others has also demonstrated the utility of the second derivative of the waveform. The group at Nara University has shown that this aspect of aPTT waveform analysis can be utilized in the assessment of factor VIII (FVIII) levels below 1.0 IU dL\(^{-1}\). Whereas conventional one-stage clotting assays are insufficiently sensitive at this level, Shima et al. [9] have shown that FVIII levels of 0.2 IU dL\(^{-1}\) can be measured reproducibly and that such accurate discrimination has phenotypic relevance and clinical significance. Furthermore, this can be utilized to monitor the effectiveness of FVIII infusions in haemophilia A patients with high responding inhibitors. In the presence of type 1 inhibitors quantified at 6–14 BU mL\(^{-1}\), 0.9 IU dL\(^{-1}\) FVIII could be detected 24 h after FVIII infusion [10]. This suggests that FVIII infusion may be continued with clinical benefit in some haemophiliacs with high responding inhibitors and that the haemostatic response can be monitored accurately and efficiently by waveform analysis. For those that do not respond to FVIII and require recombinant factor VIIa, waveform analysis can also indicate dose-dependent improvements in clot formation [11].

Thrombin potential in haemophilia

Haemophilia is a deficiency of factor VIII (FVIII) or IX (FIX) that leads to a defect in the thrombin-generating system of the blood. Although in haemophiliacs the deficient factor limits the haemostatic function, it is not its sole determinant. Its residual activity remains dependent upon the ensemble of clotting factors, platelets and the vessel wall. This is best illustrated by the observation that substitution with factor VIIa (FVIIa) instead of the lacking FVIII or FIX can restore haemostasis. In addition, the common clinical observation that haemophiliacs with identical residual levels of the missing factor can show large variations in bleeding tendency stresses this point.

It therefore seems logical to try and measure the affected function rather than the missing factor, at least that part of the function, which is accessible because it is located in the blood, i.e. thrombin generation. Indeed, it can be shown that thrombin generation in haemophilic blood is dependent on other factors than the deficient one. It is, for example, proportional to the prothrombin level and inversely proportional to the antithrombin level and augmented by inhibition of the activated protein C system. The most important practical reason for measuring thrombin generation in haemophiliacs is in fact its sensitivity to FVIII bypassing therapy with FVIIa-containing preparations.

A typical thrombin generation curve (Fig. 1) shows a lag time, a steep rising slope and a slower declining
the CAT method using 2 p
48, 30, 60 h. The thrombin generation curves are obtained with
bottom at the following times after injection: 15 min, 1, 3, 5, 24,
before injection; thin lines
Upper fat line
patient.
and the amount added. When this is corrected for,
depends upon the binding constant of the substrate
Haemophilia (2006),
(2006),
and ETP via this mechanism. This may explain the
suggests a direct link between late bleeding tendency
fibrinolytic character of much haemophiliac bleeding
resistance of the haemostatic plug to fibrinolysis. The
fibrinolysis inhibitor (TAFI) formed and thus the
also determines the amount of thrombin-activatable
activation that the signals are influenced by variables
outside the thrombin-generating system, such as
fibrinogen content, and the Ca++-dependent interac-
tion of very low-density lipoproteins and C-reactive
protein.

In contrast to the classical view, it becomes more
and more obvious that platelets are not just respon-
sible for that phase of haemostasis that occurs before
thrombin is formed. Recent experiments show clearly that thrombin starts to form within seconds
after a lesion occurs. Platelets influence the amount
and the effect of thrombin formed in at least three
ways: by forming an aggregate from which the
formed thrombin cannot be washed away, by pro-
viding the required procoagulant phospholipids and
by releasing factor V. Thrombin generation in

An approximate impression of thrombin genera-
tion can also be obtained by surrogate methods, i.e.
methods that do not render thrombin concentration
(in nM) as a function of time. Measuring the first
derivative of substrate conversion without proper
calibration gives curves that to the non-initiated looks
deeingly like a thrombogram but that gives a
distorted view and introduces important errors. The
first derivative being a function of both thrombin
concentration and substrate concentration, variation
in the latter needs to be corrected for by continuous

Other surrogate methods are based on the conver-
sion of fibrinogen into fibrin, measuring either its
turbidity or its tensile strength. Through mathemat-
ical manipulation, curves can be obtained, which
look like thrombin generation (TG) curves but are
not. Because fibrinogen is converted into fibrin long
before all prothrombin is converted into thrombin,
esentially only the lag time of the thrombelastogram
is comparable to one of the parameters of the actual
thrombin generation curve. After clotting occurs, the
signal results from the changing properties of the clot
rather than from the amount of thrombin present.
The method has the advantage that it can be carried
out in whole blood and that fibrinolysis is readily
observed. The latter, via TAFI, may be a secondary
effect of a low ETP (see above).

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platelet-rich plasma, therefore, is one step nearer again to complete physiological function testing than TG in PPP. This is especially important in von Willebrand’s disease where impaired thrombin generation can be demonstrated, that is not, or only partly, due to the lack of FVIII but must be attributed to impaired platelet activation via the fibrin–von Willebrand factor (VWF)–GPIb–platelet activation axis [12]. By comparison of the thrombin generation test in PRP to that in PPP with added phospholipids, this phenomenon allows to differentiate between the effect on thrombin generation of FVIII and of VWF.

**Experiences in comparison of waveform, thrombin generation test and bioassay in haemophilia patients**

Historically, laboratory practises adopted in the management of haemophilia in recent times have been based on the experiments conducted in the early 1950s when the activated partial thromboplastin time (APTT) method was first devised [13]. Today, a prolonged APTT test result found in a male person with an abnormal tendency to bleeding should always raise suspicion of haemophilia, and the relevant investigation will include a bioassay recording the level of factor VIII and/or factor IX (FIX) based on the APTT methods. Should the patient suffer from haemophilia, APTT assays are also employed to monitor treatment. Thus, common laboratory routines around haemophilia are closely linked with the earliest small signal of fibrin formation. However, one must be aware that the APTT method only records the beginning of the overall clotting process, leaving >95% of the reaction untold.

For this reason, methods were developed and published providing more detail of the entire course of the clotting process in haemophilia as early as 55 years ago. In principle, two avenues were followed: one type of assay recorded the thrombin activity generated during the coagulation process [14], while the other assay illustrated the formation of fibrin during clotting [15]. In the original versions, these methods were quite crude and imprecise and were not well suited for routine use and therefore were not generally adopted in clinical work.

**Thrombin generation methods**

Recent progress has refined these assays to a large extent, and the thrombin generation principle has been used more generally in haemostasis research [16,17]. As thrombin formation occurring during plasma coagulation is quite explosive by nature, slow-reacting substrates have been developed and introduced. For practical reasons, in particular when the thrombin generation test is used in the study of plasma with platelets, thrombin-induced peptide hydrolysis in a chromogenic reaction has been superseded by fluorogenic substrates [18]. In order to study continuous thrombin generation in plasma, either fibrinogen must be removed or its polymerization into fibrin must be inhibited. The continuous curve of thrombin generation is usually differentiated and derived parameters such as the lag time, the peak value and the area under the curve are employed in the interpretation of the signal. Most commonly, the activator is tissue factor in small amounts, but a variety of concentrations of tissue factor have been used, making direct comparisons quite difficult.

A major clinical and laboratory hallmark of haemophilic plasma clotting – the prolonged initiation phase – seems to be lost if too much tissue factor is used for activation [19]. Alternatively, for the study in haemophilia A, the use of activated FIX as an activator has been more successful in this regard [20].

**Fibrin formation methods**

The systems developed for recording fibrin formation, as assessed by the change in the viscoelastic properties of blood or plasma during coagulation, had to come quite some way until velocity characteristics were introduced to substitute for the classical ‘brandy-glass’ shape signature. Two systems are available today and quite comparable in performance: the thrombelastograph and the rotating thrombelastometer. Compared with Hartert’s original model, the new equipments employ single-use reaction cells and the raw continuous signal may be converted into velocity by differentiation.

With the viscoelastic recorders, the optimal activator for study in haemophilia is tissue factor in low concentrations at 0.35 pm or less [21]. These assays appear to mirror the haemophilic bleeding phenotype quite well, as the initiation of clotting is severely prolonged in severe haemophilia, and the fibrin formation signal lacks acceleration and is weakly represented [22].

**Overall haemostasis potential**

In addition, a slightly different system has been communicated, in which fibrin formation dynamics in plasma is recorded by photometry following activation with a small amount of thrombin. This method denoted the overall haemostasis; haemo-
static potential also has the convenience of measuring the fibrinolytic resistance of the clot. The most recent report demonstrates the feasibility of the method in hypocoagulable states such as haemophilia and other single factor deficiencies [23].

A study was recently reported comparing thrombin generation with fibrin formation in whole blood showing a quite convincing correlation between the two principles of recording whole blood coagulation in a continuous manner. As fibrin formation is the consequence of thrombin generation, this finding is by no means surprising [24].

Conclusions

The PFA-100 would appear to be useful in the diagnosis of different types of von Willebrand's disease (VWD) and in distinguishing VWD from platelet function. As well as showing a better correlation with the various bleeding disorders, the PFA-100 has the clear advantage over the bleeding time of practical convenience.

Analysis of the waveform of the clotting process can be carried out in a number of ways with appropriate instrumentation. The biphasic waveform is atypical in disseminated intravascular coagulation and has been found useful in diagnosing this coagulation abnormality. In haemophilia, analysis of the second derivative has allowed quantitative measurements at Factor VIII (FVIII) levels below 1 IU dL⁻¹ and has proved useful in monitoring the treatment of inhibitor patients.

The thrombin generation test can be used to monitor treatment in haemophilia and gives more information than factor assays, as it takes into account non-FVIII influences on clotting, such as thrombin-activatable fibrinolysis inhibitor. However, the concentration of tissue factor is critical for the application of thrombin generation test (TGT) in haemophilia, and an alternative activator that has been used successfully is Factor IXa. Two other tests that may be of clinical benefit are thrombelastography and the overall haemostasis potential, although these have not been studied as intensively in haemophilia as TGT and waveform analysis.

References

4 Toh CH, Samis J, Downey C et al. The biphasic transmittance waveform in the aPTT coagulation assay is due to the formation of a Ca²⁺-induced complex of C-reactive protein with very low density lipoprotein and is a novel marker of impending disseminated intravascular coagulation. Blood 2002; 100: 2522–9.


