

Towards standardization and recommendations for clot waveform analysis

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Introduction

Automated coagulation analyzers are being relied upon increasingly in response to the heightened demand for blood clotting investigations and the need for rapid results. The automated systems can provide a wealth of information in addition to that provided by conventional clotting tests such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which are often considered of limited use for clinical purposes [1-3]. One particular type of analysis, the clot waveform, which is provided by the Multichannel Discrete Analyzer (MDA series; Organon, Technika), defines changes in light transmittance which occur during the process of clot formation.

A number of recent reports have described the use of this type of automated clotting instrument for clot waveform analysis (CWA), and there appears to be significant advantages in using this system for the assessment of global coagulation function. Principally, CWA can be conveniently performed simultaneously with routine coagulation tests, and no dedicated apparatus is required in laboratories equipped with the appropriate automated analyser. In addition, quantitative measures of several novel parameters reflecting coagulation velocity and acceleration are provided alongside the qualitative observations of waveform.

Principles of Waveform

The principles of waveform analysis can be illustrated using the APTT performed on the MDA-II system utilising Platelin LS, 0.025mM CaCl₂, and Verify reference plasma. The waveform is visualized, recorded and a hard copy printed. The data are automatically processed mathematically by the MDA-II system using algorithms built into the commercial software [3]. The CWA results are accessed using exporting software. Changes in light transmittance are determined by continuous measurements during the APTT and are designated the clot waveform (Fig 1). This clotting process is categorized into the three parts; the pre-coagulation phase, the coagulation phase and post-coagulation phase. Pre-coagulation is described as the first segment

of the trace, from the beginning of the signal to the onset of coagulation. Usually, this phase is horizontal, except in waveforms in patients with DIC induced by septicemia (see below). After the onset of coagulation, light transmittance is decreased by the formation of fibrin and is defined by a slope in the waveform. At the end of coagulation, light transmittance tends to stabilize and is characterized again by a linear segment. If fibrinolysis is enhanced due to acquired or congenital pathological disturbances such as plasminogen inhibitor deficiency, light transmittance may be increase again in the post-coagulation phase.

Various commercial APTT reagents can be utilized for both CWA and routine coagulation assays. It is noteworthy, however, that selection of reagents is most important for optimal CWA results. Colourless reagents, without opacity, are recommended to detect sensitive changes in transmittance or absorbance, and for subsequent precise measurements of the various parameters. In addition, the APTT clotting time should be adjusted in order to increase sensitivity for the assessment of impaired clotting function as in coagulation factor deficiencies. Hence, the APTT time-scale may be too short using some currently available, standard APTT reagents, and these reagents should be diluted appropriately to adjust the clotting time to approximately 30-40 sec. with normal plasma and > 90 sec with FVIII deficient plasma.

Quantitative assessment of waveform parameters

In the MDA-II system, a set of nine parameters are used to describe the characteristic optical profiles obtained during APTT and PT assays [1-3]. Slope 1 is initial slope of the pre-coagulation phase before the onset of coagulation and is derived from mean slope during this phase. tmin2 is the time at the onset of coagulation, and min2 is the minimum value of the second derivative of the transmittance. The absolute value of min2 ($|min2|$) reflects maximum coagulation acceleration. tmin1 is the time at the mid-point of the coagulation phase. min1 is the minimum value of the first derivative of the transmittance. The absolute value of min1 ($|min1|$) reflects the maximum coagulation velocity. tmax2 is the time at the end of the coagulation phase, and is recorded at the maximum deceleration rate of coagulation, max2, is derived from the second derivative. slope 3 is mean slope in the post-coagulation phase. delta is amplitude of signal change. This value reflects fibrinogen concentration.

CWA in various coagulation disorders

Initial evaluation of clotting function by CWA is undertaken by qualitative assessment of the CW pattern. In particular, two characteristic CW patterns are observed in various coagulation abnormalities compared with normal reference plasma (Fig 2). In normal plasma, the pre-coagulation phase is short and the slope, reflecting the coagulation phase, is steep. In factor V (FV) deficiency and factor VIII (FVIII) deficiency, and in the anti-phospholipid syndrome (APS),

the pre-coagulation phase is prolonged but the changes in slope are different. In APS, the waveform shifts towards a normal pattern after the addition of phospholipid, Changes in slope are most evident in FVIII deficiency. In APS, the steepness of the slope is similar to that of reference plasma, although the pre-coagulation phase is prolonged. This particular pattern might provide a useful index in the investigation of APS. Thus, qualitative analysis of CW may have diagnostic value in various clinical settings with impaired clotting function.

It is very important to understand how CW patterns reflect the concentration of deficient clotting factors. This can be demonstrated by analyzing mixtures of deficient plasma and various amounts of normal plasma. In FVIII deficiency, the CW pattern changes dose dependently on the addition of FVIII (Fig 3A). As the FVIII level is decreased, the pre-coagulation phase is prolonged and slope becomes slower. Furthermore, Interestingly, this feature is most prominent in the presence of concentrations of FVIII less than 5%. At higher concentrations, the changes in CW patterns are less marked compared to that of reference plasma. Similar results are seen in FV deficiency except that differences between higher and lower concentrations of FV are not as remarkable (Fig 3B). It may be that these results reflect the likelihood that global clotting function is more abnormal in the presence of less than 5% FVIII.

Quantitative evaluation of CW parameters in factors II, V, VIII, IX, X, XI and XII deficiency shows that t_{min2} and $min2$, representing coagulation acceleration, remain within the normal range at clotting factor concentrations higher than 50%, but gradually increase at less than 50% and are markedly abnormal at less than 5%. In contrast, no changes in CW patterns are seen in factor VII and protein C deficiency [3].

Evaluation of clotting function in hemophilia A

The clinical severity in hemophilia A (HA) generally correlates with the level of FVIII activity (FVIII:C). Hence, hemophilia A is classified into three categories on the basis of FVIII:C activity; severe (FVIII:C <1.0 IU/dl), moderate (FVIII:C 1.0-5.0 IU/dl) and mild (FVIII:C >5 IU/dl). Studies have indicated that CWA in a number of patients with severe HA diagnosed by conventional clotting assays differed from patient to patient. The APTT clotting time was prolonged in all patients with severe HA, but there was variation in the slope [4]. Using mixtures of severe HA plasma and exogenous FVIII ranging from zero to 1.0 IU/dl, the slope and the APTT clot time and the $min2$ appeared to change in a dose-dependent manner. Similarly, in experiments with 36 patients with severe HA, significant correlations between $min2$ and FVIII:C were confirmed [4].

These results indicated that in some patients, the presence of very low levels of FVIII mediated higher coagulation acceleration characterized by the steeper slope, although It was possible that factors other than FVIII:C alone may have influenced clotting kinetics reflected in the waveform profile. Nevertheless, the data suggested that CWA could discriminate between different levels of FVIII:C in this critical category of severe HA defined as having <1.0 IU/dl FVIII:C by conventional

assays (Figure 4).

Some HA patients classified as severe on the basis of coagulation assay exhibit milder clinical symptoms. Similarly, some patients who may be initially identified in the moderate-mild category demonstrate a severe clinical phenotype with frequent bleeding symptoms. CWA, therefore, might provide very important data for evaluating *in vivo* clotting function in various types of hemophilia A. To validate this proposal, hemophilia A patients were divided into clinically severe and non severe groups. Severe patients were characterized by the presence of spontaneous bleeding episodes at the age of < 1 year, the onset of joint or muscular bleeding before the age of three years old, or the presence of severe bleeding such as intracranial bleeding or refractory oral bleeding. The differences between the severe and the non severe phenotype were significant for four CW parameters; clot time, maximal coagulation velocity (Min1), maximal coagulation acceleration (Min2) and maximal coagulation deceleration (Max2), These results suggested, therefore, that CW parameters reflect clinical severity (Fig. 5).

According to the cell based coagulation model, thrombin generation is considered to provide an important means for global clotting assessment. It may be valuable, therefore, to compare the results obtained from CWA with those of the thrombin generation test (TGT). CWA and TGT were performed simultaneously on FVIII-and FIX-deficient plasma, which had been reconstituted with known concentrations of FVIII and FIX. Overall, the results obtained with both assays showed good correlation with FVIII and FIX levels, confirming that the changes in CWA coincided with changes in TG. This study demonstrated, however, that the CWA was more sensitive than TGT especially at low levels of these clotting factors [5].

Biphasic pattern (BPW) in sepsis and DIC

The normal sigmoid appearance of the APTT transmittance waveform appears to be modified to a “biphasic” pattern in patients with sepsis and disseminated intravascular coagulation (DIC), CWA, therefore might provide important data for the prediction this major complication of septicaemia [7]. As demonstrated above, a normal APTT-CW on the MDA-II illustrates an initial plateau phase followed by a downward slope, providing the typical sigmoid appearance for the entire curve. This pattern is maintained in patients with clotting factor deficiencies, including those with hemophilia and inhibitors, and individuals who are receiving anticoagulant agents including heparin and plasma expanders, although the plateau phase can be prolonged in association with the degree of prolongation of the APTT [8]. The biphasic wave form profile is characterized by the development of an initial low-gradient slope followed by a much steeper slope (Fig 6) [8,9]. This biphasic pattern (BPW) persisted in sepsis even in the presence of a normal APTT and was not influenced by pre-analytical variables such as time from venipuncture and the presence or absence of platelets [8]. The wave pattern was not consistently demonstrated, however, using the PT test

except in cases with a prolonged PT [9]. In this clinical group, the distinct waveform with a greater steepness of the initial slope correlated with levels of D-dimer greater than 10mg/L but did not correlate with changes in plasma fibrinogen level. Some workers have defined the BPW as when the slope of the pre-coagulation phase exceeds a threshold value of $-0.25\%T/sec$ [10-11].

Future Vision on CWA

Although CWA is a very convenient global clotting technique, limitations with the MDA series of coagulation analysers have hampered the wider use of this method. Automated CWA is not always available, although most of the currently purchased instruments include software to process the changes in transmittance or absorbance. Even if the relevant computer software is not included with the system, however, it would be possible to perform CWA using standard programmes such as Microsoft *Excel* if raw data can be extracted. The next stage for the future development CWA is to compare the results using different analytical platforms and to standardize the evaluation based on selected test reagents and agreed parameters. It will also be important to examine this technique in various other clinical settings, such as monitoring hemostasis-bypassing therapy and inhibitor neutralization [6,12] .

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Figure legends

Figure 1. Optical transmittance vs. time for APTT assay of a normal specimen.

The upper trace shows the changes in light transmittance observed during the performance of APTT with normal reference plasma. Point "A" marks the beginning of the recording by the instrument which occurs 8 sec. after the addition of CaCl₂, Point "B" indicates the initiation of coagulation, namely fibrin formation. The clot waveform is separated into the pre-coagulation phase (A-B), the coagulation phase (B-D), and the post-coagulation phase (D-E)

Figure 2. Examples of normal and variant APTT optical data.

The light blue colored WA shows typical results in patients with anti-phospholipid antibody syndrome (APS). The yellow and green curves represent FV and FVIII-deficient plasma respectively (George King INC. Overlnd Park, KS, USA). In these instances, the pre-coagulation phase is markedly prolonged compared with that of the normal plasma, and slope 2, reflecting the coagulation phase, is relatively gentle.

Figure 3 Dose-dependent waveform changes in plasma containing various concentrations of FVIII (A) and FV (B).

Normal reference plasma was mixed with FVIII (A) or FV (B) deficient plasma to derive a series of standard factor deficient plasmas with 100, 50, 10, 5, 1 IU/dl.

Figure 4 Significance in difference in CW parameters among hemophilia A.

Hemophilia A patients were classified into three categories; a severe group with FVIII less than 0.2 IU/dl, a group with FVIII of 0.2-1.0 IU/dl, a group with inhibitor. Three CW parameters, Min1 (maximal coagulation velocity), MIn2 (maximal coagulation acceleration) and clot time of each patient group were tested and compared.

Figure 5. CWA parameters and clinical severity of hemophilia A.

The criteria for defining the clinically severe phenotype were spontaneous bleeding episode at the age of < 1 year, the onset of joint or muscular bleeding before the age of three years old, or the presence of severe bleeding such as intracranial bleeding or refractory oral bleeding.

Figure.6 Activated partial thromboplastin time (APTT) waveform patterns: (A) normal, (B) biphasic. *Dashed lines* denote transmittance level at 18 s (TL18) as the quantitative index (In (A) TL18=100 and in (B) TL18=63)

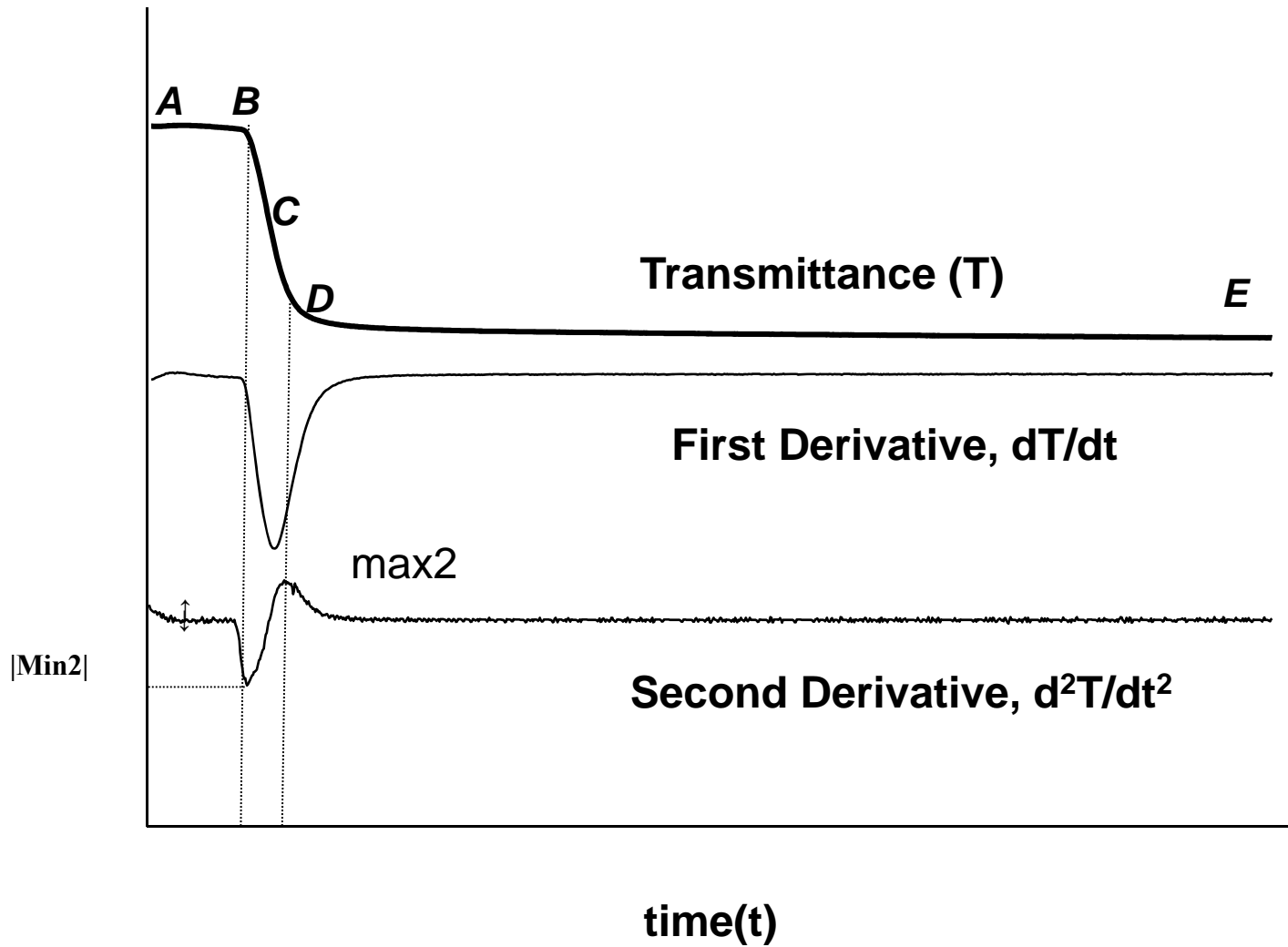


Figure 1

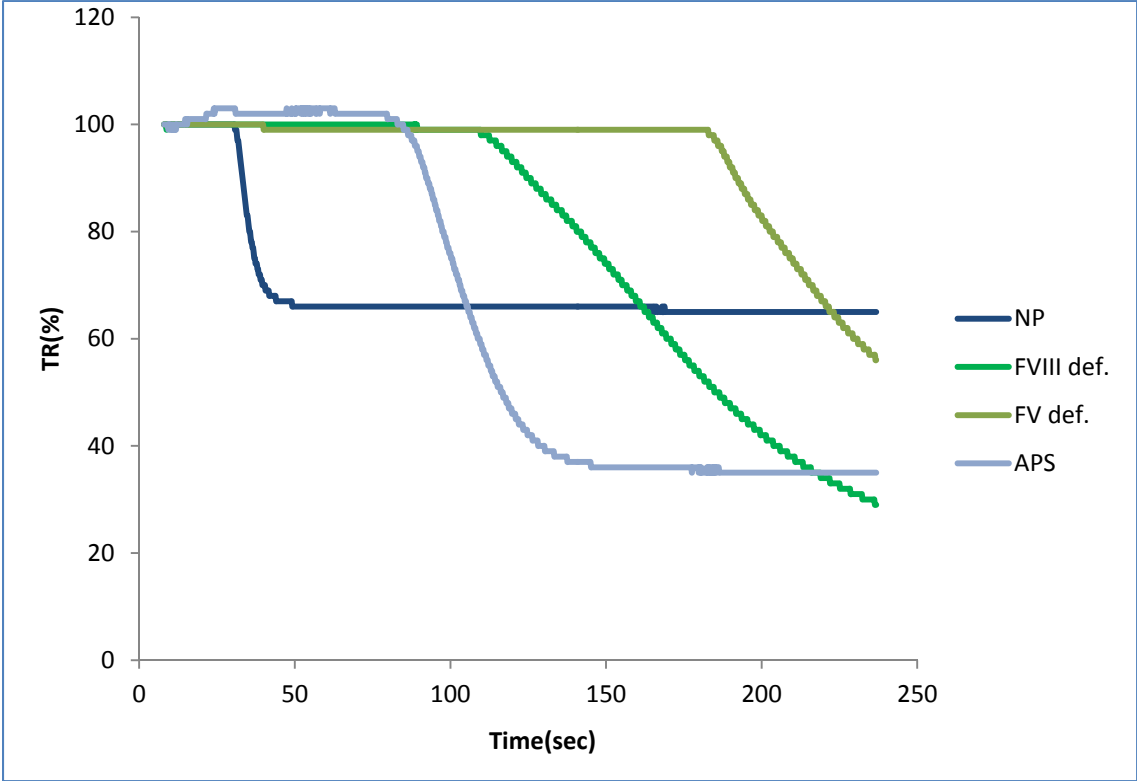
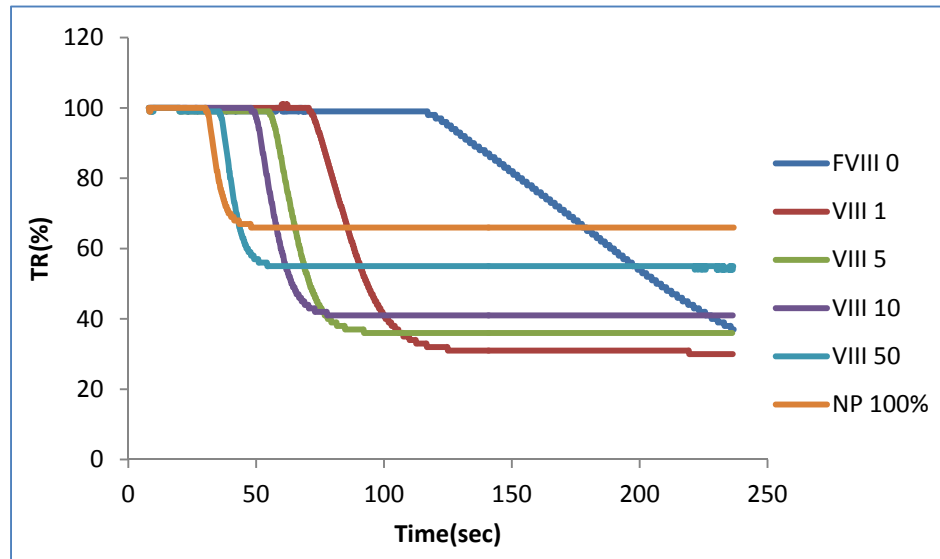


Figure 2

(A)



(B)

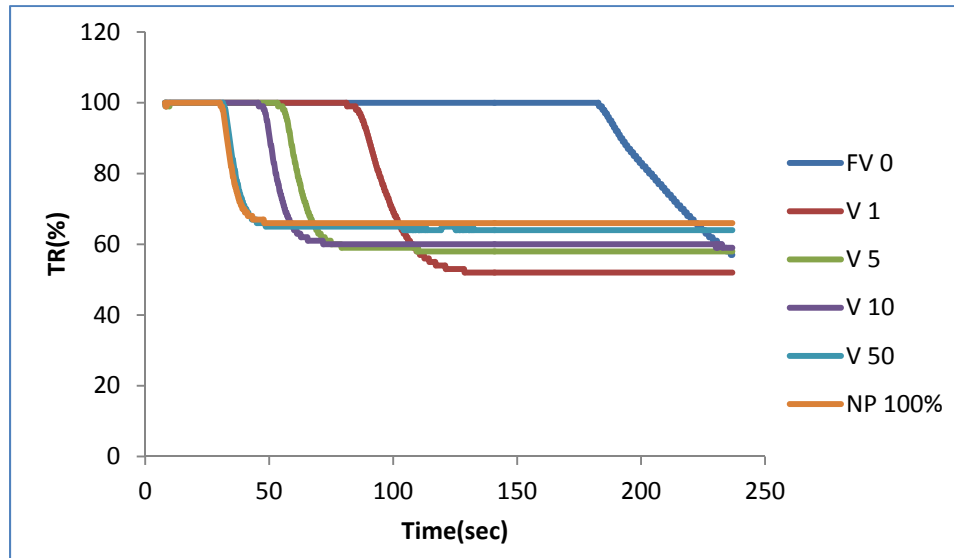


Figure 3

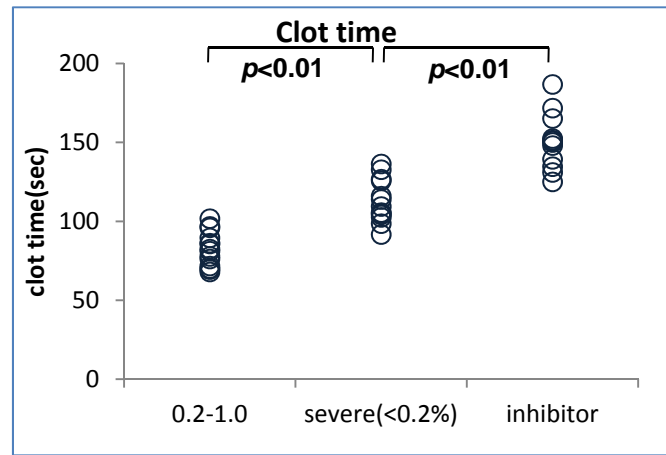
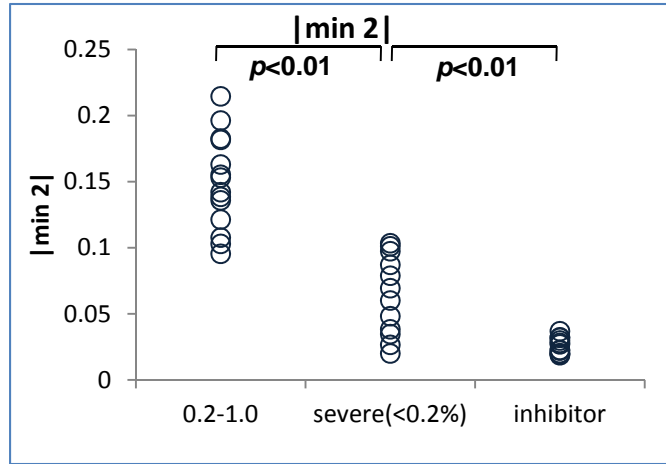
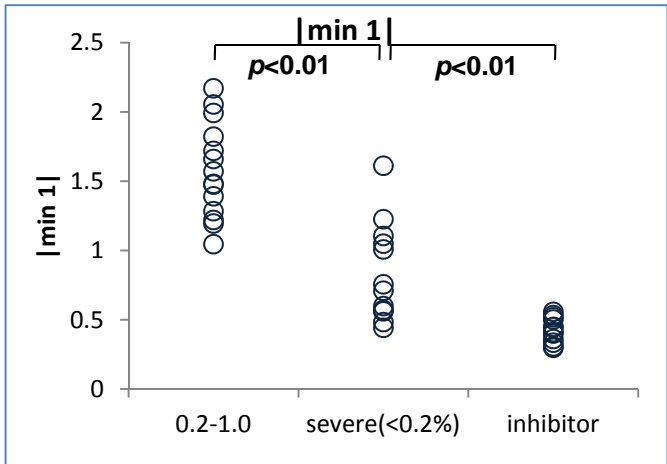


Figure 4

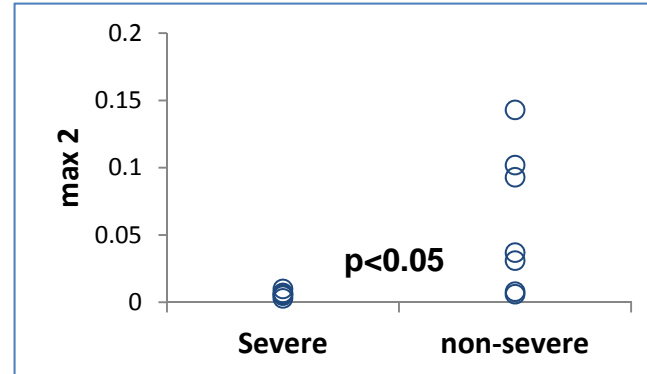
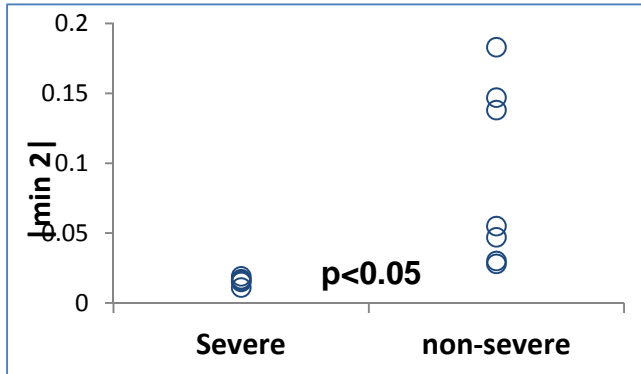
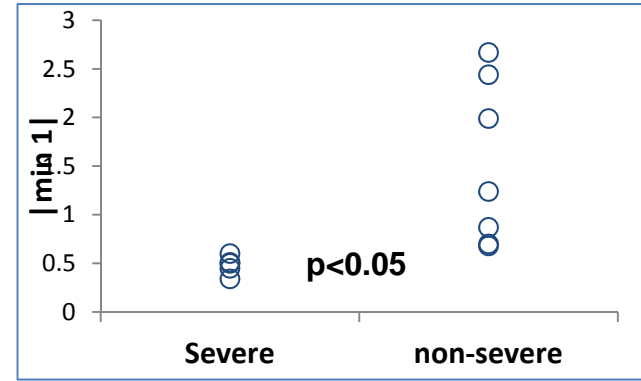
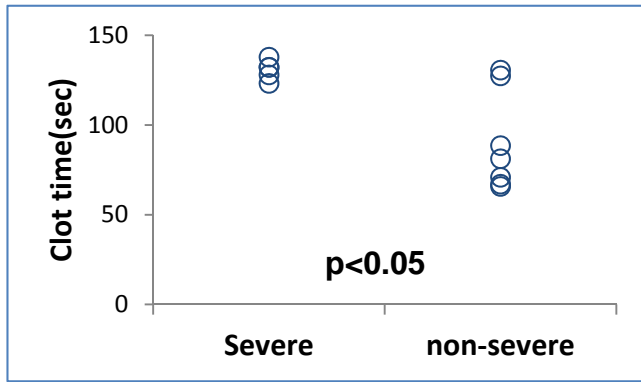


Figure 5

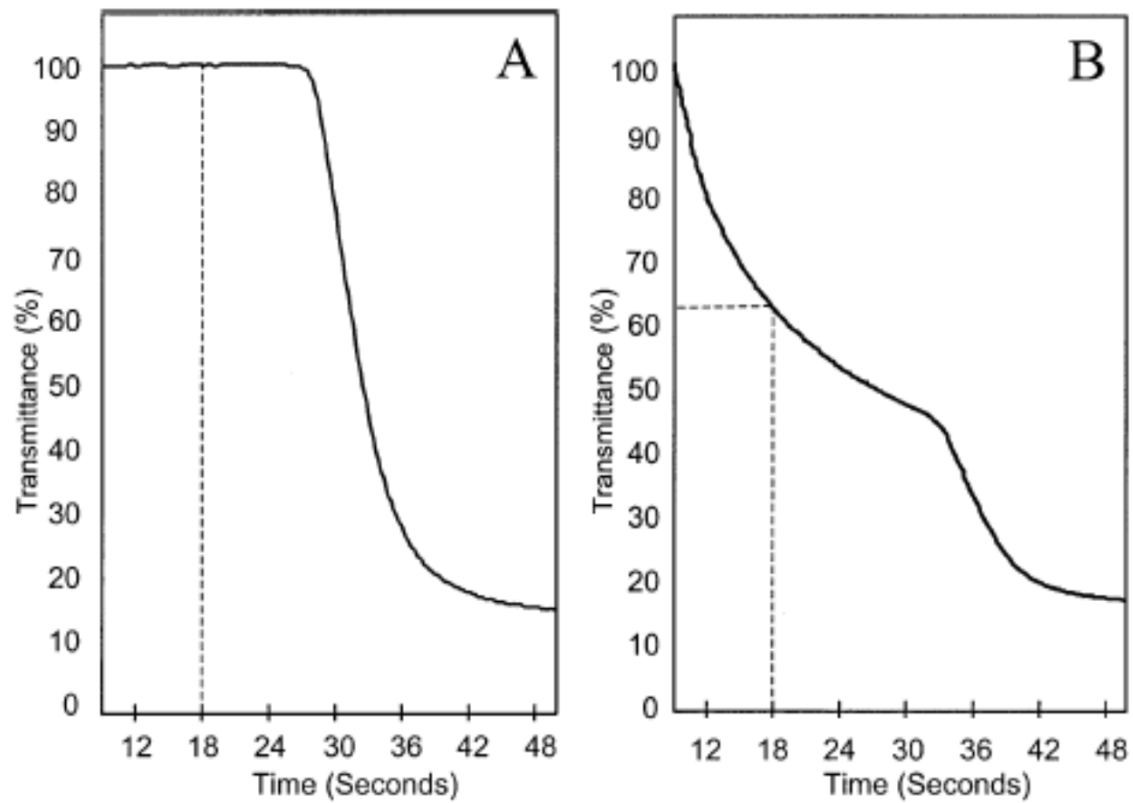


Figure 6