Clot waveform analysis in patients with haemophilia A

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Summary
Clot waveform analysis extends the interpretation of aPTT measurement curves. The curve is mathematically processed to obtain information about fibrin formation kinetics including semiquantitative determination of thrombin, prothrombinase and tenase activity. Patients, method: In this study the feasibility of clot waveform analysis for monitoring of haemophilia A was investigated using blood samples from healthy controls as well as haemophilia A patients under various clinical conditions. Results: Thrombin, prothrombinase and tenase activity show a high correlation to factor VIII levels. Tenase activity was found to exhibit a linear relationship to factor VIII levels over a very large concentration range and was able to discriminate patients with severe, moderate and mild haemophilia. Conclusion: Clot waveform analysis is an easy, fast and cheap method to access disturbances in clot formation and can be done without any additional measurements beside an aPTT.

Zusammenfassung

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An expanded interpretation of optical data retrieved during clot formation in measurements of activated partial thromboplastin time (aPTT) or prothrombin time (PT) was proposed to provide additional information about coagulation disorders (1). Using transmittance data from aPTT measurements an improved classification of factor VIII (FVIII) deficiency at levels less than 0.01 U ml⁻¹ was achieved (2). This approach, called clot waveform analysis, also allowed monitoring of FVIII substitution therapy in patients with high responding inhibitors (3). On the other hand, a biphasic waveform caused by formation of a complex of C-reactive protein and very-low-density lipoprotein is an indicator for disseminated intravascular coagulation and the degree of waveform abnormality correlates with the severity of haemostatic dysfunction (4).

The inability to create sufficient amounts of activated factor X is a hallmark of haemophilia (5). Subsequently, the amount and velocity of thrombin and fibrin formation is diminished (6). Extending the analysis of clot formation kinetics beyond the first appearance of fibrin poses a promising tool in diagnosis and monitoring of haemophilia. Compared to thrombin generation assays, clot waveform analysis is performed at a fraction of effort and cost. On the other hand, measuring absorbance excludes the use of platelet-rich plasma or whole blood.

Technical background
Considering basic physico-chemical principles, the method may provide additional information about coagulation disorders. By using an optical detection system, alteration of absorbance (or transmission) (1–4) during clot formation can be related
to fibrin concentration, and to some extent to fiber thickness (7), similar to the Beer-Lambert law (the Beer-Lambert law does not apply when light is scattered within the sample; nevertheless, attenuation of light due to scattering follows a similar relationship in respect to the concentration). The subsequent derivatives of the measured curve with respect to time can be related to fibrin formation properties through Michaelis-Menten kinetics (▶Tab. 1, ▶Fig. 1). Measured data are processed mathematically to obtain information regarding fibrin formation (measured curve), thrombin activity (1st derivative), prothrombinase activity (2nd derivative) and tenase activity (3rd derivative). The use of the first derivative to calculate thrombin activity is already used in thrombin generation assays where an artificial substrate is used instead of the natural substrate fibrinogen. Using the same rationale, prothrombin can be seen as the substrate and prothrombinase as the enzyme. The first derivative of the thrombin activity in respect to time, which is the same as the second derivative of the fibrin formation curve, leads to prothrombinase activity. Similar, the third derivative leads to tenase activity. This approach is the reversal of calculating fibrin formation from systems of differential equations represent-

Fig. 1 Clot waveform analysis interprets the time course on optical data obtained during fibrin formation in the coagulation process by calculating derivatives of the measured curve (actual sample). The y-axis exhibits a non-linear scale.

Fig. 2 Comparison between 1st derivative of the normalized fibrin formation curve and the thrombin activity (calculation according to Materials and Methods). Time course of 1st derivative and thrombin activity for a representative sample (left) and correlation between both parameters in samples from this study (right).

Fig. 3 The strong correlation between all clot waveform analysis parameters (top, exemplary tenase activity, 3rd derivative) and fibrinogen levels is eliminated upon normalization (bottom) of the underlying measured curve.
ing the waterfall sequence of the coagulation system (8, 9).

As all those processes depend on the concentration of the involved reaction partners and their mutual affinities, the data only represent semi-quantitative measurements. Furthermore, the consumption of reaction partners and substrates allows data interpretation only within the first phase of the coagulation process.

Patients, material, methods

All patients and controls were from our Center of Coagulation Disorders at the MVZ Lab Dr. Reising-Ackermann and Colleagues. Specimens were obtained after informed consent in accordance to the Declaration of Helsinki.

The measurements were performed on the fully automated ACL TOP 500 (Instrumentation Laboratory, Munich, Germany).

Synthasil (Instrumentation Laboratory), an aPTT reagent containing synthetic phospholipids, was used to activate the intrinsic coagulation cascade. Data of the measured curves were exported into a spreadsheet and derivatives were calculated according to the Savitzky-Golay algorithm (10) using Microsoft Excel (the algorithm was performed using a 3rd degree polynomial comprising 25 data points).

Thrombin activity was alternatively calculated as published by Rand (11) using reaction constant as published by Higgins (12) assuming 90% consumption of fibrinogen and compared to values of the 1st derivative.

\[
\text{Thrombin (t)} = \frac{\text{dA/dt}}{k_{\text{cat}}} \left(1 + \frac{[\text{Fibrin(t)}] + k_{M}}{[\text{Fibringogen(t)}]}\right)
\]

Total change in absorbance of each reaction curve is normalized to a fixed absorbance change (100 x 10^{-3} absorbance units). For normalized data, curves with incomplete fibrin formation (absorbance is still changing at the end of the measurement) may be excluded from further analysis. Clot waveform analysis parameters reflect the maxima of each derivative. A software (Borland Delphi) was developed to expedite data analysis.

FVIII was determined using a one-stage assay (Instrumentation Laboratory) or a chromogenic two-stage assay (Siemens Healthcare Diagnostics, Marburg, Germany).

Results

The concept of clot waveform analysis was tested in patients and controls. The 1st derivative, representing thrombin activity showed a very high correlation (r² = 0.95, Fig. 2) with the actual thrombin activity using a modified Michaelis-Menten equation including substrate inhibition. Similar comparisons between 2nd derivative and prothrombinase activity was not performed, as too many unknown variables are involved (e.g. free factor Xa or degree of factor Xa consumption). The appearance of negative values in the 2nd and 3rd derivative due to inhibition (e.g. antithrombin, activated protein C) and substrate consumption make the calculations require a high number of assumptions. Furthermore, the
assay is unable to differentiate between intrinsic and extrinsic tenase.

All investigated clot waveform analysis parameters show a significant correlation with fibrinogen levels that is completely eliminated upon normalization (Fig. 3). Constant absorbance at the end of the measured curve is mandatory, otherwise normalization leads to overestimated values for the subsequent derivatives.

Patients with haemophilia A show significantly impaired fibrin formation in clot waveform analysis. Severe haemophilia A exhibits a delayed begin of clotting, thrombin, prothrombinase and tenase activity are extremely low or not detectable. A significant improvement of clot waveform analysis parameters was found in samples from patients with moderate or mild haemophilia. The four patients with the lowest FVIII levels (around 0.01 U ml$^{-1}$) showed the lowest values in the 3rd derivative of clot waveform analysis.

The maxima of all derivatives show a high positive correlation with FVIII levels over a wide concentration range. While a logarithmic relationship is observed between FVIII level determined in the one-stage assay and the maximum thrombin activity (1st derivative), it becomes increasingly linear for prothrombinase activity (2nd derivative) and tenase activity (3rd derivative) as seen by increased correlation coefficients for a linear model. Similar results were obtained in comparison to the two-stage assay (data not shown).

FVIII substitution therapy was monitored in a patient undergoing surgery at 15 different time points. An almost identical time course of FVIII levels and tenase activity determined by clot waveform analysis was observed (Fig. 5). FVIII recovery was monitored in 11 patients. Prothrombinase activity and even more tenase activity showed good correlation with the increase of FVIII activity (Fig. 5).

Discussion

In this study, the feasibility of clot waveform analysis for monitoring of haemophilia A patients was investigated. The parameters determined by the method correlate well with conventionally determined FVIII levels over a wide concentration range. Even at very low levels of FVIII clot waveform analysis showed a very good discrimination between samples with small differences in FVIII levels, a phenomenon that was previously described by Shima (2). This study expands the analysis of aPTT measurements to the third derivative, assumed to represent tenase activity. The linear relationship between FVIII levels and the obtained tenase activity supports the validity of the presented approach. Although slightly lower correlation is observed between FVIII levels and prothrombinase activity or thrombin activity, the method underlines that haemophilia leads to a delayed and diminished generation of factor Xa and thrombin, respectively.

It could be shown that the 1st derivative correlates well with the actual thrombin concentration obtained by an advanced mathematical model (11, 12). For the subsequent derivatives such a model would contain too many assumptions to yield a reliable comparison.

The total absorbance change, and subsequently, the maxima of all derivatives of the measured curve strongly depend on the fibrinogen level in the sample. Normalization is shown to eliminate this effect and leads to a significantly improved correlation of all determined parameters to FVIII. On the other hand, fibrinogen is the natural substrate of the coagulation cascade and has itself a significant influence on clot formation in vitro and in vivo. Further studies may show, whether inclusion or exclusion of fibrinogen provides better information about a patient's coagulation state.

It is noteworthy, that clot waveform analysis is unable to discriminate between FVIII and FIX deficiencies. Furthermore, this study did not include patients with defects in other coagulation factors than FVIII. As the method uses absorption measurements for the determination of fibrin formation, platelet rich plasma or whole blood can not be used as a specimen. On the other hand, applying clot waveform analysis on thrombin generation measurement curves is conceivable. Alterations of plasma that interfere with aPTT or absor-
monitoring of absorbance changes during an aPTT measurement and can be performed on most of the modern coagulation analyzers, although additional software is required. The analysis itself is performed using a simple and fast mathematical approach, reducing the required technical effort to a minimum.

Conflict of interest
The authors declared that they have no conflict of interest.

References

Appendix

 Calculation

The Michaelis-Menten equation for the cleavage of a substrate $S$

$$v = \frac{v_{max} \cdot [S]}{[S] + k_m}$$

The reaction velocity $v$ is given by the first derivative of the substrate concentration $S$ in respect to time $t$

$$v = -\frac{d[S]}{dt}$$

The maximal reaction velocity $v_{max}$ is given by the enzyme concentration and the $k_{cat}$

$$v_{max} = [E] \cdot k_{cat}$$

Therefore,

$$-\frac{d[S]}{dt} = [E] \cdot k_{cat} \cdot \frac{[S]}{[S] + k_m}$$

As we observe the generation of the product $P$ (fibrin) instead of the cleavage of the substrate $S$ (fibrinogen) we write

$$\frac{d[S]}{dt} = -\frac{d[P]}{dt}$$

The change of the product concentration $d[P]/dt(t)$ is observed measuring absorbance change $d[\lambda]/dt(t)$ similar to Lambert-Beer which is the first derivative of the measured curve

$$\frac{d[P]}{dt} = \frac{d[\lambda]}{dt} \cdot \frac{d \cdot \varepsilon}{[S]}$$

Finally we get

$$\frac{d[\lambda]}{dt} \cdot \frac{d \cdot \varepsilon}{[S]} = [E] \cdot k_{cat} \cdot \frac{[S]}{[S] + k_m}$$

Rearranged the enzyme concentration $E$ is given by

$$[E] = \frac{\frac{d[\lambda]}{dt} \cdot \frac{d \cdot \varepsilon}{[S]} + k_m}{[S]}$$

If the substrate concentration $S$ exceeds $k_m$ or the change of the substrate concentration $S$ is insignificant $([S] + k_m)/[S]$ is considered constant, all constant values are combined

$$[E] = \frac{d[\lambda]}{dt} \cdot \frac{d \cdot \varepsilon}{[S]}$$

Now we can calculate (estimate) a thrombin generation curve from the fibrin generation curve (similar to thrombin generation where a fluorogenic substrate is measured instead of fibrin) by calculating the first derivative of the absorbance (fibrin formation) curve. In a similar way Xa generation is calculated as the first derivative of the now calculated thrombin generation curve which is the second derivative of the fibrin formation curve. Tensase activity is the first derivative of Xa activity, the second derivative of thrombin activity and the third derivative of fibrin concentration at any time point. From this method it is impossible to distinguish between extrinsic and intrinsic tensase.

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