The technique of measuring thrombin generation with fluorescent substrates: 4. The H-transform, a mathematical procedure to obtain thrombin concentrations without external calibration

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Summary

In fluorogenic thrombin generation (TG) experiments, thrombin concentrations cannot be easily calculated from the rate of the fluorescent signal increase, because the calibration coefficient increases during the experiment, due to substrate consumption and quenching of the fluorescent signal by the product. Continuous, external calibration via an in a parallel sample therefore was hitherto required for an accurate calculation of the TG curve. A technique is presented that allows mathematical transformation of experimental fluorescence intensities into "ideal" data, i.e. in the data that would have been obtained if substrate consumption and quenching by the product would not play a role. The method applies to fluorescence intensities up to 90% of the maximal fluorescent signal corresponding to total substrate conversion and thereby covers the entire region of interest encountered in practice. The first derivative of the transformed signal can then be converted into thrombin concentrations via a conventional, fixed calibration factor. This calibration factor can be obtained from a separate experiment but also by measuring the amidolytic activity of the $\alpha_2$macroglobulin-thrombin complex present in the reaction mixture ("serum") after thrombin generation is over. This method halves the amount of sample required per experiment.

Keywords

Thrombin generation, fluorogenic methods, calibration

Thromb Haemost 2009; 101: ■■■

Introduction

Thrombin generation (TG) is being increasingly used as a method to assess the over-all function of the coagulation system. In a fluorescence plate reader TG can be monitored in large series of plasma samples via the conversion of a suitable fluorogenic thrombin substrate. The problem remains of how to convert the time course of fluorescence signal (F(t)) into the time course of thrombin activity (T(t)). The calibration factor (Cf) i.e. the ratio between thrombin activity (T) and velocity of increase of fluorescence (dF/dt) varies between plasmas and increases in the course of the experiment. Neglecting this fact (1) leads to gross errors in the determination of the endogenous thrombin potential (ETP) (2).

In the calibrated automated thrombogram (CAT) method (3) this problem is solved by comparing, at every level of fluorescence, the reaction rate in the TG experiment to that in a parallel experiment where a constant thrombin activity is added. This method allows reliable determination of the course of thrombin activity but necessitates simultaneous measurement in two wells and a dedicated computer program to compare the two signals.

The measured reaction velocity per unit enzyme decreases with time because the concentration of substrate decreases and because the fluorescent product that is formed quenches the signal in a concentration dependent way. Cf therefore is dependent upon F and, as we have shown before (2), within the same experiment, upon F only.

It is possible to calculate the precise effects of substrate consumption and fluorescence quenching (4–6). We abandoned this approach because it required the calculation of more parameters than could reliably be obtained in a routine setting. Instead, we reasoned that the theoretical relationships can be approached by linear ones: Within certain limits the reaction velocity, at fixed enzyme concentration, can be assumed to be linearly proportional to the substrate concentration ($dS/dt \propto S$). Similarly, the increase of fluorescence per unit of product can be assumed to decrease in linear proportion to the level of fluorescence ($dF/dP = -dF/dS \propto k$"F). As long as these proportionalities hold, the change of fluorescence with time (always at constant thrombin activity) would then be proportional to the square of the fluorescence intensity, because $dF/dt = dF/dS.dS/dt = kF^2$. This relation provides a means to test in how far the as-

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Received: September 2, 2008
Accepted: October 15, 2008

Prepublished online: ■■
doi:10.1160/TH08-09-0562

sumption of linearity holds under our experimental conditions, because it predicts that dF/dt plotted against F would be a parabola.

In a first experimental section of the present article it is shown that with ZGGR-AMC as a substrate and under the usual conditions of a TG experiment, the approximation holds within the limits of acceptable experimental error over the whole range of practical interest.

In a second, mathematical section we used the relation $dF/dt=kF^2$ to derive the relationship between fluorescence and time at constant thrombin activity (F=f(t)). From that relation we then developed a simple mathematical procedure that allows to transform the fluorescence traces that are measured in practice into the "ideal" traces that would have been obtained if substrate consumption and inner filter effect would not influence the measurement. This "H-transform" requires the determination of one constant only (α), that can be found in any TG experiment from the boundary condition that the ideal signal is linear when the enzymatic activity is constant. Constant enzymatic activity is found in a TG experiment, when free thrombin has disappeared and only the amidolytic activity of formed α2macroglobulin-thrombin (α2M-T) remains.

In a second experimental section we apply the H-transform to fluorescence traces obtained at constant thrombin activity and confirm that, until 90–95% of the maximal fluorescence level is reached, the curved lines that are obtained in practice are transformed into straight ones with a slope equal to the initial rate of fluorescence production, i.e. that one single calibration factor applies over the entire H-transformed curve.

In the third experimental section the H-transform is applied to obtain TG curves from normal plasma and plasma from patients under oral anticoagulation and the results are compared to those obtained with the CAT method.

Material and methods

Chemicals

Ancrod, the fibrinogen clotting enzyme of the Malayan Pit Viper, was the commercial preparation Arvin (Knoll AG, Ludwigshafen, Germany). Phosphatidylserine, phosphatidyl-ethanolamine and phosphatidyl-choline were from Avanti (Alabaster, AL, USA). Recombinant relipidated tissue factor (rTF) was innovin from Dade Behring (Marburg, Germany).

The fluorogenic thrombin substrate for TG experiments was Z-Gly-Gly-Arg-aminomethylcoumarine (ZGGR-AMC) from Bachem (Basel, Switzerland). The chromogenic substrate for measuring thrombin activity was S2238 (Chromogenix, Mölndahl, Sweden). All other chemicals were commercially obtained at the highest available grade of purity.

The α2macroglobulin-thrombin complex (A2M-thrombin), i.e. the preparation of stable thrombin-like activity in plasma was prepared as described previously (3).

Reagents

Buffers:

- BSA5 for preparing dilutions of the reagents: 20 mM Hepes, 140 mM NaCl, 0.02% NaN_3 and 5 mg/ml bovine serum albumin (BSA) at pH 7.35.
- BSA60 for solution of the fluorogenic substrate: 20 mM Hepes, 0.02 % NaN_3 and 60 mg/ml BSA at pH 7.35. BSA it is brought on top of the buffer without stirring and allowed to dissolve slowly (> 1 hour!). The buffers are filtered using a corning filter system 255 ml with 0.2 µm PES (polyethersulfone) membrane and stored at –20ºC.
- A stock suspension of phospholipids (20 mol% phosphatidylserine, 20 mol% phosphatidyl-ethanolamine and 60 mol% phosphatidyl-choline) is made by sonication of the phospholipids for 2x 5 minutes (min) and brought to a final concentration of 30 µM, stored at –80ºC.
- A mixture of fluorogenic substrate and $CaCl_2$ is prepared daily. To 875 µl of buffer BSA60, at 37°C, 100 µl of 1 M $CaCl_2$ is added, then 25 µl of a dimethyl sulfoxide (DMSO) solution of 2.5 mM ZGGR-AMC is squirted in and immediately vigorously mixed (vortex).
- The trigger solution (extrinsic system) contains five volumes of 30 µM procoagulant phospholipids in Hepes buffer A and one volume of 180 pM rTF. The latter is prepared by dilution of commercial Innovin. Commercial Innovin that gives a clotting time of 17 seconds (s) with normal plasma has been found to contain 6 nM rTF, by comparison to a well characterised rTF preparation donated by Prof Y. Nemerson (Mt. Sinai, New York, NY, USA).

Blood and plasma

- Blood was obtained through antecubital venipuncture (1 volume trisodium citrate 0.13 M to 9 volumes blood) from healthy consenting individuals.
- Platelet-poor plasma (PPP) is prepared by centrifuging twice at 2,900 g for 10 min at room temperature. In order to avoid contamination with procoagulant microparticles from aging platelets, PPP is prepared within 30 min after venipuncture.
- Defibrinated PPP was prepared by adding Ancrod to PPP at a final concentration of 1 U/ml. The plasma is mixed well (vortex) and kept at 37ºC for 10 min. Then it is put on ice for 10 min. The clot is wound out at a plastic spatula.

Fluorescence-based measurement of thrombin activity

The development of fluorescence intensity from aminomethylcoumarine (AMC) was measured in a 96-well plate fluorometer at an excitation wavelength of 390 nM and emission wavelength at 460 nM. Either an Ascent reader (Thermolabsystems OY, Helsinki, Finland) with dispenser was used or a Spectramax M2^e Fluorescence microplate reader and the reaction started manually. Immulon 2HB, round-bottom 96-well plates (Dynex) were used. CAT was performed as described before (3) using dedicated software (Thrombinoscope, Maastricht, The Netherlands). A minimum of four readings was done per minute, and experiments were carried out in triple unless otherwise indicated.

To each well, 80 µl of plasma was added. Wells in which TG was measured received 20 µl of buffer, containing the trigger but no Ca^{2+}.

Wells in which constant thrombin-like activity was to be measured received 20 µl of the α2M-thrombin solution. At 1.2 µM, the final concentration of α2M-thrombin was 200 nM, which corresponds to 100 nM of thrombin-activity (3). For TG the trigger was 30 pM of recombinant tissue factor (TF) and

24 µM phospholipid preparation, i.e. at final concentrations in the reaction mixture of 5 pM TF and 4 µM phospholipids.

The plate was placed in the fluorometer and allowed to warm to 37°C (minimally 15 min). The dispenser of the fluorometer was flushed with warm 100 mM $CaCl_2$ solution, emptied, and then flushed with a prewarmed (37°C) solution of the substrate in Hepes buffer with 60 g/l BSA (FluCa). At the start of the experiment, the Ascent instrument dispenses 20 µl of FluCa to all the wells to be measured, registers this as zero time, shakes them for 10 s and starts reading.

Chromogenic determination of residual thrombin-like activity

A 10-µl aliquot of the fluid remaining in the well after the TG experiment is over was added to a tube containing 465 µl EDTA buffer and 25 µl of a 4 mM solution of S2238. The tube was prewarmed to 37°C. After 2–4 min incubation time the reaction was stopped by adding 0.3 µl of 1 M citric acid. The concentration of p-Nitroaniline was measured in a spectrophotometer at 405 nm (see [7])

Data handling

The raw data of optical density or fluorescence measurements of thrombin activity were exported to SIGMAPLOT version 9.0 (Systat Software Inc., Point Richmond, CA, USA) or to EXCEL (Microsoft® Excel XP or higher) for further mathematical operations. The hyperbolic arctangent (arctanh) is a standard function (8). Its value can be found in a spreadsheet (=atanh() in Excel) in the same way as the more familiar goniometric functions (e.g. sinus). A spreadsheet that explains the calculations to be performed on a raw fluorescence signal is to be found at www.thrombin.com.

Experimental study

Testing of the hypothesis

We determined fluorescence traces in 24-fold, at four fixed α2M-thrombin concentrations at three substrate concentrations each. The 24 traces were obtained in one run of the fluorometer. Half of the experiments was done in normal plasma, the other half in the same plasma after defibrination; these data were identical to within less than one standard deviation (SD) of each condition (n=24) so they were pooled. Figure 1 shows the ± 1 SD – limits of the experimental traces (black) obtained at the usual substrate concentration (416 µM). The results obtained at 104, 208 and 312 µM of substrate were similar except for the scale of the ordinate and are not shown.

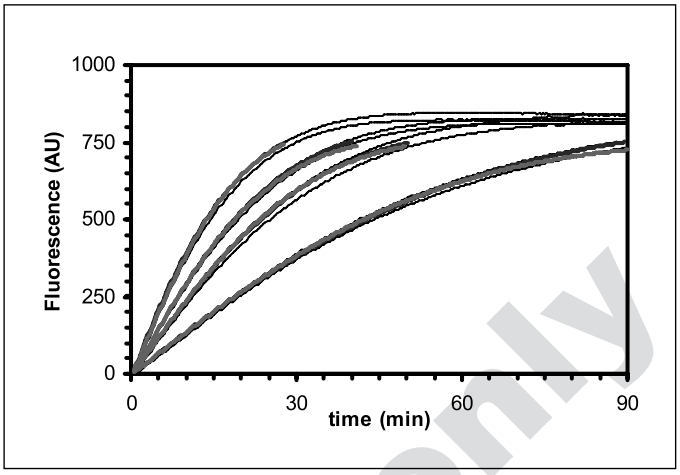

Figure 1: Fluorescence traces at different fixed enzyme concentrations. From left to right 200, 133, 100 and 50 nM. Black lines show the ± 1 SD limits of the experimental data. Blue lines: theoretical lines obtained using the constants from the best fitting parabolas in Figure 2. Red lines: Best fitting lines under the assumption that k_3=0 (see next section). Where no blue lines can be seen, they are coincident with the red ones.

The data from the fluorescence traces were then plotted as dF/dt against F. As explained in the introduction, simplifying assumptions on the relation between dF/dt and enzyme concentration apply if these curves can be described by parabolas. Because such plots allow to judge in how far this approach can be used in practice we called them "diagnostic plots". In Figure 2 it can be seen that it is possible to fit parabolas to these data within or close to the ± 1 SD (n=24) limits (Fig. 2). Using the formulas that are developed in the next section and the parameters of the fitted parabolas we then constructed the theoretical fluorescence curves that would have appeared if the experimental dF/dt=f(F) relation would indeed have been exactly rendered by the fitted parabola (blue lines in Fig. 1). These curves are again within or close to the ± 1 SD (n=24) limits up to ~ 90% of the maximal fluorescence. We conclude that, under the given conditions, the assumption of a parabolic relationship between dF/dt and F is not to be rejected as long as the fluorescence measured is < 90% of its maximal value.

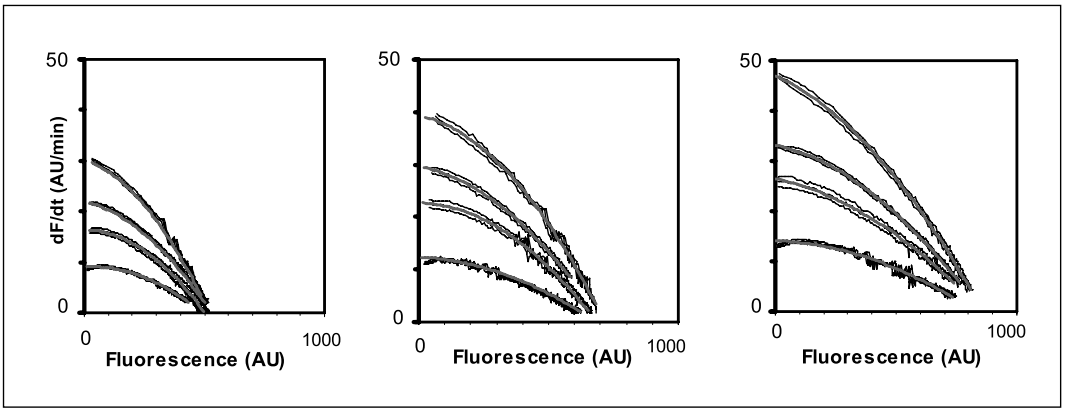

Figure 2: Diagnostic plots at three substrate and four enzyme concentrations. From left to right 208, 312 and 416 µM ZGGR-AMC. From top to bottom 200, 133, 100 and 50 nM thrombin activity, added as the double concentration of α2macroglobulin-thrombin complex. Black lines experimental data, mean ± 1 SD. Red lines: fitted parabolas.

Mathematical interlude

In this section we develop the "H-transform" i.e. the algorithm that enables to convert experimental fluorescence data to the "ideal" data that would have been obtained if substrate consumption and fluorescence quenching by the product would have no influence. Except for the last paragraph, this part can be skipped by those who want to limit themselves to the use of the formulas in practice.

If, at constant enzymatic activity, there is a parabolic relation between the experimentally obtained fluorescence signal and its derivative (Fig. 2) i.e.

$$dF_{expt}/dt = k_1^2-k_2^2(F_{expt}+k_3)^2 \quad (1)$$

then mathematical analysis shows that the signal in time is given by the function:

$$F_{expt}(t)=k_3 + k_1/k_2\tanh(k_1k_2t-\operatorname{arctanh}(k_2k_3/k_1)). \quad (2)$$

In Figure 1 (blue lines) the course of this function of time is shown, when the values of the constants are those that are obtained by fitting a parabola to the curves of formula (1).

The derivative of this function in the origin is

$F_{expt}'(0)=k_1^2-k_2^2k_3^2$ and the "ideal" signal, i.e. a signal not disturbed by substrate consumption and quenching by the product would be:

$$F_{ideal}(t)=(k_1^2-k_2^2k_3^2)t \quad (3)$$

By eliminating t from equations (2) and (3) we obtain the formula that can transform any experimentally obtained fluorescence value $F_{expt}(t)$ into its corresponding ideal value; because for any $F_{expt}(t)$

$$F_{ideal}(t)=((k_1^2-k_2^2k_3^2)/(k_1k_2))(\operatorname{arctanh}(k_2k_3/k_1)+ \operatorname{arctanh}(((F_{expt}(t)-k_3)k_2)/k_1)) \quad (4)$$

If k3=0 this formula simplifies to

$$F_{ideal}(t)=(k_1/k_2)\operatorname{arctanh}(F_{expt}(t)k_2)/k_1) \quad (5)$$

If we define $\alpha= k_1/k_2$, this formula becomes

$$F_{ideal}(t)= \alpha \operatorname{arctanh}(F_{expt}(t)/?) \quad (6)$$

This is the "H-transform" i.e. the formula that transforms experimental fluorescence data (F(t) into the data that would have been obtained in the ideal case where no substrate consumption and quenching of light by the product would occur (9).

Whether it is justified to assume that $k_3=0$ depends on the contribution of k_3 to the goodness of fit of the theoretical functions to the experimental data. If we assume that $k_3=0$, formula (2) reduces to:

$$F(t)=k_1/k_2\tanh(k_1k_2t). \quad (7)$$

When this relation, instead of formula (2), is used to reconstruct the theoretical time course of F(t), the red lines in Figure 1 are obtained. It is seen that the contribution of k_3 to the goodness of fit is minimal, so that formula (6) can be used for all practical purposes.

The value of α is the intercept of the parabola with the abscissa in Figure 2, i.e. the point where formula (2) does no longer increase with increasing F. Although in practice it may be very close to the maximal fluorescence observed, it is not necessarily identical because the experimental points do not necessarily fit the parabola at very high fluorescence values. In other words: the upper limits of the experimental fluorescence (black lines in Fig. 1) are not necessarily identical to the upper limits of the theoretical functions (red or blue lines in Fig. 1).

The H-transform is checked experimentally in the next section, by testing whether a value α can be found that transforms the fluorescence traces obtained at fixed enzyme concentration into straight lines.

In summary: In practice one can transform experimentally obtained fluorescence intensities (F_{expt}) into the intensities that would have been obtained if substrate consumption and inner filter effect would not play a role (F_{ideal}) by using formula (6): $F_{ideal}(t)= \alpha \operatorname{arctanh}(F_{expt}(t)/\alpha)$. The constant α is found as explained below. A spreadsheet in which the calculation is carried out in practice is to be found at www.thrombin.com.

Validation of the H-transform

For practical purposes the proof of the H-transform is in its performance. For the H-transform to serve its purpose, it should convert the curved line of fluorescence development at constant thrombin activity into a straight one, at least at fluorescence values < 90% of maximal. Moreover the slope of that line should be equal to the initial velocity measured. This we tested first on the same fluorescence data as those used for obtaining Figures 1 and 2.

In Figure 3 the experimental data are rendered. The white dots represent the prolongation of the initial slope. The test for the validity of the H-transform then is whether a value of α can be found that transforms the experimental data (red) into a straight line (black) with a slope identical to the experimental initial rate (white dots). The figure shows that this is possible at all substrate and enzyme concentrations tested, and within the narrow limits of ±1 SD (n=24).

It should be stressed that α is an experimentally found value, selected by virtue of its ability to transform the experimental data in "ideal" data. It is the intercept of the parabolas in Figure 2 with

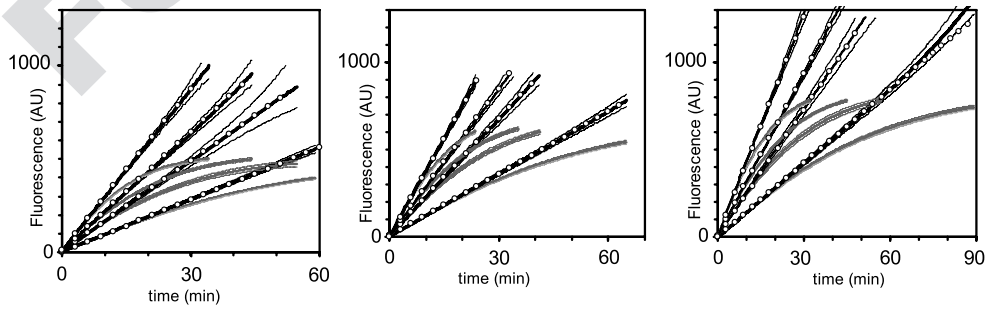

Figure 3: Application of the H-transform to data obtained at fixed enzyme concentrations. The red lines show the ± 1 SD limits (n=24) of the experimental data. The black lines give the transformed data, the white dots represent the extrapolation of the initial slope of the experimental data.

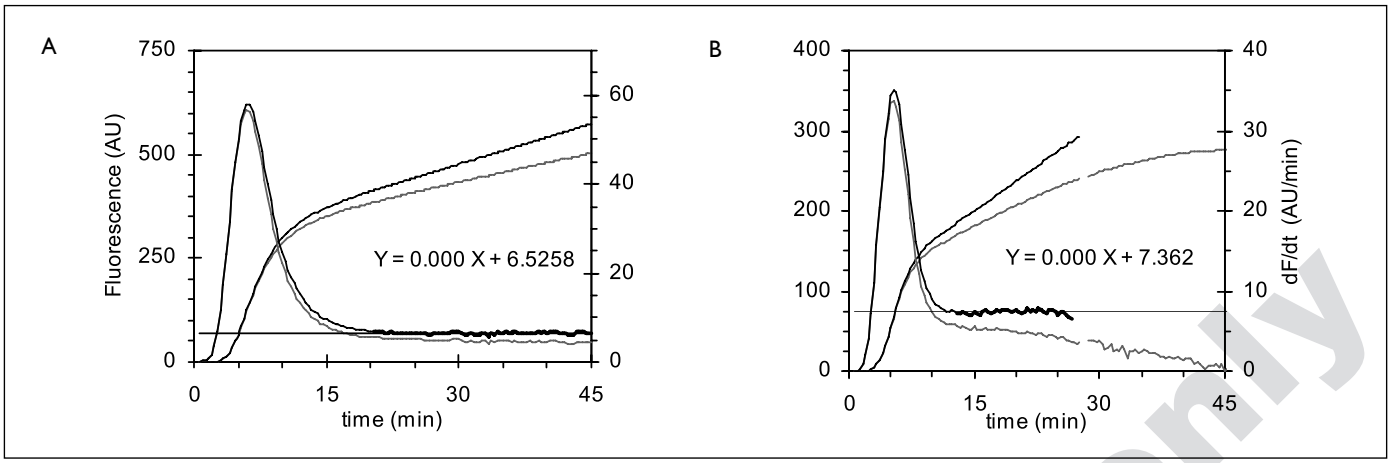

Figure 4: Application of the H-transform to thrombin generation (TG) curves. A) Normal plasma; B) Defibrinated plasma. Red lines Original data, black lines: Transformed data. Steadily increasing lines: (Transformed) fluorescence data; lines with peak: first derivative of (transformed) fluorescence data. Horizontal black lines: best fitting straight line through the end-portion of the transformed derivative. The formula is that of the trendline through this end-portion. The zero-slope indicates that the right value of α has been found and the constant indicates the level of arbitrary fluorescence units that corresponds to the amidolytic activity in the well at the end of the experiment. The data were obtained in different fluorimeters.

the abscissa and similar but not identical, to the maximal value of fluorescence that is measured at very high product concentrations.

Application of the H-transform

The H-transform can be used to obtain a correct TG curve from a single experiment. For this we first transform the experimental data into the "ideal" data with the formula $F_{ideal}=\alpha \text{arctanh}(F_{expt}/\alpha)$. The right value of α is that one that transforms a stretch of observations at a fixed amidolytic activity into a straight line. Such a set of observations is automatically at hand in the "tail" of a TG experiment. The "tail" being defined as the part where free thrombin has disappeared and only the α2M-thrombin complex persists. The start of the tail is most conveniently recognised in the first derivative, i.e. in the peaked curves in Figure 4. There are various manners to determine the start of the tail, a convenient one is to determine the time (s) that is required to descend from the top value to half the top value. At 5 x s after the top value, TG can for all practical purposes be assumed to be over. The "end tail" is either the end of the experiment or the point where the transformation formula no longer holds, i.e. where the experimental fluorescence values exceed 90% of the maximal end level (e.g. Fig. 4B).

We here show the application of this procedure to the data from a TG experiment in a defibrinated and in a non-defibrinated sample of pooled normal plasma (Fig. 4). The data were entered in a spreadsheet and the value of α was varied until the slope of the transformed "tail" equalled zero.

We determined the end amidolytic activity in the same normal- and defibrinated normal plasmas in 12-fold. In the non-defibrinated plasma we found 31.2 ± 1.7 nM thrombin activity in the normal plasma and 85.2 ± 3.6 nM in the defibrinated plasma. We had not to reckon with instability of the residual α2M-thrombin activity because in the calibrator wells the α2M-thrombin added (50, 100, 133 or 200 nM) was quantitatively (97 ± 4.6 %, n=12) recovered after 60 min reaction time.

The end level of the transformed tail, i.e. 6.5258 AU/min therefore corresponds to 31.2 nM thrombin, i.e. the scaling factor for the non-defibrinated plasma therefore is 31.2/6.53 = 4.78 nM/AU. The scaling factor for the non-defibrinated plasma is 11.6 AU/min. When these scaling factors are applied to the right ordinates in Figure 4, the transformed lines represent the actual amidolytic activity curves. In order to obtain the free thrombin curves the α2M-thrombin component has still to be subtracted (see [10]).

The H-transform was applied in the same manner to a pooled normal plasma that was tested (n=4), 16 times in the course of one month. The peak value found was 318 ± 21 nM (coefficient of variation [CV] 6.8%) and the ETP 1621 ± 70 nM.min (CV 4.3%). This compared well to the values found with the CAT method: peak 300 ± 92nM (CV 8%) and ETP 1599 ± 81 nM.min (CV 5%).

Comparison of the new methodology with the CAT method shows that the curves obtained with the two methods compare well but are not necessarily identical. Further research will have to show whether there are systematic differences and if so why. See further in the discussion.

Discussion

The simple observation that a fixed amount of amidolytic activity of the thrombin molecule does not cause a linear increase of fluorescent signal (Fig. 1), whereas the enzymatic activity is found back quantitatively after the experiment, indicates that one cannot simply calculate thrombin activities from the velocities of fluorescence increase. Evidently the ratio between enzyme concentration and reaction velocity (Cf) varies during the experiment. This problem is sometimes neglected and a fixed initial Cf has been employed during the whole of the experiment (1, 11).

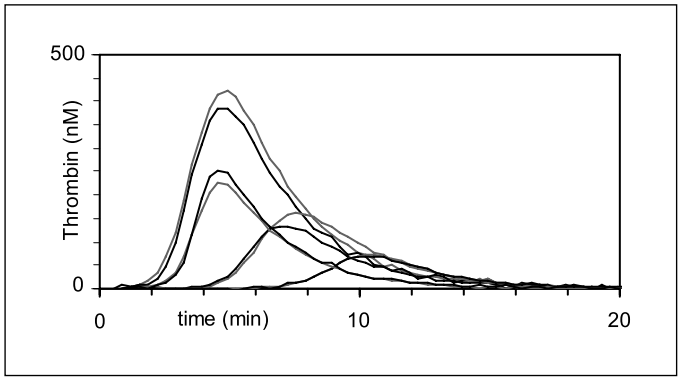

Figure 5: Comparison of internal and external calibration. In the order of peak height: Normal plasma and plasma from orally anticoagulated patients with international normalized ratios (INRs) of 1.3, 3.0 and 4.1. Red lines: Internal calibration as presented in this article, Black lines: CAT method. N=4 for each curve.

This may lead to significant errors (2), unless it can be demonstrated, as in the case of some chromogenic substrates (12), that a sufficiently small fraction of the substrate is consumed during the reaction. The curvature is due to substrate consumption in combination with the non-linearity of the fluorescent signal with the concentration of the fluorophore (inner filter effect). The transformation proposed is valid in the range where these two disturbances can be supposed to be linear, which is shown to be the case over the whole range of signal covered by a TG experiment. If there is only one disturbing factor, as is e.g. the case with chromogenic substrates or with fluorogenic substrates measured in thin layers where the inner filter effect does not occur, then another transformation formula applies, i.e. $F_{ideal}=-\alpha \ln (1-F_{expt}/\alpha)$ (proof not shown).

In practice three solutions have been proposed to cope with the curvature of the calibration line.

What is known on this topc?
- In fluorogenic thrombin generation experiments thrombin concentrations cannot reliably be obtained from the fluorescence trace by using a fixed calibration constant
- A parallel experiment in the same plasma without thrombin generation but with a fixed amount of thrombin activity added allows correct calibration but requires the double amount of sample and reactants.

What does this paper add?
- The formula $F_{ideal} = \alpha \text{arctanh}(F_{exp}/\alpha)$ converts experimental data into ideal data the first derivative of which can be converted into thrombin concentrations via a single calibration factor
- The correct value of α can be found because it yields a fixed end amidolytic activity.
- The calibration factor can be found by measuring the amidolytic activity in the material ("serum") remaining after the thrombin generation experiment is over.

- In the first type, a mathematical model of the combined effect of quenching by product and substrate consumption is constructed based on the known laws that govern these processes. The parameters of that model are then established by curve fitting from experimental data. Once these parameters (at least three) are known the model can be used to determine unknown enzyme concentrations (4–6). This approach requires a thorough kinetic analysis of the system and advanced mathematics to establish the parameters. It therefore is applicable to fundamental research but less practical for routine analysis.
- A second type of solution is continuous external calibration, so as used in the CAT method (3, 13, 14). Here observed fluorescence values are converted into ideal values by continuous comparison to a simultaneously measured, calibrator curve, obtained by adding a fixed, known amount of stable thrombin activity. This curve allows to establish the calibration factor at each level of fluorescence.
- A third, common type of solution is that the curvature of the non-linear calibrator curve is obtained experimentally in one situation and the reference curve is assumed to be identical in all experiments except for the scaling factor of the ordinate (e.g. [15]). The scaling factor is obtained from a single measurement under the actual situation. This solution has recently been proposed for the application to the measurement of TG by Giesen en Van Asten (16). In plasma, where differences in scaling factor are caused by the absorption and quenching properties of the individual plasma sample, the scaling factor can be found by adding known amounts of the fluorophore (17–19).

This last approach has the drawbacks that the curvature of the reference curve is experimentally determined in one situation only, fitted to an *ad hoc* mathematical expression and assumed to be identical in all other curves except for the scaling factor. In mathematical terms: A curve $F_{ideal}=f(F_{expt})$ is fitted to experimental data and another set of data is assumed to fit to the function $F_{ideal}=k.f(F_{expt})$, where k is the scaling factor. As long as this assumption holds this approach is a practical shortcut that has been shown to work in certain situations (15).

In the present article we prove that, within all practical limits, the reference curve is of the type $F_{expt}(t)=k_1/k_2 \tanh(k_1k_2t)$ (formula 7). The fundamental difference with the method discussed above being that $k_1/k_2 (= \alpha)$ is not a scaling factor but influences both the scale and the curvature of the curve. More important, the formula is based on formula (1) and experimentally proven to hold between 0 and 90% of the maximal possible signal. In fact this covers the entire region of interest in a TG experiment, because in the 90–100% region the increase of the signal per unit of enzyme activity per unit time is so small that accurate measurements become impossible anyhow.

Comparison of the present method with the CAT method (Fig. 5), applied to the same observations, shows that the results are comparable within the limits of accuracy of a routine experiment. This does not mean to say that the results are identical. Further research will have to show whether there are systematic differences. This could not be done in the context of the present article because it appeared that the CAT procedure is very sensitive to small shifts in temperature and that differences found

could be due to this sensitivity. The underlying phenomenon is the fact that temperature dependency of TG in a fibrin clot is fundamentally different from that of substrate conversion by the calibrator in free solution (20). An extensive analysis of temperature dependency is being prepared for publication in this series of articles.

In contrast to other methods, the present approach allows to visualise directly the extend of the correction that is required. In non-defibrinated normal plasma correction appears to be minimal (Fig. 4B). We applied the H-transform to 36 more normal plasmas with essentially similar results, i.e. the differences were so small that one might as well have refrained from correction. Previously we have observed that useful results can sometimes be obtained by direct comparison of uncorrected data from normal plasmas to a reference plasma (2). This broaches the interesting question of whether correction for substrate consumption and fluorescence quenching by the product is always required and if not, under what conditions it can be abandoned. This question is referred to a separate article of this series.

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