Disciplines of enzyme kinetics applied to data obtained in clotting tests yield results that cannot on first sight be discarded as meaningless. This implicates the necessity of a theoretical evaluation of the enzyme kinetics characteristic of clotting tests. An article presenting the basic assumptions and formulae has already been published (1).

In the preceeding article (2) it was deduced that a protein competitively inhibiting prothrombin conversion is found in the plasma of vitamin K-deficient patients. This article discusses the kinetics of competitive inhibition in clotting tests.

Our basic assumptions are:

1. In a one stage clotting-time estimation, either a "prothrombin-time" estimation or a specific test for any one factor of the extrinsic coagulation system, the clotting time is proportional to the inverse of the reaction velocity of the rate-limiting step. In a "prothrombin-time" estimation factor X is the rate-limiting factor, provided that factors I and V are added in excess (3).

2. The assumptions used in the derivation of the formulae of steady-state enzyme kinetics (4) hold for coagulation reactions except for the modification made by assumption 3.

3. In coagulation-tests substrates and inhibitors (if any) cannot be assumed to be present in excess over the enzyme.

We have already shown (1) that under these conditions the formula for clotting time in an uninhibited system becomes

$$t_c \cdot h = \frac{1}{E} + \frac{1}{S} + \frac{K_m}{E. S.} \qquad (1)$$

where t_c = clotting time
  h = a constant
  E = enzyme concentration
  S = initial substrate concentration
  K_m = the Michaelis constant of the interaction between E and S.

This formula was shown to be valid under the limiting condition $(E + S + K_m)^2 \geqslant 2ES$, which implies that either $2E < S + K_m$ or $2S < E + K_m$, which is not an unlikely assumption.

When we dilute a plasma sample, the concentration of the clotting factors after dilution is inversely proportional to the dilution factor (D), D being defined as the ratio of the volume of the sample after dilution to the original volume.
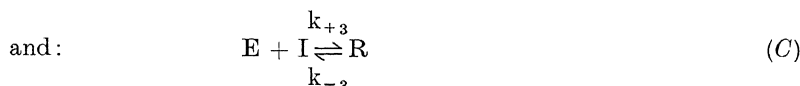
This modifies formula (*1*) into:

$$t_c \cdot h = \left(1 + \frac{K_m}{E}\right) \frac{D}{x} + \frac{1}{E} \qquad (2)$$

where D = dilution

x = the concentration of clotting factor in the given plasma, expressed as a fraction of a standard amount.

The reaction formulae for a case of competitive inhibition are:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C \qquad (A)$$

$$C \overset{k_{+2}}{\rightarrow} E + P \qquad (B)$$

and: $$E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} R \qquad (C)$$

(where C is the enzyme-substrate complex and R the enzyme-inhibitor complex).

The conservation equations are: $E = E_{free} + C + R \qquad (3)$
$$S = S_{free} + C \qquad (4)$$
$$I = I_{free} + R \qquad (5)$$

Here E, S, and I stand for the total amount of enzyme, substrate, and inhibitor added to the reaction medium. The amount of substrate converted into product is not taken into account since the discussion is limited to initial reaction velocities.

The initial steady-state equations are:

$$\frac{dC}{dt} = k_{+1} (S - C) (E - C - R) - (k_{-1} + k_{+2}) (C) = 0 \qquad (6)$$

$$\frac{dR}{dt} = k_{+3} (I - R) (E - C - R) - k_{-3} (R) = 0 \qquad (7)$$

We further define a Michaelis constant and an inhibitor constant:

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (8) \quad K_i = \frac{k_{-3}}{k_{+3}} \qquad (9)$$

From (*6*), (*7*), (*8*), and (*9*) it follows that

$$(S - C) (E - R - C) - K_m C = 0 \qquad (10)$$
$$(I - R) (E - R - C) - K_i R = 0 \qquad (11)$$

It has already been shown (*1*) that equations of the type

$$(\alpha - x) (\beta - x) - \gamma x = 0 \qquad (12)$$

yield the solution

$$x = \frac{\alpha \cdot \beta}{\alpha + \beta + \gamma} \qquad (13)$$

provided that

$$\frac{(\alpha + \beta + \gamma)^2}{\alpha \cdot \beta} \geqslant 2 \qquad (14)$$

Application of (14) to formulae (10) and (11) gives:

$$C = \frac{S\,(E - R)}{S + K_m + (E - R)} \text{ and} \tag{15}$$

$$R = \frac{I\,(E - C)}{I + K_i + (E - C)} \tag{16}$$

provided that

$$\frac{(E - R + S + K_m)^2}{(E - R)\,S} \gg 2 \tag{17}$$

and

$$\frac{(E - C + I + K_i)^2}{(E - C)\,I} \gg 2 \tag{18}$$

which is true under the extra conditions: $2R \ll E + S + K_m$ $\qquad(19)$
$$2C \ll E + I + K_i \tag{20}$$

This is a reasonable assumption, because $R < E$, $R < S$, $C < E$ and $C < I$. So formulae (15) and (16) can be considered to be valid.

Now, from these formulae it can be deduced that

$$\frac{1}{C} = \frac{1}{E} + \frac{1}{S} + \frac{K_m}{E.\,S.} + \frac{I}{S} \cdot \frac{1}{E} \cdot \frac{K_m + E}{K_i + E} \tag{21}$$

Since the reaction velocity equals $k_{+2} \cdot C$ and clotting time is assumed to be inversely proportional to reaction velocity, this can be written as

$$t_c \cdot h = \frac{1}{E} + \frac{1}{S} + \frac{K_m}{E.\,S.} + \frac{I}{S} \cdot \frac{1}{E} \cdot \frac{K_m + E}{K_i + E} \tag{22}$$

In the experiments described in the preceeding article, variation in S is achieved by dilution of the sample, in which an unknown concentration (x) of factor X was present. When the dilution factor is D, S will equal x/D, so that $1/S = D/x$. Both the inhibitor present in the original sample and factor X will be diluted, so the ratio of I/S will not change with dilution. This ratio of I over S will be called n.

Introduction of these symbols into formula (22) gives:

$$t_c \cdot h = \frac{1}{E} + \frac{D}{x} + \frac{K_m}{E} \cdot \frac{D}{x} + n \cdot \frac{1}{E} \cdot \frac{K_m + E}{K_i + E} \tag{23}$$

and, since $1/E$, K_m, and K_i are considered to be constant, this is a formula of the form

$$t_c \cdot = \frac{D}{x} \cdot \alpha + \beta + n \cdot \gamma \tag{24}$$

(α, β and γ being constants)

When no inhibitor is present [compare formula (2)] this modifies into:

$$t_c = \frac{D}{x} \cdot \alpha + \beta. \tag{25}$$

Comparison of the formulae (24) and (25) shows that the slope of the $t_c - D$ graph indicates the magnitude of x, regardless whether or not the inhibitor is present.

The intercept with the Y-axis in the case of absence of an inhibitor will be β. This β is the $t_{min.uninh.}$ discussed in the preceeding article.

When $t_c \cdot h = \beta$ in the case of an inhibited plasma, it follows that
$$\beta = \alpha \cdot D/x. + \beta + \gamma \, n. \text{ and so}$$

$$- D = n. \, x. \, \frac{\gamma}{\alpha}$$

Here, x indicates the amount of factor X in the original sample and n the ratio of inhibitor to factor X. Therefore, n. x. indicates the amount of inhibitor in the original sample. This is the reason why the length of the horizontal line drawn through $t_{min.uninh.}$ parallel to the X-axis until it meets the $t - D$ graph of an unhibited plasma, is proportional to the amount of a competitive inhibitor present in that plasma.

Summary

The enzyme kinetics of competitive inhibition under conditions prevailing in clotting tests are developed and a method is given to measure relative amounts of a competitive inhibitor by means of the $t - D$ plot.

Résumé

La théorie de la cinétique enzymatique de l'inhibition compétitive appliquée sous les conditions rencontrées dans les tests de coagulation est developpée et une méthode est donnée pour mesurer la quantité relative d'un inhibiteur compétitif au moyen d'une méthode graphique utilisant la relation temps/dilution.

Zusammenfassung

Es wird die Enzymkinetik der kompetitiven Hemmung unter den Bedingungen, die bei Gerinnungstesten vorliegen, entwickelt und es wird eine Methode angegeben, welche es ermöglicht, die relativen Mengen eines kompetitiven Hemmstoffes mit Hilfe der Gerinnungszeit – Verdünnungsgrafik zu messen.

References

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