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(54) Title: IMPROVEMENT OF SPECIFICITY IN THE DETERMINATION OF ANTITHROMBIN

(57) Abrégé/Abstract:

The invention concerns a method for determining antithrombin III (AT) in body fluids by adding an AT binding partner to the sample and determining the free AT binding partner and it also concerns a reagent that is suitable for this method.



Abstract

The invention concerns a method for determining antithrombin III (AT) in body fluids by adding an AT binding partner to the sample and determining the free AT binding partner and it also concerns a reagent that is suitable for this method.

Improvement of specificity in the determination of antithrombin

Description

The invention concerns a method for determining antithrombin III (AT) in body fluids by adding an AT binding partner to the sample and determining the free AT binding partner and it also concerns a reagent that is suitable for this method.

AT is a factor of the blood coagulation system which plays a regulatory role. Blood coagulation is initiated by a cascade-like interaction of various proteases. The last of the successive activation steps releases thrombin which in turn generates fibrin monomers which associate to form a thrombus. The most important regulator is AT which can form a complex with thrombin and also with other proteases involved in blood coagulation, that blocks the active center. The AT content in the blood of healthy humans is within a relatively narrow range. Reduced AT contents may be due to consumptive coagulopathy, a severe liver disease or they may be hereditary. A reduced AT content is nowadays generally regarded to be a risk for thrombosis. Hence in some cases the AT content is even reduced in an acute thrombosis. Therefore the AT content is a valuable parameter in clinical diagnostics.

Various methods are already known for detecting AT in which an AT binding partner is added to a sample under conditions that allow an interaction of the AT binding partner with AT present in the sample, and subsequently determining the amount of free AT partner. Such determinations may for example be based on immunological methods or use chromogenic substrates. In the latter case thrombin or activated factor X is for example added to the sample which interacts with the AT present in the sample. Excess thrombin is then determined by incubation with a chromogenic substrate which forms a coloured substance due to the action of thrombin and

evaluation of the colour generation where the AT content is indirectly proportional to the colour formation. Methods for determining AT are described for example in Bergmeyer, Methods of Enzymatic Analysis, 3rd edition, "Verlag Chemie", vol. 5, p. 441 — 448; 1. Witt, ed., "Neue Methoden der Gerinnungsanalyse mit chromogenen Substraten", Stormorken, "Neue Methoden der Gerinnungsanalyse", page 119 — 121; Odegard et al., Haemostasis 7: 202-209 (1978); Fareed et al., Chromogenic Peptide Substrates (eds. M.F. Scully and V.V. Kakkar) Churchill Livingstone (1979) 183-191 and Abildgaard et al., Thromb. Res. 11, 549-553 (1977).

A disadvantage of known methods for detecting AT by adding thrombin is that a false high AT value is obtained in the presence of interfering factors e.g. drugs such as hirudin that can themselves interact with thrombin. This disadvantage can be avoided by using activated factor Xa instead of thrombin. However, at present several factor Xa inhibitors are under development as therapeutic agents (Ostrem et al., Biochemistry 37 (1998), 1053-1059; US Patent 5,783,421; US Patent 5,721,214; WO 96/40679; US Patent 5,693,641; WO 97/46523; JP-96-191434 etc.). When these agents come onto the market, the same problems will occur with a factor Xa-based detection method as with the thrombin-based test.

The present invention aims to improve known detection methods and to provide a method that leads to a reliable measured result even in the presence of interfering factors in the sample to be tested.

In one aspect, there is provided a method for detecting antithrombin HI (AT) in a sample which may contain an interfering factor comprising the steps:

- (a) contacting the sample with a first reagent R1 containing an AT binding partner under conditions where the AT binding partner essentially does not interact with AT but interacts with the interfering factor,

- (b) adding a second reagent R2 for a first determination of the free fraction of the AT binding partner,
- (c) adding a third reagent R3 to change the conditions such that the AT binding partner interacts with AT and carrying out a second determination of the free fraction of AT binding partner and
- (d) determining the AT content in the sample from the difference between the first and second determination of the free fraction of the AT binding partner.

In one aspect, there is provided a method for detecting antithrombin III (AT) in a sample comprising the steps: (a) contacting the sample with a first reagent R1 comprising thrombin under conditions where the thrombin does not substantially interact with AT but does interact with an interfering factor when such is present in the sample; (b) adding a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin for a first determination of the free fraction of the thrombin; (c) adding a third reagent R3 comprising heparin to change the conditions such that the thrombin interacts with AT and carrying out a second determination of the free fraction of thrombin; and (d) determining the AT content in the sample from the difference between the first and second determination of the free fraction of the thrombin.

In another aspect, there is provided a method for the detection of antithrombin III (AT) in a sample based on determining the interaction of thrombin with AT present in the sample, wherein a first determination of the thrombin is carried out without AT interaction and subsequently a second determination of the thrombin is carried out with AT interaction and the AT content of the sample is determined from the difference between the first and second determination.

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In another aspect, there is provided a reagent kit for the quantitative detection of antithrombin (AT) in a sample comprising: (a) a first reagent R1 containing thrombin; (b) a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin to determine the free thrombin; and (c) a third reagent R3 comprising heparin, where the third reagent R3 is separate from the first reagent R1.

In another aspect, there is provided a method for determining the antithrombin III (AT) content in a sample that may contain one or more pharmaceutical compounds that inhibit thrombin, the method comprising: (a) providing a reaction mixture by contacting the sample with a first reagent R1 comprising thrombin, under conditions wherein the thrombin essentially does not interact with AT but interacts with the one or more pharmaceutical compounds that inhibit thrombin, if present, (b) adding to the reaction mixture a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin, and determining the amount of thrombin activity in the reaction mixture, (c) changing the conditions of the reaction mixture by adding to the reaction mixture a third reagent R3 comprising heparin such that thrombin interacts with AT, (d) conducting a second determination of the thrombin activity in the reaction mixture, wherein the determination comprises using the reagent R2, (e) determining the difference between the first and second determinations of thrombin activity, and (f) determining the AT content of said sample from said difference determined in step (e).

In another aspect, there is provided a method for determining the amount of antithrombin III (AT) in a sample containing one or more pharmaceutical compounds that inhibit thrombin, the method comprising: (a) providing a reaction mixture by mixing the sample with thrombin and an antagonist for heparin under conditions wherein the thrombin essentially does not interact with AT but interacts with the one or more pharmaceutical compounds that inhibit thrombin, (b) adding to the reaction mixture a chromogenic substrate which is a peptide substrate for thrombin, and

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conducting a first determination of the thrombin activity in the reaction mixture, (c) changing the conditions of the reaction mixture by adding heparin to the reaction mixture such that thrombin interacts with AT, (d) conducting a second determination of the thrombin activity in the reaction mixture using the chromogenic substrate, (e) determining the difference between the first and second determinations of thrombin, and (f) determining the AT content of said sample from said difference determined in step (e).

The method according to the invention comprises the detection of AT in a sample, in particular in a body fluid such as blood or plasma based on determining the interaction of an AT binding partner with AT present in the sample wherein a first determination of the AT binding partner is carried out without AT interaction and subsequently a second determination of the AT binding partner is carried out with AT interaction and the AT content of the sample is determined from the difference between the first and second determination.

The method according to the invention is based on determining the free fraction of AT binding partner in the sample under different conditions. A first determination of the AT binding partner is carried out without AT interaction i.e. under conditions where AT that is present essentially does not react with the AT binding partner i.e. there is no interaction or only to an extent that does not substantially impair the determination due to the fact that AT is for example not present in the sample can interact with the AT binding partner by for example adding a suitable reagent to set up conditions under which the interaction, e.g. complex formation between AT and AT binding partner, is accelerated. The subsequent second determination of the remaining free (and active) AT binding partner allows an inference about the AT content of the sample.

The free AT binding partner can basically be determined by any method. Chromogenic determinations of activity are preferred in which for example the proteolytic activity of AT binding partners such as thrombin or factor Xa is determined or an immunological determination is carried out in which for example antibodies are used which are specifically directed towards an AT binding partner that is not complexed (with AT) and which do not interfere with the subsequent complex formation between AT and AT binding partners.

A particularly preferred embodiment of the method according to the invention consists of determining the proteolytic activity of an AT binding partner. In this method the proteolytic activity of the AT binding partner remaining after reaction with an interfering factor is determined under conditions where AT present in the sample cannot react or can only slightly react with the AT binding partner. Subsequently the formation of the complex between AT and AT binding partner is accelerated by for example activating the AT. Complexes are formed in this process from activated AT and the binding partner. Such a complexed binding partner has essentially no more proteolytic activity. Subsequently the activity of the binding partner is again determined. The difference between the first and the second activity corresponds to the amount of AT in the sample.

The AT binding partner is a detectable substance and preferably a substance with protease activity that can form a complex with AT which preferably results in its inhibition. Examples of suitable AT binding partners are thrombin and factor Xa. Thrombin is particularly preferably used.

The AT binding partner is preferably detected by means of a chromogenic substrate which forms a colour due to the action of the AT binding partner and measurement of the resulting colour. Examples of preferred substrates are peptidic substrates, for example the thrombin substrate Tos-Gly-Pro-Arg-p-nitroaniline (Chromozym®TH)

which is converted by thrombin to Tos-Gly-Pro-Arg-OH and p-nitroaniline. However, other substrates that are accepted by corresponding AT binding partners are of course also suitable.

In contrast to methods of the prior art a first determination of the activity of the AT binding partner occurs in the method according to the invention under conditions where AT present in the sample cannot or can only to a slight extent complex the binding partner and inhibit its activity. Hence it is expedient to carry out the first determination in the absence of substances such as heparin which accelerate complex formation between AT and AT binding partner. Antagonists for the accelerator such as heparin antagonists e.g. polybrene can be optionally added in small amounts. The addition of heparin antagonists is especially expedient when a patient has been previously treated with heparin such that a (low) heparin concentration present in the sample leads to an undesired acceleration of the complex formation between AT and AT binding partner. The addition of antagonists can at least partially prevent this undesired acceleration.

After the first determination of the free AT binding partner, another reagent is preferably added to the reaction mixture which contains an accelerator of complex formation such as heparin. Subsequently a second activity is determined under conditions where AT present in the sample can complex the AT binding partner. The AT content in the sample can be determined from the difference between the first and the second determination. The measured signal is inversely proportional to the AT concentration in the sample. If required, the third reagent can also contain additional AT binding partners. In addition additional substrate can be pipetted in another step if too much substrate has already been consumed in the first determination.

The AT binding partner is determined by methods that are basically known for example as described in the antithrombin III test from Roche Diagnostics GmbH,

Mannheim, Germany. The determination can for example comprise a kinetic test or a two point determination.

The invention also concerns a reagent kit for the quantitative detection of AT in a sample comprising:

- (a) a first reagent R1 containing an AT binding partner,
- (b) a second reagent R2 to determine the free AT binding partner and
- (c) a third reagent R3 containing an accelerator for the interaction between AT and AT binding partner where the third reagent R3 is separate from the first reagent R1.

The first reagent R1 is free of an accelerator for the interaction, which is for example a complex formation, between AT and AT binding partner. The first reagent can optionally also contain an antagonist for such an accelerator. The second reagent R2 for determining the AT binding partner can be a suitable reagent for a chromogenic determination which for example contains a substrate for the AT binding partner. Furthermore the second reagent may also be suitable for an immunological determination and for example contain antibodies against a free unbound AT binding partner and optionally other reagents for carrying out an immunological test e.g. a latex test. The third reagent R3 is separate from the first reagent R1 and contains an accelerator for the interaction, such as a complex formation, between AT and AT binding partner and is preferably heparin.

The determination can be carried out on conventional automated analysers such as the Hitachi, e.g. the Hitachi model 911 and Integra.

The method according to the invention is further illustrated by the following example:

Determination of antithrombin III in the presence of Lepirudin

1. Reaction scheme

3 µl sample solution was pipetted into a measuring cuvette. 175 µl reagent R1 was added by pipette. The reagent R1 consists of 100 mM Tris-HCl, 270 mM NaCl, 12 mM EDTA, 10 g/l polyethylene glycol 6000, 1 g/l bovine serum albumin, 0.5 NIH/ml bovine thrombin and a suitable amount of a fibrin polymerization inhibitor such as GPAP, pH 8.10. It was subsequently incubated for 5 min and then 75 µl reagent R2 (Chromozym TH 1.9 mM) was added. Then the first thrombin activity was determined in a kinetic test by a continuous bichromatic measurement at 415 and 700 nm (primary and secondary wavelength).

Finally 175 µl reagent R3 (100 mM Tris-HCl, pH 8.1; heparin 2 USP-U/ml; bovine thrombin (3.5 NIH/ml; 140 mM NaCl) was added and the second thrombin activity was determined in a kinetic test by continuous measurement. The AT content was determined from the difference between the second and first thrombin activity according to the instructions of the antithrombin III kit of Roche Diagnostics GmbH, Mannheim.

This determination was carried out in the presence of different amounts of hirudin (Lepirudin®, Behringwerke) (0, 1, 2, 4 and 8 µg/ml).

The results are shown in the following table.

Lepirudin® (µg/ml)	AT concentration (%) found (invention)	deviation (%) with the test according to the invention	deviation (%) with the test according to the prior art
0	100	--	--
1	100	0	5

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2	99.9	0	10
4	102.5	3	25
8	130.0	30	52

The table shows that the Lepirudin® interference can be completely (deviation < 5 %) eliminated up to a concentration of 4 µg/ml. The therapeutic concentrations when administering Lepirudin® are usually within this range and hence the method according to the invention reliably determines the AT content even in the presence of drugs that may potentially interfere.

CLAIMS:

1. Method for detecting antithrombin III (AT) in a sample comprising the steps:
 - (a) contacting the sample with a first reagent R1 comprising thrombin under conditions where the thrombin does not substantially interact with AT but does interact with an interfering factor when such is present in the sample;
 - (b) adding a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin for a first determination of the free fraction of the thrombin;
 - (c) adding a third reagent R3 comprising heparin to change the conditions such that the thrombin interacts with AT and carrying out a second determination of the free fraction of thrombin; and
 - (d) determining the AT content in the sample from the difference between the first and second determination of the free fraction of the thrombin.
2. The method of claim 1, wherein the first reagent R1 additionally contains an antagonist for heparin.
3. The method of claim 2, wherein the first reagent R1 contains polybrene as a heparin antagonist.
4. The method of any one of claims 1 to 3, wherein the third reagent R3 also contains an additional thrombin.
5. The method of any one of claims 1 to 4, wherein the determination of the thrombin comprises a kinetic determination.
6. A method for the detection of antithrombin III (AT) in a sample based on determining the interaction of thrombin with AT present in the sample, wherein a first determination of the thrombin is carried out without AT interaction and subsequently a second determination of the

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thrombin is carried out with AT interaction and the AT content of the sample is determined from the difference between the first and second determination.

7. A reagent kit for the quantitative detection of antithrombin (AT) in a sample comprising:
 - (a) a first reagent R1 containing thrombin;
 - (b) a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin to determine the free thrombin; and
 - (c) a third reagent R3 comprising heparin, where the third reagent R3 is separate from the first reagent R1.
8. The reagent kit of claim 7, wherein the second reagent R2 is for chromogenic determination of the thrombin.
9. Use of a reagent kit as claimed in any one of claims 7 to 8 in a method as claimed in any one of claims 1 to 6.
10. A method for determining the antithrombin III (AT) content in a sample that may contain one or more pharmaceutical compounds that inhibit thrombin, the method comprising:
 - (a) providing a reaction mixture by contacting the sample with a first reagent R1 comprising thrombin, under conditions wherein the thrombin essentially does not interact with AT but interacts with the one or more pharmaceutical compounds that inhibit thrombin, if present,
 - (b) adding to the reaction mixture a second reagent R2 comprising a chromogenic substrate which is a peptide substrate for thrombin, and determining the amount of thrombin activity in the reaction mixture,
 - (c) changing the conditions of the reaction mixture by adding to the reaction mixture a third reagent R3 comprising heparin such that thrombin interacts with AT,
 - (d) conducting a second determination of the thrombin activity in the reaction mixture, wherein the determination comprises using the reagent R2,

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- (e) determining the difference between the first and second determinations of thrombin activity, and
 - (f) determining the AT content of said sample from said difference determined in step (e).
11. The method of claim 10, wherein the first reagent R1 further comprises an antagonist for heparin.
12. The method of claim 11, wherein the antagonist is hexadimethrine bromide.
13. The method of any one of claims 10 to 12, wherein the third reagent R3 further comprises additional thrombin.
14. The method of any one of claims 10 to 13, wherein said first and/or second determination of thrombin comprises a kinetic determination.
15. The method of any one of claims 10 to 14, wherein the sample contains one or more pharmaceutical compounds that inhibit thrombin.
16. A method for determining the amount of antithrombin III (AT) in a sample containing one or more pharmaceutical compounds that inhibit thrombin, the method comprising:
- (a) providing a reaction mixture by mixing the sample with thrombin and an antagonist for heparin under conditions wherein the thrombin essentially does not interact with AT but interacts with the one or more pharmaceutical compounds that inhibit thrombin,
 - (b) adding to the reaction mixture a chromogenic substrate which is a peptide substrate for thrombin, and conducting a first determination of the thrombin activity in the reaction mixture,
 - (c) changing the conditions of the reaction mixture by adding heparin to the reaction mixture such that thrombin interacts with AT,

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- (d) conducting a second determination of the thrombin activity in the reaction mixture using the chromogenic substrate,
 - (e) determining the difference between the first and second determinations of thrombin, and
 - (f) determining the AT content of said sample from said difference determined in step (e).
17. The method of claim 16, wherein the first reagent R1 comprises hexadimethrine bromide.
18. The method of claim 16 or 17, wherein the third reagent R3 further comprises additional thrombin.
19. The method of any one of claims 16 to 18, wherein said first and/or second determination of thrombin comprises a kinetic determination.