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(71) Applicant (*for all designated States except US*): **OCTAPHARMA AG** [CH/CH]; Seidenstrasse 2, CH-8853 Lachen (CH).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **KARLSSON, Göran** [SE/SE]; Siltgränd 29, S-17840 Ekerö (SE). **WINGE, Stefan** [SE/SE]; Langhalsvägen 17, 2 tr, S-12050 Arsta (SE).

(74) Agents: **MEYERS, Hans-Wilhelm** et al.; P.O. Box 10 22 41, 50462 Cologne (DE).

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(54) Title: METHOD OF SEPARATION

(57) **Abstract:** A method for separation of native antithrombin and pre-latent antithrombin present in a sample from each other is provided. The method comprises performing, in the order given, the following steps: a) application of a sample containing native antithrombin and pre-latent antithrombin to an affinity material with affinity for antithrombin, wherein said application is performed under conditions such that binding of native antithrombin and pre-latent antithrombin to the affinity material is achieved; b) application of a solution of an elution salt S_1 to said affinity material at a concentration C_1 of elution salt, for elution of pre-latent antithrombin, said concentration C_1 is in the range of 30-95 % of the concentration needed for elution of native antithrombin with the elution salt S_1. In the method, the concentration C_1 is such that pre-latent antithrombin is eluted and native antithrombin is retained.

METHOD OF SEPARATION

Field of the invention

The present invention relates to a method of separation of plasma proteins, in particular separation between native antithrombin and pre-latent antithrombin.

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Background of the invention

Antithrombin III (in the following often referred to as AT) is a plasma glycoprotein with a total molecular weight of 58.1 kDa (Lebing *et al,* Vox Sang. 67, 117-124, 1994), which, in its native form, inhibits serine proteases in the
10 coagulation cascade and thus plays a major role in the regulation of blood clotting. Antithrombin III is the major inhibitor of coagulation factor IXa, coagulation factor Xa and thrombin, and also interferes with other enzymes involved in coagulation and fibrinolysis, such as factor XIa, factor XIIa, kallikrein and plasmin. Thus, AT regulates clot formation in different stages of
15 the coagulation cascade. A decrease of the AT content in the blood is associated with an increased risk of thromboembolism. AT concentrates are used in the prophylaxis and treatment of thromboembolic disorders in patients with acquired or hereditary antithrombin deficiency. In addition, it has been reported that AT has functions in many other aspects of the human body, for
20 example in angiogenesis and inflammatory responses. The function of AT in these physiological processes is not fully understood.

When antithrombin III is prepared according to Wardell *et al* (Biochemistry 36, 13133-13142, 1997), a form of AT known as the latent form (in the following often referred to as L-AT), is produced. A selectively elastase
25 cleaved variant has also been identified. These have both been shown to have a strong antiangiogenic activity, and also to suppress tumor growth in mice injected subcutaneously with a human neuroblastoma cell line (O'Reilly *et al*, Science 285, 1926-1928, 1999, and PCT publication WO00/20026). Both the latent and the cleaved forms of antithrombin have been shown to have their

reactive loops irreversibly inserted into the â-sheet of the protein (Carrell *et al*, Nature 353, 576-578, 1991).

Recently, Larsson *et al* (J Biol Chem 276, 11996-12002, 2001) have reported the antiangiogenic and anti-tumor effects of a newly discovered form of antithrombin, denoted pre-latent antithrombin (in the following often referred to as P-LAT), in mouse models. This P-LAT was shown to be formed during heat treatment in the virus inactivation step in the manufacturing process of antithrombin. Larsson *et al* concluded that it is likely that all commercial antithrombin products that have been heat treated during the virus inactivation process contain AT in the special P-LAT form. This conclusion was based on analyses of commercial AT preparations, which were all shown to contain P-LAT to some extent. AT that had not been heat treated did not show the corresponding antiangiogenic or anti-tumor effects.

Purification of AT with affinity chromatography, using purified heparin as the solid phase bound ligand, is well known in the art. Miller-Andersson *et al* (Thrombosis Research 5, 439-452, 1974 and US patent 3 842 061) disclose the use of heparin sepharose to purify human AT. This chromatographic system has also been useful for the separation between native AT and L-AT. A decreased affinity of heparin for L-AT compared to that for native AT enabled resolution between the two components, as described by Chang and Harper (Thrombosis and Haemostasis 77, 323-328, 1997). Larsson *et al* (*supra*) report the separation of P-LAT from L-AT using a heparin affinity chromatography system. However, according to the disclosure of Larsson *et al*, no separation between P-LAT and native AT has been achieved, and the affinity of P-LAT and native AT for a heparin chromatography column is the same.

In pharmaceutical antithrombin products, it is desired that only native antithrombin is present. The reasons for avoiding P-LAT in particular in the final product is that (i) pre-latent antithrombin may be transferred to the latent form, which has a documented effect to inactivate native antithrombin by forming a so-called heterodimer, especially with the beta-form of AT, thus decreasing the antithrombotic properties of native AT *in vivo* (Zhou *et al*,

Blood 94, 3388-3396, 1999), (ii) the inhibition of angiogenesis, as an effect of P-LAT, may be harmful for some patients by slowing down the wound healing process, and (iii) it makes the product inhomogeneous.

5 Description of the invention

The main object of the present invention is to enable the separation between native and pre-latent antithrombin in a sample.

A related object of the present invention is to achieve enrichment of either native or pre-latent antithrombin in a sample, through the selective
10 removal of one or the other of these two components.

Yet a related object of the present invention is to provide a method which makes it possible to obtain a solution of native, heat-treated antithrombin, which solution is essentially free from pre-latent antithrombin. This is desirable in particular with regard to the negative effects of P-LAT in AT
15 samples described above.

These and other objects which will be apparent to the skilled man from the following disclosure are met by the invention as claimed. Thus, the present invention provides a method for separation of native antithrombin and pre-latent antithrombin present in a sample from each other, which method
20 comprises performing, in the order given, the following steps:

a) application of a sample containing native antithrombin and pre-latent antithrombin to an affinity material with affinity for antithrombin, wherein said application is performed under conditions such that binding of native antithrombin and pre-latent antithrombin to the affinity material is achieved;
25 b) application of a solution of an elution salt S_1 to said affinity material at a concentration C_1 of elution salt, for elution of pre-latent antithrombin, said concentration C_1 is in the range of 30-95 % of the concentration needed for elution of native antithrombin with the elution salt S_1;
in which method said concentration C_1 is such that pre-latent antithrombin is
30 eluted and native antithrombin is retained.

Thus, the present invention is based on the unexpected finding that the pre-latent form of antithrombin has a slightly lower affinity than the native

form, when a sample containing both forms is treated using a material with affinity for native antithrombin, e. g. a matrix having a ligand with affinity for native antithrombin coupled thereto. This finding makes it possible to meet the objects of the invention, through exploiting the difference in affinity in a
5 method of separation.

The first step, step a), of the method according to the present invention is the application of a sample containing native antithrombin and pre-latent antithrombin to an affinity material with affinity for antithrombin. The sample may for example be the result of a conventional process for the commercial
10 production of antithrombin, but the method is applicable on any sample in which the two forms of antithrombin in question are present. The application of the sample to the affinity material is performed under conditions such that binding of both the native and the pre-latent forms of antithrombin to the affinity material is achieved. Such conditions are readily established by the
15 person skilled in the field of chromatography, and typically involve buffer solutions having a low ionic strength, as well as a pH of about 7-8 in a buffered solution. The elution salt which is to be used in subsequent step of the method is suitably present at low concentrations or not at all during this step of binding to the affinity material.

20 The next step, step b), is the application to the affinity material of a solution of an elution salt S_1. Said elution salt should be applied in a concentration C_1, which is such that the pre-latent antithrombin bound to the affinity material is released and eluted, whereas the native antithrombin is retained on the material. The concentration C_1 is in the range of 30 - 95 % of
25 the concentration of the salt S_1 needed for elution of native antithrombin.

In effecting elution of pre-latent antithrombin in this manner, the separation between the two forms of antithrombin is achieved. It may subsequently, however, be advantageous also to elute the bound native antithrombin from the affinity material, in order for example to reuse the
30 affinity material for separation of P-LAT and native AT, or just to recover the native AT. Thus, the method of the invention may comprise a further step c). This third step is the application to the affinity material of a solution of an

elution salt S_2, which is the same elution salt as, or a different elution salt from, said elution salt S_1. The elution salt S_2 is applied at a concentration C_2, which is such that native antithrombin is eluted.

The method of the invention is performed in the batch mode.

5 The underlying purposes in carrying out the method of separation according to the invention may differ in different situations. Thus, it is possible that the purpose is to prepare a commercial product containing native antithrombin with as little pre-latent antithrombin as possible. In such a product, the disadvantages of P-LAT presence are minimized accordingly. 10 Conversely, the purpose may be the preparation of a sample containing as much pre-latent antithrombin as possible, in order for example to study further the properties of this newly identified, and highly interesting, form of antithrombin, or to produce a pharmaceutical preparation thereof. Thus, it may be of interest, after step b) of the method according to the invention, to 15 collect the P-LAT eluted from the affinity material in step b), and/or to collect the native AT eluted from the affinity material in step c) after this step, when present.

L-AT is, due to the relatively large difference in heparin affinity, easy to separate from native AT by for example analytical or preparative heparin 20 affinity chromatography. In contrast, as discovered by the present inventors, P-LAT has an affinity for heparin that is very close to the affinity for heparin of native AT, which has made an analytical, and especially a preparative, separation much harder to accomplish.

Established technology enables the purification of antithrombin using e g 25 affinity chromatography. Through these methods, the skilled person has a knowledge about what concentrations of various elution salts bring about the elution of antithrombin. In this text, the minimum concentration of a given elution salt that is necessary for elution of native antithrombin is regarded as "100% of the concentration needed for elution of native antithrombin" with 30 that elution salt.

As to what concentrations of elution salt or salts should be used as concentrations C_1 and optionally C_2 in the method according to the invention,

what is said above implies that the concentration C_2 is at least 100% of the concentration needed for elution of native antithrombin with the elution salt S_2, when step c) is present. The concentration C_1 of the elution salt S_1 in step b) of the method is between 30 and 95 % of the concentration needed for elution of native antithrombin with that elution salt. A narrower concentration range for the concentration C_1 of the elution salt S_1 is between 30 and 90 % of the concentration needed for elution of native antithrombin with that elution salt. An even more restricted concentration range which may be used is a concentration C_1 of the elution salt S_1 of between 60 and 85 % of the concentration needed for elution of native antithrombin with that elution salt.

The method according to the invention is not critically dependent on what salt is used as elution salt S_1, or as elution salt S_2 when present. When both are used, the elution salts S_1 and S_2 may be the same or different salts. In certain cases, it may be preferable to use the same salt as both elution salt S_1 and S_2, for example in a case where the concentrations C_1 and C_2 of elution salt are applied to the affinity material using a concentration gradient of elution salt.

Examples of possible salts for use as elution salt or salts in the method according to the invention are NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$, BaCl, sodium acetate, Na_2SO_4, $NaHSO_4$, $MgSO_4$, K_2SO_4, $KHSO_4$, sodium citrate, potassium citrate, Na_3PO_4, Na_2HPO_4, NaH_2PO_4, K_3PO_4, K_2HPO_4 and KH_2PO_4. However, other elution salts are known to the skilled person, and may be used in carrying out the process according to the invention.

In the method according to the invention, an affinity material is used to effect the separation between native AT and P-LAT. This affinity material has an affinity for antithrombin, and may be one that is used conventionally for the separation of antithrombin from other proteins or for the cleaning of antithrombin samples from impurities. Suitable materials for use as the affinity material include heparin and heparan sulfate, including unfractionated, fragmented or selected forms thereof, optionally immobilized to a solid support. The form of the affinity material may be any known form of

separation media, including a gel for column or batch chromatography, or an affinity membrane.

Different affinity matrices have different affinities for native antithrombin. Commercial heparin chromatography columns usually require about 0.5-1.2 M NaCl for elution of native AT. Differences between affinity matrices are due to differences in e g particle and pore size and properties of the gel matrix, including the coupling properties used, as well as the molecular weight and sulfation degree of the ligand coupled to the matrix, for example in the case where the ligand is heparin or heparan sulfate. A smaller pore size implies a larger binding surface in the gel, which in turn generally results in a stronger retention, necessitating the use of a more concentrated elution solution. Also, a higher molecular weight and/or sulfation degree of ligands such as heparin increases the ligand's affinity for AT, and in accordance, the concentration of elution salt needed is correspondingly increased. Factors such as coverage degree of the gel particles and pores are also important, in the case where a gel is used as affinity material. The volume of eluting buffer in e g a step-wise eluted chromatographic system, preferably measured in number of column volumes, is of course also of high concern for the determination of the required elution conditions, in that a larger volume of eluting buffer generally means that a lower concentration of elution salt is needed.

In the following, the invention will be described through the recital of experiments conducted in accordance therewith, where appropriate with reference to the accompanying figures. The experiments and figures are not to be seen as limiting in any way of the scope of the invention as claimed.

Brief description of the figures

Figure 1. Analytical heparin affinity chromatography of pre-latent antithrombin. (A) water blank; (B) native antithrombin, not heat treated; and (C) antithrombin, heat treated for 24 h at 60 °C in 0.5 M sodium citrate. Elution times of native antithrombin (AT), latent antithrombin (L-AT), and pre-latent antithrombin (P-LAT) have been indicated.

Figure 2. Analytical heparin affinity chromatography of pre-latent antithrombin. (A) purified antithrombin, not heat treated; (B) purified antithrombin, heat treated; and (C) purified antithrombin after heat treatment and a first heparin affinity chromatography step for removal of latent
5 antithrombin. Elution times of native antithrombin (AT), latent antithrombin (L-AT), and pre-latent antithrombin (P-LAT) have been indicated.

Figure 3. Analytical heparin affinity chromatography of pre-latent antithrombin. Commercial pharmaceutical antithrombin preparations from four different manufacturers; (A) Antithrombin III-Alpha® (Grifols, Langen,
10 Germany),(B) AT III Kybernin® (Aventis Behring, Marburg, Germany), (C) Antithrombin III Immuno® (Baxter, Unterschleissen, Germany) ,(D) Atenativ® (Octapharma, Stockholm, Sweden). Elution times of native antithrombin (AT), latent antithrombin (L-AT), pre-latent antithrombin (P-LAT), human serum albumin (HSA) and acetyl tryptophan (Ac-Trp) have been
15 indicated.

Figure 4. Native electrophoresis. Antithrombin, 0.25 µg protein per lane was loaded on a 12.5% homogeneous Phast gel. (1) Antithrombin, unpasteurized, (2) Antithrombin III-Alpha (Grifols, Langen, Germany), (3) AT III Kybernin (Aventis Behring, Marburg, Germany), (4) Antithrombin III
20 Immuno (Baxter, Unterschleissen, Germany), and (5) Antithrombin, pasteurized, corresponding to Atenativ (Octapharma, Stockholm, Sweden), but without added albumin. The cathode (-) is at the top, and the anode (+) is at the bottom of the gel.

Figure 5. (A) Micro scale preparation of pre-latent antithrombin by
25 heparin affinity chromatography. The horizontal bracket signs denote collected and pooled fractions of P-LAT and L-AT. (B) Analytical heparin affinity chromatography of the pooled fractions of pre-latent antithrombin from (A). (C) Analytical heparin affinity chromatography of the pooled fractions of latent antithrombin from (A).

30 Figure 6. SDS polyacrylamide gel electrophoresis under reducing conditions of antithrombin samples partially digested with thermolysin at 37 °C for 120 min. A Phast 12.5% homogeneous gel was used in the analysis. The

gel was loaded with 0.2 μg protein, in 1 μL, per lane, and silver-stained after running. (Lane 1 and 4) purified AT, non-heat-treated, (Lane 2 and 5) P-LAT, from the micro scale purification procedure, (Lane 3 and 6) LAT, from the micro scale purification procedure. Lanes 1 - 3 contain samples that were not
5 exposed to thermolysin and lanes 4-6 contain samples that were exposed to thermolysin. Molecular weights of marker proteins (in kDa) are indicated in the figure.

EXAMPLE 1

10 ## Analytical heparin affinity chromatography

Equipment and chromatography conditions:

A HPLC system equipped with a TSK-GEL® Toyopearl® Heparin column (Tosohaas, Stuttgart, Germany) having the dimensions 7.5 i.d. x 75 mm, 10
15 μm, 1000 Å, was used. The buffers used were:

Mobile phase A – 20 mM Tris/HCl, pH 7.4, and
Mobile phase B - 2.5 M NaCl in 20 mM Tris/HCl, pH 7.4.

20 For the experiments, a segmented gradient was run according to the following:

Time (min)	% B in A	[NaCl] (M)
0-25	6-29	0.15-0.73
25-30	29	0.73
30-40	29-100	0.73-2.5
40-45	100	2.5
45-80	6	0.15

The column was not temperated, and the analysis was run at room
25 temperature (i e about 22 °C). However, the samples were kept in the temperated sample container at about 6 °C during the analysis. The flow rate

was 0.4 ml/min, and detection was carried out by measurement of UV absorbance at 280 nm. The injection volume was 35 μl. The concentration used for the analysis of antithrombin samples was 1 mg antithrombin per ml, achieved by dilution in 20 mM Tris/HCl with 150 mM NaCl, pH 7.4.

5

Experiment I:

In this experiment, the following three samples were analyzed using the conditions set out under "Equipment and chromatography conditions" above:

A) Water blank (negative control);

10 B) Native antithrombin (not heat treated); and

C) Heat treated antithrombin.

The preparation of C) was performed according to the preparation of L-AT (Wardell *et al, supra*), but with an incubation for a longer period of time, in accordance with Larsson *et al* (*supra*). A laboratory sample of antithrombin, 5

15 mg/ml, was transferred to 0.5 M trisodium citrate in 10 mM Tris/HCl, pH 7.4, which was incubated in glass tubes for 24 h at 60 °C, and finally desalted on a NAP-5® gel filtration column (Amersham Biosciences, Uppsala, Sweden) to a solution containing 20 mM Tris/HCl, 150 mM NaCl, pH 7.4.

The chromatograms obtained from the analysis of samples A)-C) are

20 shown in corresponding Figure 1A-C. The elution positions of native antithrombin (AT), latent antithrombin (L-AT) and pre-latent antithrombin (P-LAT) have been indicated. The baseline disturbance visible at about 7 min in the chromatograms, i e in the void volume, was due to some remaining citrate, and the disturbance at 54 min was due to the gradient. For Figure 1C,

25 the integrated area of L-AT and P-LAT was 31 % and 9 %, respectively. This experiment shows that native AT that has not been heat treated elutes as a single peak, while AT that has been heat treated according to Wardell *et al* contains 31 % latent, 9 % pre-latent and 60 % native antithrombin.

30 Experiment II:

The following three antithrombin samples were prepared using the protocol in Example 1 of international patent publication WO00/78792, and

were analyzed using the conditions set out under "Equipment and chromatography conditions" above:

A) Purified antithrombin (not heat treated);

B) Purified antithrombin after heat treatment for virus removal (at 60 °C for 10 h in the presence of stabilizers); and

C) Purified antithrombin after heat treatment for virus removal (at 60 °C for 10 h in the presence of stabilizers) and a preliminary heparin affinity chromatography step for removal of latent antithrombin.

The chromatograms obtained from the analysis of samples A)-C) are shown in corresponding Figure 2A-C. The elution positions of native antithrombin (AT), latent antithrombin (L-AT) and pre-latent antithrombin (P-LAT) have been indicated. The integrated area of the L-AT and P-LAT peaks in Figure 2B were 3 % and 6 %, respectively, and the area of the P-LAT peak in Figure 2C was 5 %. This analysis demonstrates that no P-LAT is present before heat treatment in the virus inactivation process (Figure 2A), and that there has been a formation of about 3 % L-AT and 6 % P-LAT during the heat treatment (Figure 2B). After an additional heparin affinity chromatography step, the L-AT formed in the heat treatment step is removed, but 5 % P-LAT remains (Figure 2C).

Experiment III:

The following four commercially available pharmaceutical preparations of antithrombin from four different manufacturers were analyzed using the conditions set out under "Equipment and chromatography conditions" above:

A) Antithrombin III-Alpha® (Grifols, Langen, Germany)

B) AT III Kybernin® (Aventis Behring, Marburg, Germany)

C) Antithrombin III Immuno® (Baxter, Unterschleissen, Germany)

D) Atenativ® (Octapharma, Stockholm, Sweden)

The chromatograms obtained from the analysis of samples A)-D) are shown in corresponding Figure 3A-D. The elution positions of native

antithrombin (AT), latent antithrombin (L-AT), pre-latent antithrombin (P-LAT), human serum albumin (HSA) and acetyl tryptophan (Ac-Trp) have been indicated. The integrated area of the P-LAT peak was 2 %, 6 %, 1 % and 5 % for samples A), B), C) and D), respectively. The integrated area of the L-AT
5 peak was 4 % and 11 % for samples B) and C), respectively. This analysis demonstrates that four commercial pharmaceutical antithrombin products contain 1-6 %, with a mean of 4 %, P-LAT, and that two of these products also contain L-AT.

10 Summary:

Experiments I-III show the separation of P-LAT from other forms of antithrombin, using a concentration gradient of NaCl. The elution positions for the three forms of antithrombin studied under these conditions were as shown in Table 1. Also shown in Table 1 is the concentration of the elution salt at the
15 elution position. As compared to the horizontal axis of the chromatograms in Figures 1-3, a correction of six minutes to account for the dead volume of the HPLC system and the void volume of the column has been applied.

TABLE 1

Elution times of AT forms in analytical chromatography

Species	Elution time (min)	[NaCl] (M)
L-AT	7	0.3
P-LAT	28	0.72
AT	35	0.9

20 Thus, in this example, the elution of P-LAT is effected by application of an elution salt concentration which is 0.725/0.888 = 82 % of the concentration needed for the elution of native antithrombin.

EXAMPLE 2

25 Micro scale preparation of P-LAT by heparin affinity chromatography

Equipment and chromatography conditions:

The same HPLC system, column and mobile phases were used as in Example 1, and at the same conditions, with the exception that the flow rate was 0.6 ml/min. The incubation of antithrombin for generation of P-LAT was performed as described for sample C) in Experiment 1 of Example 1. The initial antithrombin concentration was 12 mg/ml, and 100 µl of the desalted antithrombin sample was injected in each chromatography run. A segmented gradient was run according to the following:

Time (min)	% B in A	[NaCl] (M)
0-25	6-27	0.15-0.68
25-28	27	0.68
28-33	100	2.5
33-45	6	0.15

The result is shown in Figure 5A. The elution positions of native antithrombin (AT), latent antithrombin (L-AT) and pre-latent antithrombin (P-LAT) are indicated. The elution positions for the three forms of antithrombin studied under these conditions were as shown in Table 2. Also shown in Table 2 is the concentration of the elution salt at the elution position. As compared to the horizontal axis of the chromatograms in Figure 5A, a correction of four minutes to account for the dead volume of the HPLC system and the void volume of the column has been applied.

TABLE 2

Elution times of AT forms in micro scale preparation

Species	Elution time (min)	[NaCl] (M)
L-AT	4	0.2
P-LAT	24	0.7
AT	30	2.5

Thus, in this example, the elution of P-LAT is effected by application of an elution salt concentration of approximately 0.7 M. In the gradient used in this example, however, the concentration of NaCl is immediately brought to 2.5 M after elution of P-LAT. This is done out of process economy considerations in order to achieve a faster elution of native antithrombin, but the elution could be achieved at a lower concentration. Thus, the lowest concentration of NaCl that will elute native antithrombin from the column in question is approximately 0.9 M, as known by the skilled person and demonstrated by Example 1. Using this value, the concentration needed for elution of P-LAT is 0.654/0.9 = 73 % of the concentration needed for elution of native antithrombin. In Figure 5A, the horizontal bracket signs denote fractions containing L-AT and P-LAT that were collected for repeated injections. The pooled fractions were desalted, concentrated and transferred to 20 mM Tris/HCl with 150 mM NaCl, pH 7.4, using a Centricon micro concentrator from Amicon (Danvers, MA, USA) with a 10 kDa cut-off. The obtained P-LAT and L-AT samples were then subjected to analytical heparin affinity chromatography as described in Example 1. The resulting chromatograms are shown in Figure 5B (pooled P-LAT fractions) and Figure 5C (pooled L-AT fractions). The integrated area of the P-LAT peak in Figure 5B is 66 % of the total integrated area. The experiment shows that P-LAT can be purified to a purity of about 65 % by a slight modification of the analysis method. Also, L-AT was purified to a purity of 100 % in the same procedure (Figure 5C).

EXAMPLE 3

Preparation of purified, native AT

A laboratory sample of AT, > 90 % pure, was obtained from Plasma Products, Biovitrum, Sweden. The laboratory sample was analyzed according to the analytical method of Example 1, and the amounts of P-LAT, L-AT and native AT were found to be 8 %, 0 % and 92 %, respectively. This laboratory

sample was used as the initial material for the following experiment, with the aim of reducing the content of P-LAT in the AT product.

An FPLC system (Amersham Bioscience, Uppsala, Sweden) with a 30 ml column packed with Heparin Sepharose FF (No 126-355, batch 11178-51, Amersham Bioscience) was used. The column was an XK16-column (Amersham Bioscience) with an approximate bed height of 15 cm. Before sample application, the column was equilibrated with 160 mM NaCl and 10 mM Na_3PO_4, pH 7.0.

7 ml of the reconstituted laboratory antithrombin sample (50 mM sodium citrate and 1 % sucrose, pH 7.4, 50 IU antithrombin/ml) was applied to the equilibrated column (flow rate: 7 column volumes/h). The column was then washed with 30 ml of equilibration buffer, after which 150 ml of one of four alternative washing buffers (see below) was applied to the column. Subsequently, elution of the remaining antithrombin was performed using 150 ml of an elution buffer containing 2.0 M NaCl and 10 mM Na_3PO_4, pH 7.0. The fraction collected during elution was denoted eluate. The process was performed at room temperature (about 22 °C). The absorbance at 280 nm UV was measured for detection of proteins. Four runs, identical except for the ionic strength of the washing buffer, were performed. The resulting fractions were analyzed for protein (A_{280}) and P-LAT content. The proteins in those samples (eluates) that were analyzed for P-LAT were first concentrated using a Centricon micro concentrator (to a protein content of A_{280} = 4-16) to increase the detection range of the analytical method. The following four washing buffers were tested:

A) 380 mM NaCl and 10 mM Na_3PO_4, pH 7.0
B) 410 mM NaCl and 10 mM Na_3PO_4, pH 7.0
C) 440 mM NaCl and 10 mM Na_3PO_4, pH 7.0
D) 470 mM NaCl and 10 mM Na_3PO_4, pH 7.0

As evident from Table 3, it is possible to decrease the P-LAT content in the antithrombin product with a suitable washing buffer. A decrease in total

protein recovery of about 35 % is observed when reducing the P-LAT content about four times (washing buffer D).

TABLE 3

Purification of native antithrombin

Sample	Protein recovery (%)	% AT	% P-LAT
Initial sample	100	92	8
A eluate	98	92	8
B eluate	100	93	7
C eluate	90	95	5
D eluate	65	98	0

5

EXAMPLE 4

Preparation of enriched P-LAT and pure native AT in pilot scale

A sample of antithrombin, > 90 % pure, was obtained from Plasma
10 Products, Biovitrum, Sweden. The sample was analyzed according to Example 1, and the amounts of P-LAT, L-AT and native AT were found to be 8 %, 0 % and 92 %, respectively. This laboratory sample was used as the initial material for the following experiment, with the aim to make an enriched P-LAT product.

A Bioprocess-system (Amersham Bioscience) with a 3 l column packed
15 with Heparin Sepharose FF (126-355, 14229-51, Amersham Bioscience) was used. The column was a Bioprocess-column (Amersham Bioscience) with an approximate bed height of 39 cm. The column was equilibrated with 130 mM NaCl and 10 mM Na_3PO_4, pH 7.0, before sample application. 1.3 l of the initial material (50 mM sodium citrate and 1 % sucrose, pH 7.4, 50 IU
20 antithrombin/ml) was applied to the equilibrated column, which was then washed with 9 l of equilibration buffer, whereupon 6 l of washing buffer 1 (375 mM NaCl and 10 mM Na_3PO_4, pH 7.0) was applied to the column. The solution that passed through the column during this procedure was called "wash 1" and contained minor amounts of L-AT. Following this, elution of a fraction enriched

in P-LAT was performed, using 15 l of washing buffer 2 (480 mM NaCl and 10 mM Na_3PO_4, pH 7.0). The solution that passed through the column during this procedure was called "wash 2" and contained a P-LAT enriched fraction. Then, the column was washed with 700 mM NaCl and 10 mM Na_3PO_4, pH 7.0 (washing buffer 3) for removal of remaining P-LAT. The fraction collected during this step was called "wash 3". The remaining AT molecules were then eluted from the column using elution buffer (2000 mM NaCl and 10 mM Na_3PO_4, pH 7.0). The fraction collected during elution was called "2M", and contained mainly AT. The process was performed at room temperature (about 22 °C). Proteins were detected by measurement of the absorbance at 280 nm. Table 4 shows the protein content recovered in the different fractions.

TABLE 4

Protein recovery in production of AT and P-LAT

Sample	Protein recovery (%)
Initial sample	100
Wash 1 (L-AT)	<1
Wash 2 (P-LAT)	5
Wash 3 (P-LAT + AT)	41
2M (AT)	54

The above chromatographic process was repeated five times and the "wash 2" (P-LAT) and "2M" (native AT) samples from different runs were collected and pooled to one P-LAT and one native AT sample. Both pooled samples were concentrated using two systems for ultra-filtration: a larger system (5 x 0.5 m^2 Biomax-5® Millipore Corporation), in which the volume was reduced to about 3 l, and a smaller system (3 x 0.1 m^2 Biomax-5®, Millipore Corporation), in which the volume was reduced to about 75 ml. The samples were analyzed, and the results are shown in Tables 5 and 6. As can be seen in the tables, the sample denoted "wash 2" contained enriched P-LAT, while the sample denoted "2M" contained essentially pure, native antithrombin.

In the tables, "IU" denotes International Units of antithrombin activity, measured as the thrombin inhibition activity in the presence of heparin, using a chromogenic substrate (Handeland et al, Scand J Haematol 31:427-436 (1983)). Furthermore, "ag" is the concentration of antithrombin as measured
5 nephelometrically, accounting for both active and inactive forms of the protein (adaptation of Stenberg, Clin Chem 23:1456-1464 (1977)).

TABLE 5

Sample	Hep.cof (IU/ml)	Protein (mg/ml)	AT:ag (IU/ml)	% L-AT	% P-LAT	% AT
Initial sample	9.6	1.5	9.3	<1	8	92
Wash 2 (P-LAT)	105	17	111	<1	56	44
2M (AT)	185	26	175	<1	<1	99

TABLE 6

Sample	Protein (IU/mg)	Ratio (IU/ag)	Purity (% AT)	Heparin binding ability
Initial sample	6.4	0.97	>99	>95%
Wash 2 (P-LAT)	6.2	0.95	90	>95%
2M (AT)	7.1	1.06	>99	>95%

10

CLAIMS

1. Method for separation of native antithrombin and pre-latent antithrombin present in a sample from each other, which method comprises
5 performing, in the order given, the following steps:

a) application of a sample containing native antithrombin and pre-latent antithrombin to an affinity material with affinity for antithrombin, wherein said application is performed under conditions such that binding of native antithrombin and pre-latent antithrombin to the affinity material is achieved;

10 b) application of a solution of an elution salt S_1 to said affinity material at a concentration C_1 of elution salt, for elution of pre-latent antithrombin, said concentration C_1 is in the range of 30-95 % of the concentration needed for elution of native antithrombin with the elution salt S_1;

in which method said concentration C_1 is such that pre-latent
15 antithrombin is eluted and native antithrombin is retained.

2. Method according to claim 1, which further comprises collection of the pre-latent antithrombin eluted in step b).

20 3. Method according to claim 1, in which said concentration C_1 is in the range of 30-90 % of the concentration needed for elution of native antithrombin with the elution salt S_1.

4. Method according to claim 3, in which said concentration C_1 is in the
25 range of 60-85 % of the concentration needed for elution of native antithrombin with the elution salt S_1.

5. Method according to any one of the preceding claims, which further comprises, after step b):

30 c) application of a solution of an elution salt S_2 to said affinity material at a concentration C_2 of elution salt, for elution of native antithrombin;

in which method said concentration C_2 is such that native antithrombin is eluted, and said elution salt S_2 may be the same elution salt as, or a different elution salt from, elution salt S_1.

5 6. Method according to claim 5, which further comprises collection of the native antithrombin eluted in step c).

7. Method according to any one of claims 5 and/or 6, in which said elution salts S_1 and S_2 are the same elution salt.

10

8. Method according to claim 7, in which the application in steps b) and c) of said concentration C_1 and said concentration C_2 is achieved through the application of a concentration gradient of the elution salt to the affinity material.

15

9. Method according to any one of the preceding claims, wherein said elution salts S_1 and S_2 are each chosen from the group consisting of NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$, BaCl, sodium acetate, Na_2SO_4, $NaHSO_4$, $MgSO_4$, K_2SO_4, $KHSO_4$, sodium citrate, potassium citrate, Na_3PO_4, Na_2HPO_4, NaH_2PO_4, K_3PO_4,
20 K_2HPO_4 and KH_2PO_4.

10. Method according to any one of the preceding claims, in which the affinity material comprises a substance selected from heparin, a fragment of heparin, fractionated heparin, heparan sulfate, a fragment of heparan sulfate
25 and fractionated heparan sulfate.

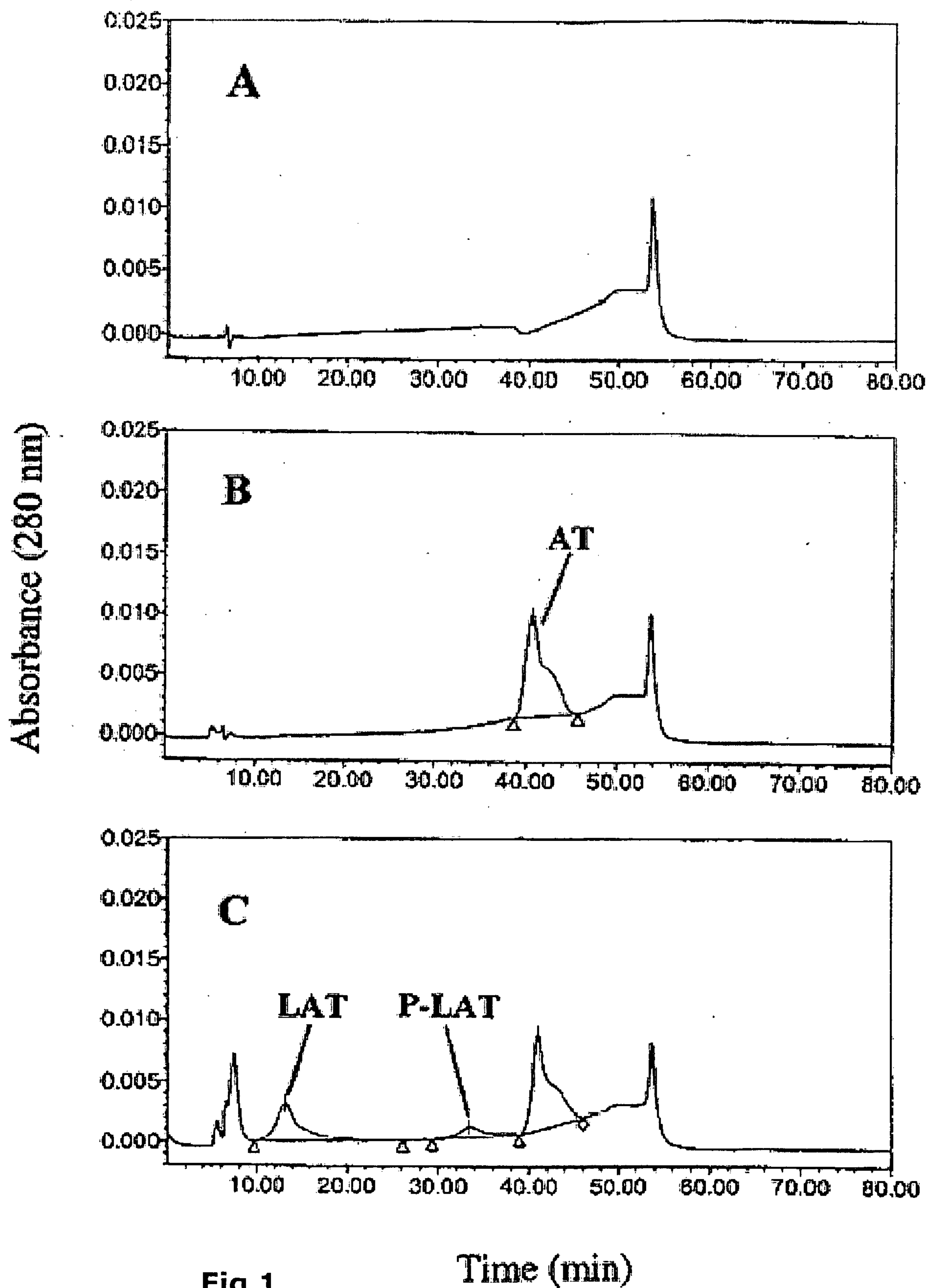

Fig.1

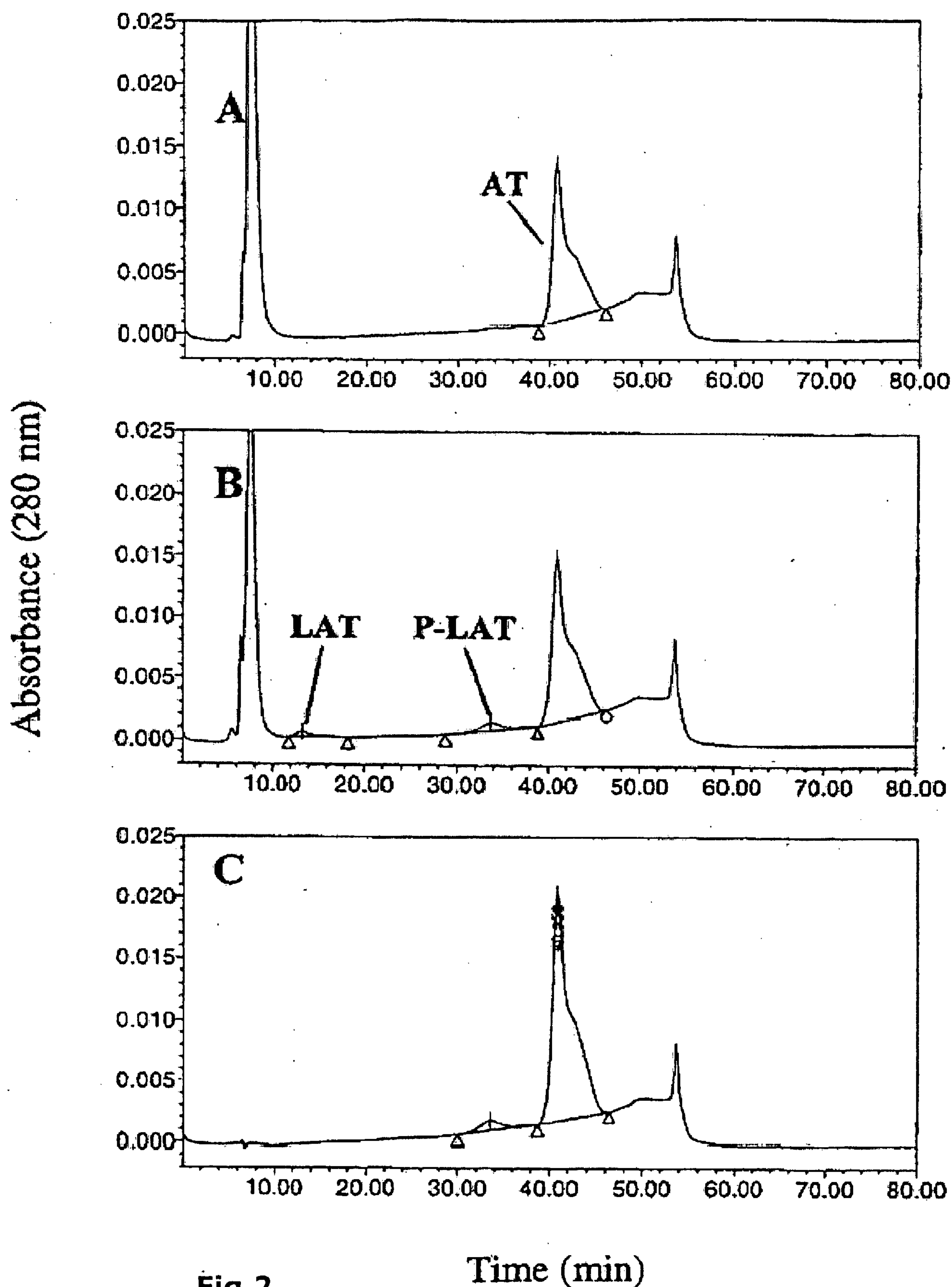

Fig.2

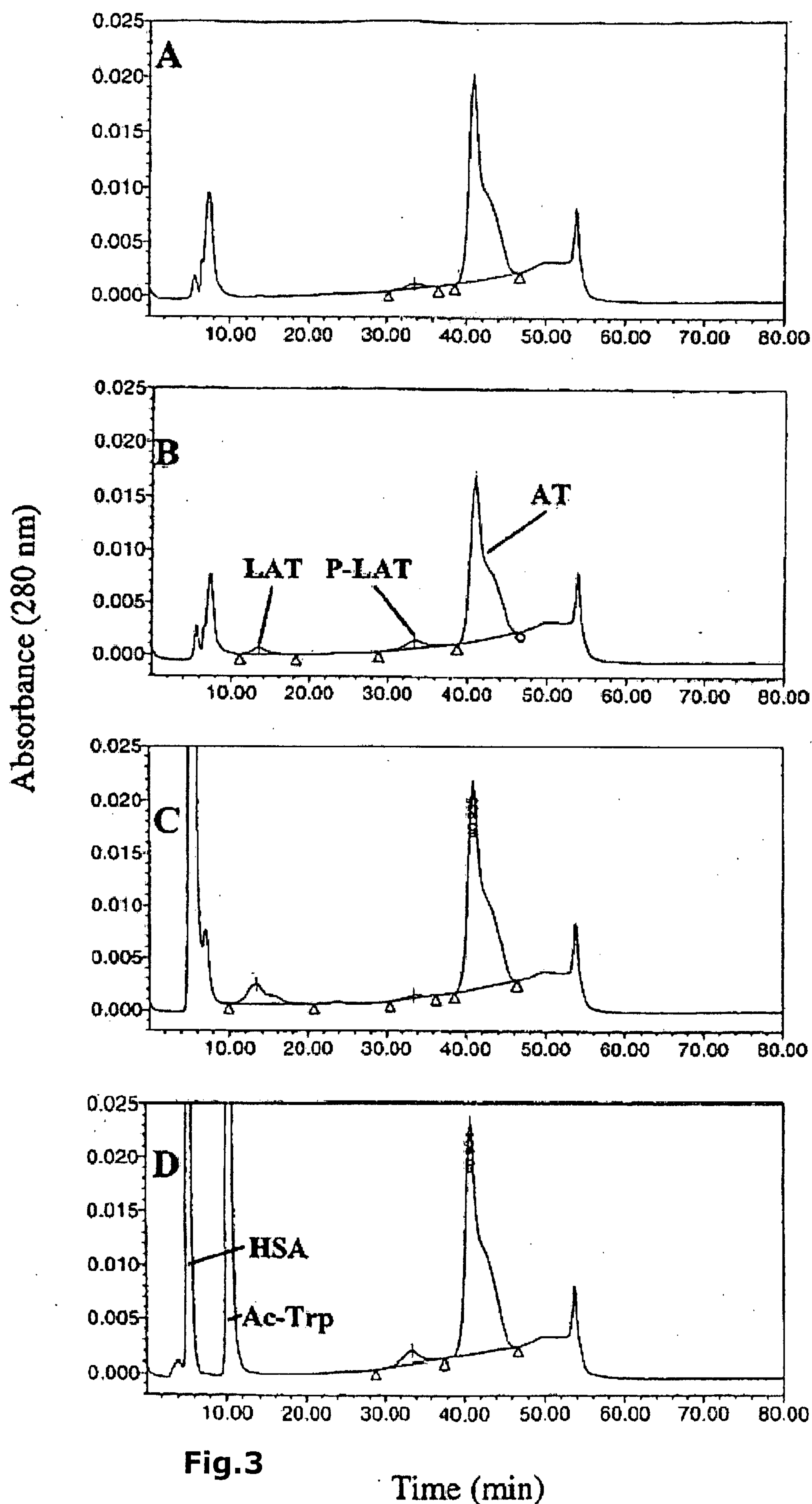

Fig.3

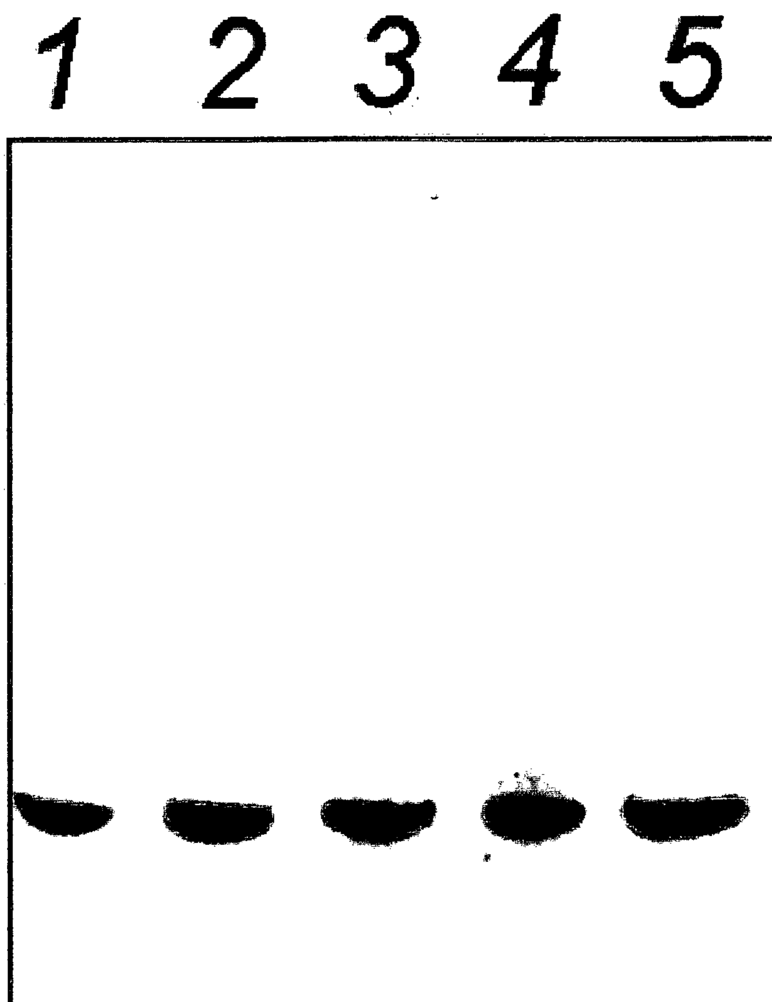

Fig.4

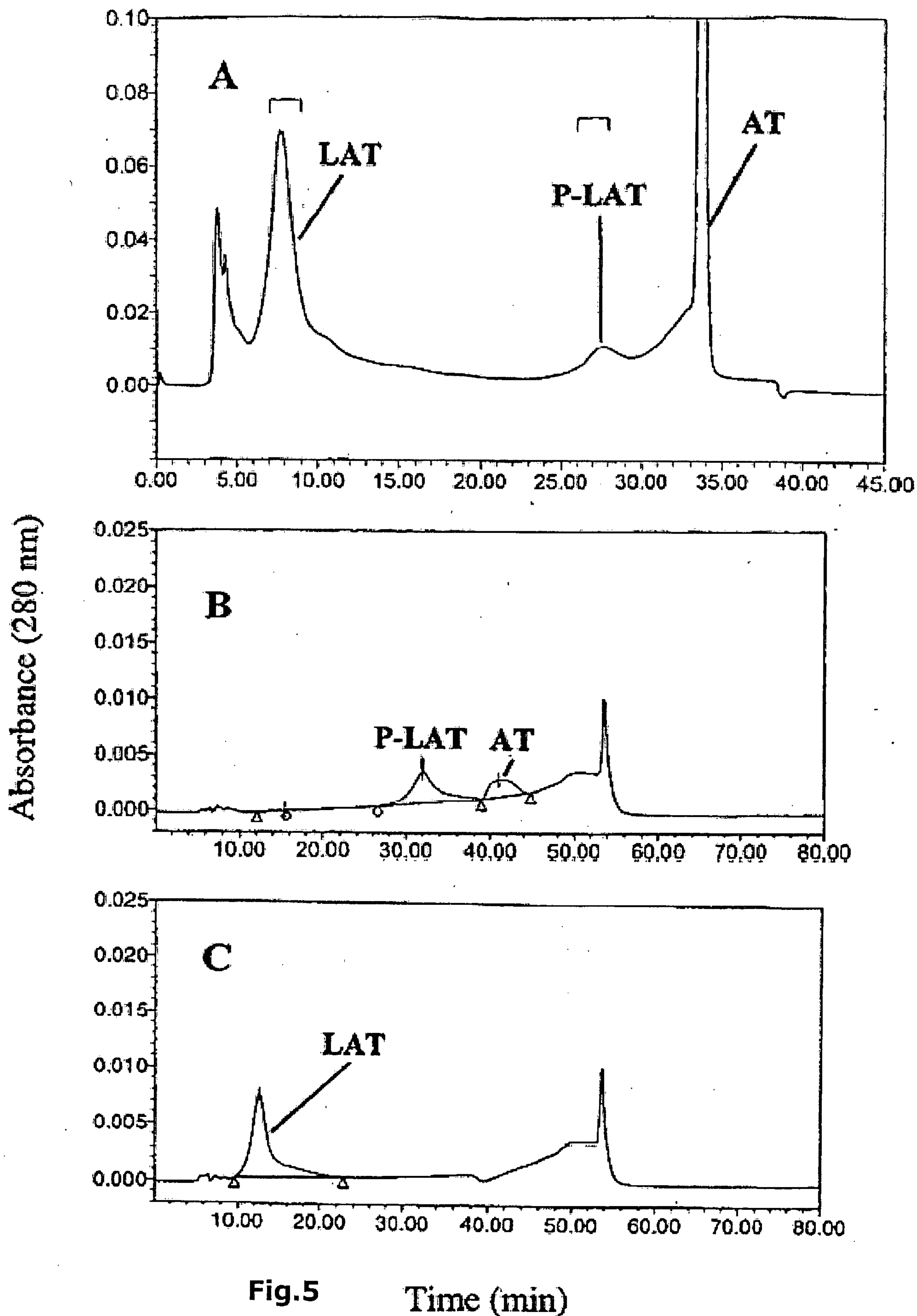

Fig.5

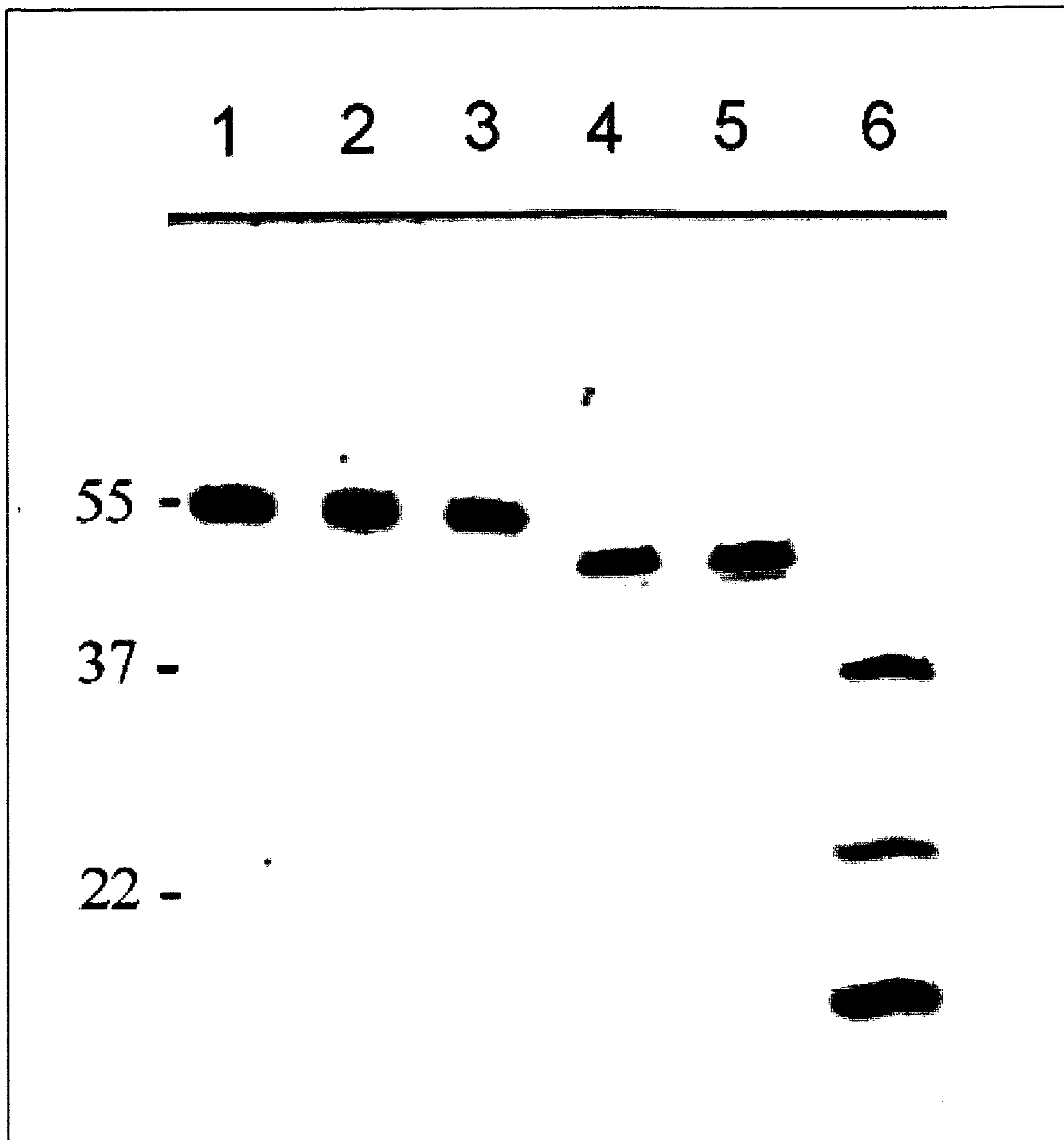

Fig.6