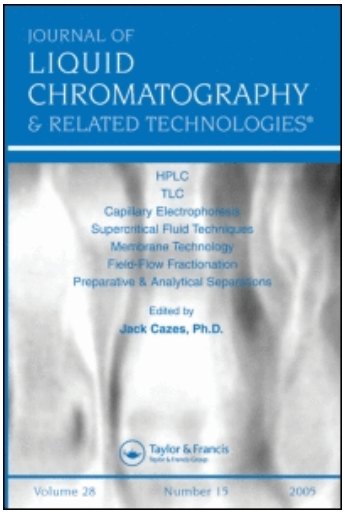

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High Performance Affinity Chromatography for Analysis of Human Antithrombin III

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HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY FOR ANALYSIS OF HUMAN ANTITHROMBIN III

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SUMMARY

Antythrombin III (AT III) is one of the most important regulators of the coagulation system. Improper concentration of AT III in blood is a great risk and often leads to thrombotic complications.

In routine laboratories there is a choice between two types of methods (functional and immunology) of AT III examination which are time consuming.

The paper deals with the AT III analysis by means of high performance affinity chromatography (HPAC). The results presented in the paper indicate high sensitivity of HPAC for AT III analysis which can be performed in a few minutes.

INTRODUCTION

α_2-globulin, known also as Antithrombin III (AT III) is the main inhibitor of blood coagulation [1]. This glycoprotein, synthetized in the liver is characterized by molecular weight from 58.000 to 67.000 and consists of a single polipeptide chain and about 15 % of carbohydrate [2,3]. The correct

concentration of At III in the blood plasma ranges from 85 to 115 % of the standard amount which equals 25-30 mg/100 cm^3 [2,3].

Reduced concentration of AT III in blood is a great and well established risk and can lead to many thrombotic complications resulting in the blood clot formation in a vascular bed [4].

The decrease of AT III biosynthesis is innate or caused by chronic acute liver diseases or by medical treatment with drugs like L-asparginase and oestrogen-progestogen [5]. The recognition of AT III deficit due to its increase consumption has also been demonstrated in sepsis and major trauma [6,7].

The functional methods (amidolytic or coagulative ones) and immunology methods (includig immunoelectrophoresis, laser nephelometry and radioimmunodiffusion on NOR-Plate-Partigen-Antithrombin III) are the main methods employed for AT III level determination in the routine analyses [8-10].

The comparison of the fibrinogen clotting time in the investigated and standard plasmas (which contains the correct AT III amount) is the coagulation method principle [8]. This measurement takes about 2 hours and requires at least 2 ml of plasma.

The amidolytic method is based on the thrombin deactivation process by AT III in the presence of heparin [9]. The AT III amount is estimated indirectly from the residual (not blocked) thrombin activity. The latter is analyzed in the second step, titrating an incubation mixture by a chromogenic substrate which reacts specifically with this enzyme. The experiment lasts at least 24 hrs even with the applicaton of the automated Hitachi 717 analyzer [11].

The competition of labelled and non-labelled antigen molecules with a limited number of bonding centers in the antibody molecule is the principle of the immunology method [10]. It is assumed that the labelled and non-labelled antigen exhibits the same skill at binding antibody. During the analysis the amount of antibody and labelled antigen is always constant. Only the amount of the investigated antigen changes. A typical immunology procedure demands about 7.5 µl of the investigated plasma and 4.4 µl of the labelled antigen (which is rather expensive substance). When electrophoresis is used for separation of the immunology mixture the analysis takes about 45 hrs.

As results from the above the analysis time of AT III by one of the described routine methods ranges from a few to several hours. The accuracy of these

methods is about 3 % which corresponds to about $\pm$ 1 mg of AT III per 100 cm^3 of blood at normal AT III blood level.

It is commonly known that antithrombin III neutralizes the active coagulation factors presented in plasma [1-3]. This process is accelerated by heparin. The affinity of antithrombin III to heparin is also revealed in the artificial system in which heparin is chemically bonded with a support surface [12,13]. In other words AT III can be estimated directly in plasma by means of affinity chromatography [14]. In the well selected chromatography system and conditions the precision of analysis can be considerably high and analysis time relatively short.

The results discussed in the paper not only show the possibility of AT III analysis directly in blood by means high performance affinity chromatography (HPAC) but also allow to conclude about the time consumption and sensitivity of this method.

EXPERIMENTAL

Materials

Controlled porous glass (CPG) preparation. Vycor glass composed of 10 % Na_2O, 35 % B_2O_3 and 55 % SiO_2 was used as the starting material for preparation of controlled porous glass. A portion of this glass (fraction 40-80 μm) was heated at 557 °C for 72 hours. The thermally treated glass was next converted into porous sorbent by proper leaching with 3 N H_2SO_4 and 0,5 N NaOH according to the procedure previously described [15,16]. The final material was characterized by the following data: the specific surface area S_{BET} = 72.6 m^2/g, the pore volume V_p = 1.13 cm^3/g and the pore diameter D = 60.0 nm.

Preparation of silica-based supports. By the deposition of polysaccharide on silica surface the advantages of the traditional soft gel affinity supports with the excellent mechanical properties of silica supports were combined. The introduction of a hydrophilic and non-ionic polymeric layer on the silica surface minimizes the specific adsorptions and gives a high concentration of activable hydroxylic functions. The obtained porous glass was covered for this reason with a double layer of polysaccharide. The starting material was preliminarily impregnated with positively charged DEAE-dextran to neutralize the

cation exchange capacity of CPG. 1.5 g DEAE-dextran (Pharmacia Fine Chemicals) (MW = 500.000) in 25 ml bidistilled water (pH had been adjusted to 11.5) was added to 10 g of CPG at room temperature. After water evaporation (in vacuum conditions) the support was dried at 80 °C for 15 hours. The former layer was cross-linked by diethylenglycol diglycidyl ether (EDG-2) (IChP, Warsaw, Poland) in the aqueous media. The solution consisting of 10 ml 1 N NaOH, 0.56 ml EDG-2 and 0.023 g $NaBH_4$ in 100 ml bidistilled water was used for cross-linking. The mixture was stirred at 40 °C for 30 minutes. After the reaction the material was washed and dried. The second layer of polysaccharide was introduced following the procedure described above, but instead of DEAE-dextran, pure dextran (Polfa, Kutno, Poland) (average molecular weight = 110.000) was employed and only 0.14 ml EDG-2 was applied during the cross-linking procedure. Evaporation of water was performed at 80 °C at the atmospheric pressure.

Before heparin bonding the prepared support was activated by EDG-2 according to the conditions described elsewhere [17].

Heparin immobilization. In order to immobilize high molecular weight heparin (Polfa, Poland), 5 g of the modified CPG was treated with the solution consisting of 5 ml 0.1 M Na_2CO_3 buffer, pH = 8.7 and 2 ml heparin. The material with heparin was stirred at room temperature for 48 hours. After washing and drying the obtained affinity sorbent was put into the chromatographic column.

Preparation of blood plasma. A standard procedure was used for blood preparation [18]. Blood taken from vein was immediately mixed with sodium citrate (concentration 3.8 %) at the volume ratio 9:1. Stabilized in such a way blood was next centrifuged at 1.500 - 2.000 rpm over 10 - 15 min. The separated plasma was directly injected on the column.

Methods

Pore volume and mean pore diameter in the obtained CPG were calculated from Mercury porosimetry data. Measurements were performed with a Mercury porosimeter Type 2000 (Carlo Erba, Milan, Italy).

The specific surface area investigations were carried out by the BET method (low temperature adsorption of nitrogen). A nitrogen Sorptomat (type 1800 from Carlo Erba) was used for this purpose.

The chromatographic investigations were carried out with a Shimadzu liquid chromatograph (Japan). The HPLC apparatus consisted of an LC-9A high pressure pump, a gradient system and UV-VIS photodiode array detector SPD-MGA connected to an IBM AT PC PCD-2M data system from Siemens (Germany).

100 µl volumes of AT III standard solution (Institute of Blood-Derivatives, Production Division of Immuno AG, Vienna, Austria) or blood plasma were injected (by means of Rheodyne 7125 injection valve) onto a column (120 x 4 mm) packed with affinity sorbent and pre-equilibrated with initial buffer (0.02 M disodium phosphate, pH 7.4 - buffer A) at a room temperature and at a flow rate 1 ml/min. The adsorbed AT III was then eluted using a salt gradient (0.02 M disodium phosphate, 2 M sodium chloride, pH 7.4 - buffer B). In these conditions the AT III and accompanying substances were eluted on their respective affinity sorbents.

RESULTS AND DISCUSSION

Controlled porosity glasses represent siliceous materials which are characterized by a very narrow pore size distribution. Due to this feature their porous structure is far better penetrated by the chromatographed molecules compared to the structure of silica gel. Controlled porosity glass applied in the investigations possesses the mean pore diameter equal 60 nm. Thus the structure of such a material is accessible for AT III molecules even after deposition of dextran layers (which changes the CPG surface character and screen the denaturative interaction between the separated molecules and bare CPG surface) and coupled heparin molecule.

The chromatogram of AT III standard obtained with the application of the sorbent described above is presented in Fig. 1. The first part of the chromatogram is connected with substances which accompany the AT III and do not exhibit the affinity for the immobilized heparin. The second concentration zone with the maximum at 14.9 min. corresponds to AT III. It appears about 4 minutes after the beginning of NaCl gradient rise (NaCl gradient starts in the 10-th minute). Table I contains the retention data of AT III standard after successive injections. These values prove the reproducibility of the analysis.

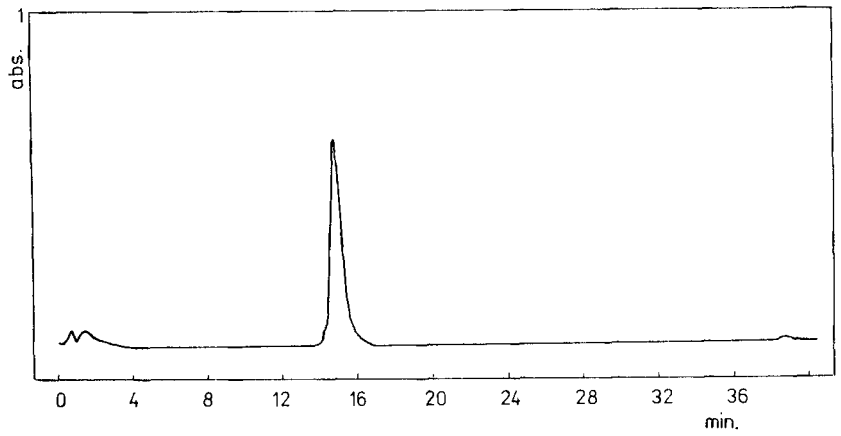

Figure 1. Elution of human antithrombin III from a column packed with the sorbent composed of heparin chemically bonded to a dextran layer immobilized on CPG.

Column: 120 x 4 mm,

Sample: 100 µl of AT III standard (50 I.U./ml),

Buffers to the elution: Buffer A - 0.02 M disodium phosphate, pH 7.4

Buffer B - 2.0 M NaCl in 0.02 Na_2HPO_4, pH 7.4

Elution: 0.0 - 10.0 min - buffer A,

10.0 - 30.0 min - linear gradient from 0 to 100 % of buffer B

Flow rate: 1 ml/min,

Detector wave length: 280 nm.

As mentioned before the possibility of the prepared sorbent employment in the AT III analysis in a human plasma was one of the main tasks of the presented investigations. The blood plasma is a complex mixture containing substances which can affect the separation shown in Fig. 1. Fig. 2 presents the chromatogram of a human plasma obtained on the same column as in Fig. 1. As in Fig. 1, in Fig. 2 two concentration zones are distinctly seen. The second peak with the retention time 14.8 min corresponds to AT III contained in the plasma. The first one represents the other compounds of plasma which do not show the affinity for chemically bonded heparin. In comparison to the previous

Table I. Retention data and features of AT III zones obtained after successive injections.

N°	Retention time [min]	Concentration [%]	Peak purity index	Max. wave length [nm]
1	14.85	93.745	0.9475	231
2	14.83	94.917	0.9037	233
3	14.85	93.743	0.9472	230
4	14.81	94.931	0.9107	230
5	14.86	94.989	0.9487	237
6	14.70	93.435	0.9245	220
7	14.73	92.498	0.8984	233
8	14.77	93.263	0.8883	226
9	14.87	95.327	0.8924	234
10	14.77	93.427	0.8706	225

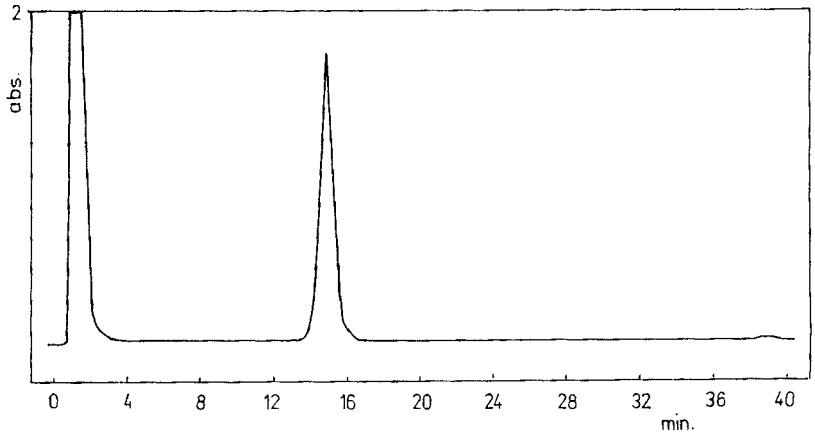

Figure 2. Elution of human plasma (sample 100 µl).

Sorbent, column and conditions - see Figure 1.

chromatogram it is significantly larger which can be seen if one takes into account a number and concentration of plasma compounds (except AT III). The result seen in Fig. 2 proves the utility of high performance affinity chromatography (with immoblized heparin in the column) for AT III analysis even in such a complex mixture as blood (after centrifugation of red cells).

The conditions and system used in the separations shown in Figures 1 and 2 allow for AT III analysis in 30 minutes. In the first 10 minutes the isocratic elution with initial buffer (buffer A) runs. From the 10-th to the 30-th minute the NaCl concentration rises (linear gradient from the initial buffer to the same buffer containing 2 M NaCl - the linear concentration increase of buffer B in buffer A). The column regeneration (the flushing of the column with the initial buffer) begins in the 30-th minute. As appears from the chromatograms (Figures 1 and 2) nothing is eluted from the column between the 3-rd and 13-th minute. The scanning from 195 to 380 nm leads to the same conclusion. This suggests the possibility of the analysis time decrease e.g. by the earlier start of the NaCl gradient in the mobile phase. Figure 3 presents the chromatogram of AT III standard where the gradient of buffer B starts in the 3-rd minute. In the 8-th minute a mobile phase contains 50 % of B, and in the 9-th minute buffer B reaches 100 %.. As it is seen the chromatogram is similar to that in Figure 1. The significantly shorter analysis time (less than 8.5 minutes) is the only difference. The retention time of the AT III peak maximum equals 7.33 minutes and the AT III zone starts to appear about 6.7 minutes after its injection. Considering the chromatogram in Figure 3 it is seen that the AT III analysis can be carried out even in shorter time and using lower NaCl concentration. The latter conclusion is confirmed by the chromatogram shown in Figure 4 where the gradient of B eluent is steeper and in 7-th minute the mobile phase contains 50 % of B. For the next 2 minutes the mobile phase composition comes back to the initial buffer. Comparing Figures 3 and 4 similarity of the both chromatograms is shown. Still shorter AT III retention time is the only difference. Almost the same peak areas (representing AT III) indicate the same amounts of the AT III eluted.

Figure 5 presents the chromatogram of AT III at the very steep NaCl gradient. It starts in the 3-rd minute and finishes in the 6-th minute (100 % of B). The

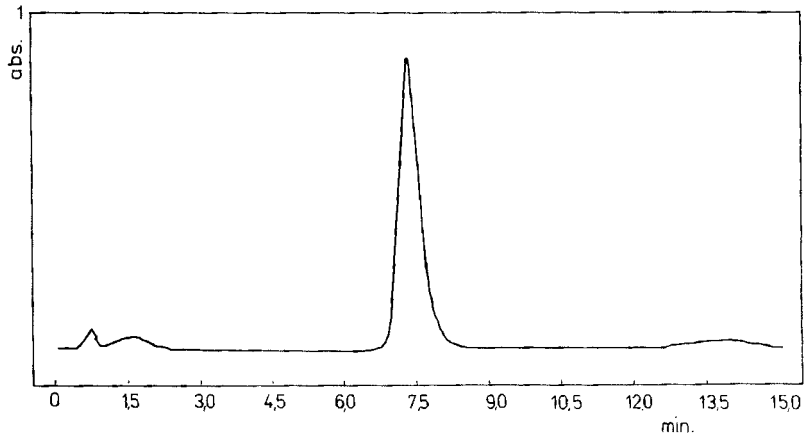

Figure 3. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 8.0 min - linear gradient from 0 to 50 % of buffer B,

8.0 - 9.0 min - linear gradient from 50 to 100 % of buffer B.

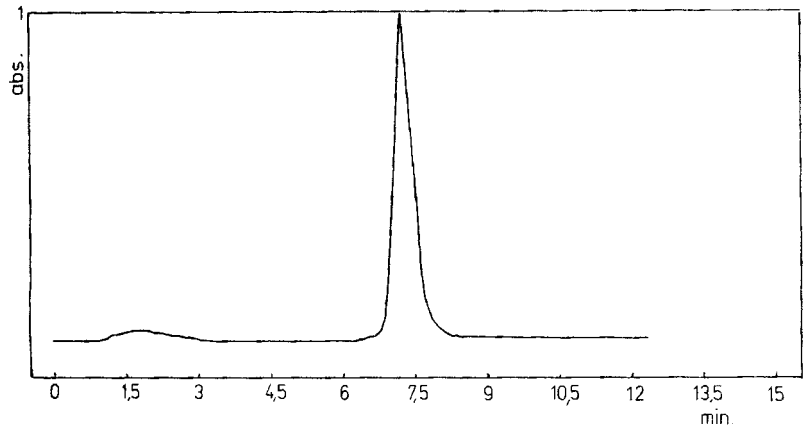

Figure 4. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 7.0 min - linear gradient from 0 to 50 % of buffer B.

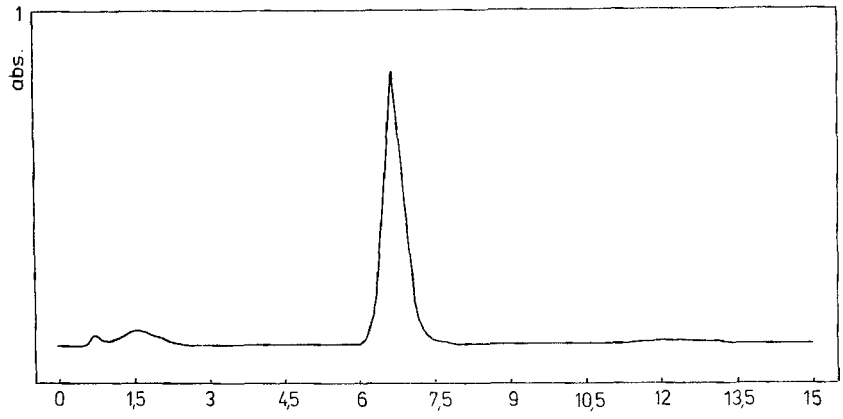

Figure 5. Elution of AT III standard.

Sorbent, column and conditions - see Figure 1.

Elution: 0.0 - 3.0 min - buffer A,

3.0 - 6.0 min - linear gradient from 0 to 100 % of buffer B.

retention time of AT III equals 6.68 minutes. Practically it is the shortest retention time that can be observed with the column and the flow rate used in the presented experiments and at the beginning of the NaCl gradient in the 3-rd minute (just after the elution of the substances which accompany the AT III). The further diminution of the analysis time is possible by the decrease of the column length (e.g. to 3 or 5 cm) and by the increase of the mobile phase flow rate.

Taking into account the last remarks and the results shown in Figures 3 - 5 it is seen that the AT III analysis can be carried out by means of high performance affinity chromatography within less than 5 minutes. It is a considerable advance compared to the quickest, classic methods described above [8-11].

The chromatograms shown in Figures 1 - 5 were performed employing 0.02 M Na_2HPO_4, pH 7.4 buffer as the initial mobile phase. It can be easily found that besides Na ions, which are contained in both eluents, blood possesses, among others, Cl ions, which are the components of the AT III eluting buffer. Their concentration in blood (95 - 107 mmol/dcm^3) should not practically influence the separation, all the more the small plasma volume (100 µl in the case of the

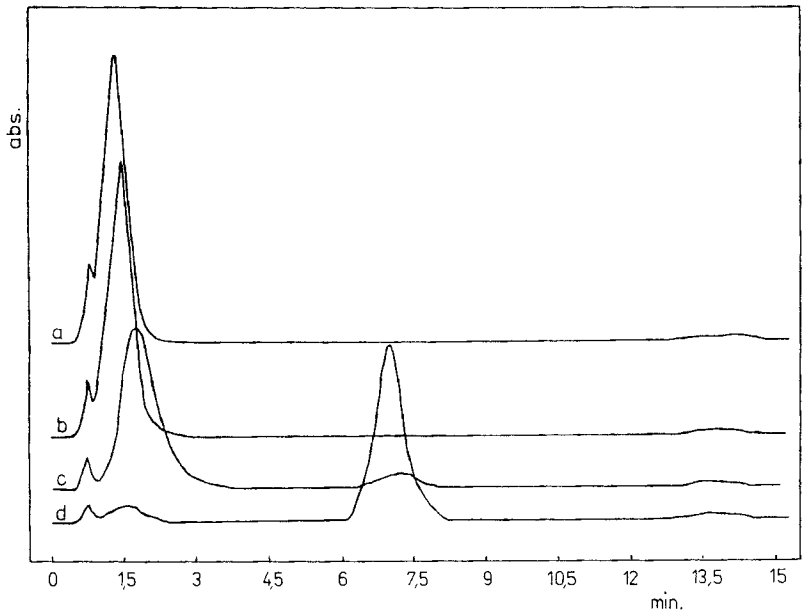

Figure 6. Chromatograms of AT III standard.

Sorbent, column and conditions - see Figure 1.

a) Elution: 0.0 - 3.0 min - 15 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 15 to 100 % of buffer B.

b) Elution: 0.0 - 3.0 min - 10 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 10 to 100 % of buffer B.

c) Elution: 0.0 - 3.0 min - 5 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 5 to 100 % of buffer B.

d) Elution: 0.0 - 3.0 min - 2 % of buffer B in buffer A,
3.0 - 8.0 min - linear gradient from 2 to 100 % of buffer B.

above shown chromatograms) is diluted in the mobile phase after injection. However, the problem appears what the maximal NaCl concentration in the initial buffer is which does not change the separation.

Figure 6 presents the chromatograms of AT III obtained at the same shape of gradient (the increase of B buffer begins in the 3-rd minute of analysis time) but with the various concentrations of NaCl in the initial buffer. (The latter is expressed as the percent of B buffer in buffer A). As results from Figure 6, at the 15 and 10 % of B buffer in buffer A (chromatograms a and b) the separations are very bad and not acceptable from the analytical point of view. A similar situation is in the case when the initial buffer contains 5 % of B. The appearance of a small concentration zone after 7.2 minutes is the only main difference among a, b and c chromatograms. The concentration 2 % of B in the initial eluent leads to the chromatogram d (Figure 6) which is very similar to those in Figures 3 - 5.

The quantitative analysis is based on the relation between the amount the injected substance and the dimension of the signal response which shows the presence of the substance leaving the chromatographic column. The linearity of such a relationship is very useful, especially in the routine analysis. The dependences of AT III peak area vs. the volume of the injected AT III standard or plasma are shown in Figures 7 and 8 respectively. These relationships were performed at 280 nm wave length and at the same detector sensitivity as well as the same gradient of the mobile phase. As results from the last Figures the deviation from the linearity of these plots occurs for the samples exceeding 50 µl. Thus, employing the system used the sample volumes lower than 50 µl should be injected or the dilution of the analysed preparate is desirable.

Table II lists the dilution degree of AT III standard (Table II a) or human plasma (Table II b), the elution volumes of AT III peak, the concentration of AT III in the eluted zone and the properties of AT III zone. These data indicate the independence of the retention of α_2-globulin vs. dilution.

The investigations of the sensitivity of the HPAC method, carried out using the equipment described in Experimental, shows that the AT III can be examined in the human plasma diluted more than 10.000 times. In other words AT III can be analyzed using less than 10 nl of blood, hence in the much smaller amount than the classical method requires.

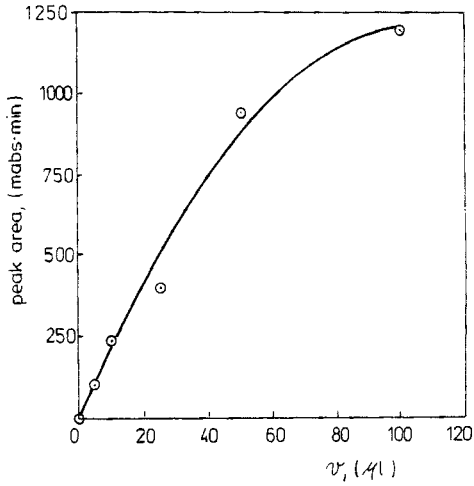

Figure 7. AT III peak area vs. the sample volume of AT III standard (activity - 50 I.U./ml).

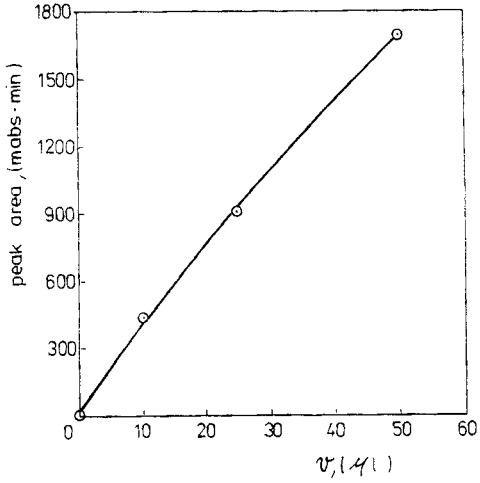

Figure 8. AT III peak area vs. the volume of human plasma.

Table II. Retention data of AT III peak in the diluted AT III standard or human plasma and features of AT III zone

Table II a. Data for AT III standard.

N°	Dilution degree	Retention time [min]	Concentration [%]	Peak purity index	Max. wave length [nm]
1	Pure standard	7.24	99.8	0.9276	225
2	2 times	7.21	100.0	0.9124	223
3	4 times	7.12	100.0	0.9410	227
4	10 times	7.27	100.0	0.9085	222
5	20 times	7.27	99.5	0.9659	219

Table II b. Data for human plasma

1	Pure plasma	7.19	99.5	0.9194	221
2	2 times	7.17	99.7	0.9212	224
3	4 times	7.19	99.8	0.9164	218
4	10 times	7.32	100.0	0.9202	227

CONCLUSIONS

1. Heparin chemically bonded to a dextran layer immobilized on CPG exhibits affinity for the human antithrombin III.
2. The other substances presented in blood plasma do not disturb the elution of AT III.
3. The application of the proper NaCl gradient and short chromatographic column can reduce significantly the AT III analysis time.
4. The sensitivity of high performance affinity chromatography in relation to AT III analysis exceeds many times that of classical methods

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