

DETERMINATION OF ANTITHROMBIN III HEPARIN-BINDING CAPACITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. H.A. Nunez, Michigan Department of Public Health, Blood Derivatives Section, Lansing, Michigan 48909

The heparin-binding capacity of purified human antithrombin III (AT) both native and after a variety of denaturing conditions was measured using a high performance liquid chromatographic (HPLC) system.

An exclusion chromatography, TSK-3000, 0.75 (ID) x 50 cm column equilibrated with 0.1M NaCl in 0.02M potassium phosphate buffer pH 7.35, at a flow rate of 1 ml/min was used. Under these conditions two essentially resolved peaks were obtained at 10.5 to 14 min. The peak corresponding to the unbound AT, or native AT in the absence of heparin (H), is sharp; that of the AT-H complex is broad due, probably, to the size heterogeneity of the H used in the tests. Without H, aggregates of AT are also resolved by this technique. The areas under the curve of the protein peaks monitored spectrophotometrically in the absence and in the presence of an excess of H are used to quantitate these species. A titration of AT with increasing amounts of H indicates the concentration of H sufficient to bind all the H-binding AT present in a given AT containing sample.

The results of the HPLC method correlate well with those obtained by two-dimensional or crossed immunoelectrophoresis (CIEP), a technique that can be used to estimate the H-binding capacity of AT. Thus, for example, in non-pasteurized, pasteurized and heated AT samples, for which the HPLC method indicated that 5, 25 and 80% of the AT had lost its H-binding capacity, the CIEP gave similar results.

The results indicate that the HPLC method can advantageously be used to determine quantitatively the H-binding capacity of AT and, if present, AT aggregates, thus appearing more informative than CIEP. The analysis time is less than 30 minutes and uses inexpensive reagents.

STUDIES ON THE DYNAMICS OF ANTITHROMBIN III DURING HEPARIN INFUSION BY RADIOIMMUNOASSAY. S. Urano, M. Nakagawa, T. Kitani, Y. Maeda, M. Watada, S. Kanayama, H. Iijichi, 1st Dpt. of Medicine Kyoto Prefectural University of Medicine, Kyoto, Japan.

A radioimmunoassay method for antithrombin III (ATIII) was developed in order to detect the AT III levels correctly in plasma and tissues and the effect of heparin infusion was investigated on rat using this method and  $^{125}\text{I}$  labeled ATIII. Rat AT III was purified from rat defibrinated plasma by heparin sepharose affinity chromatography and gel filtrations. This purified AT III was used for the preparation of specific AT III antiserum. Labeling of AT III with  $^{125}\text{I}$  was performed according to the method by Hunter and Greenwood. Plasma level of AT III were significantly decreased in the treated group with heparin for 6 hours, although significant difference was not observed in AT III contents in various organs. The behavior of i.v. injected AT III labeled with  $^{125}\text{I}$  in the normal control and treated groups proved the difference on the half life of AT III. Control group gave 52 hours and it was shortened in the treated group. The percent radioactivity per ml plasma after 6 hours of heparin infusion was  $1.16 \pm 0.51$ , and  $2.01 \pm 0.38$  in the control group, and significant difference was observed ( $p < 0.05$ ). On the contrary the percent dose radioactivity per g tissue wet weight was significantly increased in the liver, lungs, and large intestine on the heparin treated group. The decreased amount of the intravenously injected labeled AT III appears to be trapped and metabolized in the various organs mainly in the liver during heparin infusion. The decrease of plasma AT III levels on the patients treated with heparin may be explained from these experimental results.

RELATIVE CONTRIBUTIONS OF THROMBIN AND ANTITHROMBIN III AFFINITIES OF HEPARIN FRACTIONS TO THE RATE OF INACTIVATION OF THROMBIN BY ANTITHROMBIN III. A.L. Cerskus, K.J. Birchall, F. Ofosu, M.A. Blajchman and J. Hirsh. Department of Pathology, McMaster University, Hamilton, Ontario, Canada.

Heparin enhances the rate of inactivation of thrombin (IIa) and other coagulant proteases by antithrombin III (AT III). The anticoagulant activity of heparin is associated with heparin moieties which have high affinity to AT III. However, the contribution of the IIa affinity to the anticoagulant activity has not been as clear. Standard porcine mucosal heparin was fractionated on affinity columns consisting of purified human AT III and IIa immobilized on agarose to determine the effect of heparins of various affinities on the second order rate constant ( $K''$ ) for the inactivation of IIa by AT III. Results are expressed as the rate enhancement factor (REF) which is defined as the ratio of the  $K''$  in the presence of 50 ng/ml heparin to the  $K''$  in the absence of heparin. The REF for unfractionated heparin was 9.5. Chromatography on either AT III-agarose or IIa-agarose resulted in elution of three heparin fractions corresponding to a void volume fraction, a low affinity fraction and a high affinity fraction. The REF's of the fractions eluted from AT III-agarose were 1.3, 1.4 and 18.8, respectively while the REF's for the corresponding IIa fractions were 5.6, 14.9 and 18.1, respectively. Rechromatography of the high affinity fraction from AT III-agarose on the IIa-agarose column resulted in the elution of three fractions with REF's of 17.9, 21.1 and 27.0, respectively. Conclusions: 1. Heparin affinity to AT III is critical for anticoagulant activity as all of the activity is found in the high affinity fraction. 2. Heparin affinity to IIa also contributes to anticoagulant activity but is not critical as significant activity is observed in low affinity fractions. 3. High affinity to both IIa and AT III results in greater activity than that seen with fractions of high affinity to either protein alone.

FRACTIONATION OF HEPARIN BY HUMAN PF-4 AFFINITY RESIN. Martha Aiken, Eduardo Novoa, Jawed Fareed and Daniel A. Walz Wayne State University School of Medicine, Detroit and Department of Pathology, Loyola University of Chicago, USA.

Platelet factor 4 (PF-4) is a high affinity heparin binding protein, specifically released from the alpha granule upon platelet activation. We have coupled purified human PF-4 to Sepharose and utilized salt gradients to fractionate commercial porcine sodium heparin. Under these conditions, all of the applied heparin bound to the PF-4, unlike heparin fractionation by antithrombin but similar to heparin fractionation by thrombin or protamine. Three heparin fractions can be distinguished, eluting at approximately 0.5, 0.7 and 0.85 M NaCl. When each fraction was evaluated for its specific activity, no differences were apparent between the starting product and these fractions for USP units. However, the three fractions were each capable of prolonging the activated PTT clotting time of citrated pooled monkey plasma. The final fraction, 0.85 M, gave a four-fold longer APTT time than non-heparinized control plasma. There was no resolution of the antithrombotic and anticoagulant heparin activities. Each of these fractions is capable of competing effectively with PF-4 and its purified proteoglycan (PG) carrier to cause dissociation of the natural PG-PF-4 complex. These systems indicate that PF-4 whether released singularly or in complex form, will readily bind to natural heparin-like compounds with a high affinity and will function as a competitive inhibitor of the antithrombin of plasma. Prior resolution of heparin, by chromatography on thrombin or protamine resins does not result in the purification of a more highly active fraction than was obtained by PF-4 chromatography alone. Experiments are underway to determine whether such procedures yields heparin with significantly longer animal survival times.