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THE RELATIONSHIP OF LIPIDS TO ANTITHROMBIN CONSUMPTION DURING CLOTTING. J. H. Winter, B. Bennett, F. McTaggart* and A. S. Douglas. Department of Medicine, University of Aberdeen, Scotland and *Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Cheshire, U.K.

The relationship between serum lipids and the plasma and serum antithrombin activity was studied in blood samples from 18 patients who had suffered myocardial infarction 1-3 years previously and 18 age matched controls without clinical evidence of vascular disease. Progressive antithrombin activity was measured by the technique of Abildgaard et al (1970) and antithrombin III levels were measured by radial immunodiffusion using Partigen plates.

Plasma specific antithrombin activity was defined as the ratio of progressive antithrombin activity to the antithrombin III level measured by immunoassay. Serum was separated from blood incubated for 1 hour at 37°C in glass tubes.

Serum tryglycerides were determined using a commercial kinetic enzymatic method, heparin precipitable lipoproteins (VLDL and LDL) by $a_2$nephelometric method, and high density lipoprotein by a Mn^{2+} and heparin precipitation method.

A negative correlation was found between plasma specific antithrombin activity and serum triglycerides ($R = -0.35$ $p < 0.05$). Significant correlations were found between the consumption of antithrombin activity during clotting and serum triglyceride ($R = -0.53$, $p < 0.001$) heparin precipitable lipoproteins ($R = -0.48$, $p < 0.01$) and high density lipoprotein ($R = + 0.40$, $p < 0.01$).

These data indicate that blood lipids influence antithrombin III - coagulant interactions, possibly protecting the activated coagulant factors from inhibition by antithrombin. The data concerning antithrombin-lipid interactions clearly merits expansion.

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INACTIVATION OF HEPATITIS B VIRUS BY HEAT IN ANTITHROMBIN III STABILIZED WITH CITRATE. Edward Tabor, Genesio Murano, Philip Snoy, Robert J. Gerety. Hepatitis & Coagulation Branches, Division of Blood & Blood Products, & Division of Product Quality Control, Bureau of Biologics, Bethesda, MD, USA.

The recent report that antithrombin III (AT-III) prepared in solution with 0.5 M sodium citrate can withstand heating at 60°C for 10 hours suggested that this method of preparation could permit the heat-inactivation of hepatitis B virus (HBV) which might be present. Although heating at 60°C for 10 hours will inactivate HBV in albumin, this will not inactivate HBV in whole plasma or in some purified hepatitis B surface antigen (HBsAg) preparations. HBV, subtype ayw, hepatitis B e antigen positive, containing $10^{3.5}$ chimpanzee infectious doses per ml, was added to each of two vials containing 690 plasma units each of AT-III. One was kept at 4°C for 10 hours (unheated AT-III), the second was heated at 60°C for 10 hours. The contents of both vials were separately dialyzed and inoculated intravenously into chimpanzees with no prior exposure to hepatitis B virus and with no serologic evidence of prior hepatitis B infection. No evidence of hepatitis B was detected in the chimpanzee inoculated with the heated AT-III during six months of evaluation. Aspartate and alanine aminotransferase levels (AST, ALT) remained within or near the normal range. Neither HBsAg, antibody to hepatitis B core antigen (anti-HBc), nor antibody to HBsAg (anti-HBs) was detected in any of the weekly serum samples. The chimpanzee inoculated with the unheated AT-III developed hepatitis B. AST and ALT became elevated 13 weeks after inoculation; HBsAg (subtype ayw) was detected six weeks after inoculation and remained detectable; anti-HBc became detectable 12 weeks after inoculation. Liver biopsies obtained 20 and 23 weeks after inoculation showed histologic changes of acute hepatitis. This study shows that the application of heat at 60°C for 10 hours to AT-III stabilized with 0.5 M sodium citrate can inactivate > 1,000 chimpanzee infectious doses of HBV.

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PASTEURIZATION OF ANTITHROMBIN III: STABILIZATION BY HEPARIN AND LYOTROPIC ANIONS. Thomas F. Busby, Donald H. Atha and Kenneth C. Ingham. American Red Cross Blood Services Laboratories, Bethesda, Md. 20014.

Pasteurization of Antithrombin III (AT III) for 10 hours at 60°C is necessary to reduce the risk of transfusion hepatitis. Addition of appropriate stabilizers can largely prevent the loss of antithrombin activity which otherwise occurs during pasteurization. Studies of the mechanism of denaturation and stabilization have been facilitated by the use of 1,8-anilinonaphthalene sulfonate (ANS) which binds weakly to the inhibitor and whose fluorescence undergoes a sigmoidal response to increasing temperature as the protein unfolds. The extent of the increase in ANS fluorescence correlated roughly with the loss of antithrombin activity and with the extent of protein aggregation as determined by high pressure exclusion chromatography. The midpoint, T_d, of the thermal denaturation curve increased by 13 and 19°C in the presence of 0.5 M and 1.0 M sodium citrate respectively. Phosphate, sulfate, and EDTA were also strong stabilizers while the chaotropic anions, iodide and thiocyanate were potent destabilizers. Heparin, at 10 mg/ml, increased T_d by 7°, presumbly through a direct binding mechanism. Reducing agents increased ANS fluorescence by an amount similar to that seen with thermally denatured samples, an effect which was inhibited by heparin but not by citrate. Furthermore, incorporation of ^{14}C-iodoacetamide into AT III during thermal titration was coincident with the increase in ANS fluorescence suggesting that disulfide cleavage is the event which triggers the unfolding of the protein. Samples pasteurized for 10 hours at 60°C in the presence of 0.5 M and 1.0 M citrate retained full antithrombin activity but exhibited evidence of minor alterations in the ability to bind heparin.

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APPLICATION OF ENZYME KINETIC ANALYSIS IN THE DEVELOPMENT OF AN AUTOMATED ANTITHROMBIN III ASSAY. J. F. Perry, J. A. Wehrly, P. L. Coleman, E. I. du Pont de Nemours & Co., Inc. Experimental Station, Wilmington, Delaware 19898 USA.

The need for adherence to basic enzyme kinetics principles in the measurement of coagulation enzymes with synthetic substrates has recently been addressed by the ICTH Subcommittee on Synthetic Substrates. We describe an assay for antithrombin III (AT III) that was developed through characterization of the system kinetic parameters and their subsequent cooptimization. A large field of thrombin substrates and reaction buffers was surveyed for combinations yielding lowest K_m values. Those selected were further analyzed by multi-variate response surface analysis to maximize reaction k_{cat} while simultaneously minimizing K_m. The resultant optimized system provides a kinetically valid means to measure thrombin and forms the basis for the AT III determination. The addition of the AT III activator heparin leads to the final optimized assay design. The analytical performance of the automated method evaluated through studies on precision, linearity, interference and correlation, was acceptable. The linear range of the method is 0-150% Normal Human Plasma (NHP). Day-to-day precision at the medical decision level of 80% NHP shows a CV of 3%.

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