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DIAGNOSTIC PROCEDURE

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1 Claim. (Cl. 23—230)

The present invention relates to diagnostic procedures for determining the presence of abnormal or pathological states or conditions. More particularly it relates to novel diagnostic procedures and reagents for the determination of the amount of serum lipids in blood.

Recent observations that serum lipid abnormalities are associated with atherosclerotic disease has created considerable interest in the development of methods for the detection of these abnormalities. Cholesterol and the serum lipids, each cholesterol esters, phospholipids and triglycerides, are present in the blood stream both independent of and in combination with proteins. When combined with proteins, these lipids are characterized as lipoproteins. The beta-lipoproteins are believed to play a significant role in the etiology of atherosclerosis. Additionally, there is a significant beta-lipoprotein imbalance associated with diabetic disease states, and beta-lipoprotein quantitation is a useful diagnostic tool for this pathology.

The methods which have heretofore been available for quantitatively measuring beta-lipoproteins are primarily dependent upon solubility differences, electrophoretic behavior, differential flotation during ultracentrifugation and specific immunochemical precipitation. All of these techniques have involved expensive and time consuming operations, and their use has required significantly large quantities of specimen material.

It is an object of the present invention to disclose novel diagnostic procedures and reagents which make possible a simple, quick, inexpensive quantitative determination of beta-lipoprotein in blood. It is a further object to provide means for determining the extent to which serum cholesterol is combined in the form of beta-lipoprotein.

It is another object to provide a beta-lipoprotein diagnostic reagent which requires the use of minute quantities of specimen, e.g. one drop of serum, and which, therefore, negates the necessity for withdrawal of blood by syringe.

The above and still further objects are obtained by the present invention which involves the immunological precipitation of beta-lipoprotein and the subsequent volumetric quantitation of the resultant precipitate.

In the preparation of the beta-lipoprotein antiserum employed in the practice of the present invention, it is desirable that the immunizing beta-lipoprotein be immunologically free of other antigenic material such as albumin and gamma globulin, the most common contaminants. To this end, beta-lipoprotein is obtained by conventional ultracentrifugation procedures at an appropriate density, time and speed. The beta-lipoprotein thus obtained can be rendered immunologically free of possible albumin and gamma globulin contaminants by absorbing these contaminants, if present, on appropriate antiserum. When this beta-lipoprotein is injected into a suitable animal using conventional immunological techniques, the animal will respond by producing anti-serum which is specific only to the beta-lipoprotein.

In the preferred practice of the present invention a beta-lipoprotein antiserum is prepared by conventional techniques in a suitable animal (e.g. rabbit, sheep, goat or horse) and combined with the human serum to be tested. The antiserum should be capable of precipitating all of the beta-lipoprotein in human serums having from 300 to 1500 mg. of beta-lipoprotein per 100 ml. of blood serum. Preferably, there will be present an excess of

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antibody in order to assure complete precipitation of all of the beta-lipoprotein contained in the serum specimen.

This combination is thoroughly mixed, and the mixture is immediately drawn into a standard capillary tube. The end of the capillary tube is then sealed. The capillary tube and its contents are centrifuged for about 5 to 10 minutes. The length of the resultant column of precipitate is then measured as, for example, with a low power microscope and eyepiece micrometer. The length of the total fluid column is also measured. The beta-lipoprotein immunocrit is then determined by the following formula:

Beta-lipoprotein immunocrit=

$$\frac{\text{length of column of precipitate}}{\text{length of column of fluid}} \times 100$$

The value thus obtained is then compared to the immunocrit of a normal human being of the same sex, within the same age group.

If measured capillary tubes are employed, so that the length of the column is a constant, the length of the column of precipitate can be translated directly into a serum beta-lipoprotein value by reference to a control standard. This control standard is prepared by assaying by differing techniques (e.g. analytical ultracentrifugation, polyanion precipitation or paper electrophoresis and the method of this invention) a number of serum samples having different levels of beta-lipoprotein and by relating the amounts of beta-lipoprotein as determined by direct assay of the beta-lipoprotein to the column heights of precipitate resulting from the practice of this invention.

The practice of the present invention is further illustrated by reference to the following example.

EXAMPLE

Preparation of the antigen

A pool of human sera was used for preparation of beta-lipoprotein with standard ultracentrifugal techniques. The serum specimen was adjusted to a density of 1.063 and centrifuged at 30,000 r.p.m. for 16 hours (5 ml. of serum plus 4 ml. saline, density 1.1310). The top-most layer was carefully removed and dialyzed against normal saline. Immuno-electrophoretic analysis indicated slight traces of albumin and gamma globulin.

Preparation of the antiserum

The beta-lipoprotein fraction was administered to rabbits via an initial intramuscular injection of the material in Freund's adjuvant. Subsequent subcutaneous injections were given at 4 week intervals, 7 days before each bleeding. The injections were continued until the beta-lipoprotein precipitin titer was such that two volumes of undiluted serum would precipitate all of the beta-lipoprotein in one volume of each of a number of human serums ranging over the normal and the abnormally high and low beta-lipoprotein levels which could be expected to be encountered in routine diagnosis, i.e. from 300 to 1500 mg. of beta-lipoprotein per 100 ml. of serum. The sera from interval bleedings of rabbits with high precipitin titers were pooled and analyzed by immuno-electrophoresis for precipitin content. One pool contained a significant amount of both albumin and gamma globulin precipitins, which were selectively absorbed with Cohn fractions II and V. Other pools were contaminated with only albumin precipitins which were removed by absorption with Cohn fraction V. The resultant absorbed antiserum pools gave one intense line in the characteristic beta-lipoprotein area upon immuno-electrophoresis against a pool of normal human sera.

Diagnostic test

A small quantity of human test serum was drawn into a capillary tube (dia. 1.2-1.4 mm. length 75 mm.), held in the vertical position, and one drop of this serum was allowed to fall passively onto a clean glass plate. To this drop of serum was added two drops of the above described antiserum using a Pasteur pipette (length of capillary stem, 120 mm., mean terminal dia 0.82 mm.). The serum and antiserum were mixed with a wooden applicator stick, using a circular motion. The resultant mixture was immediately drawn to a column height of 60 mm. into a capillary tube described above) and the end sealed by a flame. After standing at room temperature for 15 minutes, the capillary tubes were centrifuged in a micro-hematocrit centrifuge for 10 minutes. The length of the resultant column of precipitate was measured with a low power microscope and eyepiece micrometer and found to be three mm. Reference to a control standard prepared for capillary tubes having these dimensions and for this column height of fluid indicated that the human test serum contained 900 mg. of beta-lipoprotein per 100 ml. of serum.

The beta-lipoprotein immunocrit, by calculation, was found to be 5. As a crosscheck the human test serum of this invention was assayed by the more complex, expensive and time-consuming polyanion precipitation and paper electrophoretic methods, and a close correlation was obtained. The polyanion precipitation and the paper electrophoretic methods gave values of 875 and 910 mg. of beta-lipoprotein per 100 ml. of serum, respectively.

For the particular system of this example (e.g. for this size capillary tube, the amounts of serum and antiserum employed, and the column height of fluid in the capillary tube) there is essentially a linear relationship between the height of the precipitate column in the capillary tube and the quantity of beta-lipoprotein normally contained in human serum specimens. Over the range of 300 to 1500 mg. of beta-lipoprotein per 100 ml. of blood, 1 millimeter of precipitate, in this system, represents 300 mg. of beta-lipoprotein per 100 ml. of blood, this being the control standard for the system. That is, a precipitate column height of 2 mm. indicates that the sample specimen contained 600 mg. of beta-lipoprotein per 100 ml. of blood, a column height of 3 mm. represents 900 mg. of beta-lipoprotein, etc.

It is of course readily apparent that in the commercial embodiment of the present invention the preparation of

the antigen and the antiserum, and the preparation of control standards will be done by a biological manufacturer, and the diagnostic test itself will be performed at the physician's office, the hospital or the clinic.

The practice of the present invention in addition to requiring minimal equipment, and technical skill allows for the determination of beta-lipoprotein levels while employing extremely small volumes of blood (0.1 ml.). This degree of sensitivity permits the obtention of test blood by relatively painless and simple means, e.g. a finger prick, instead of by the otherwise requisite relatively complex procedures, e.g. withdrawal of blood from a vein by syringe and needle. Also, if laboratory facilities are not immediately available, the blood can be drawn into a capillary tube which has been treated with a preservative, e.g. phenol. The tube would then be sealed at both ends and sent to the laboratory for analysis.

It will be likewise apparent that many modifications and changes may be made without departing from the spirit and scope of the present invention.

The embodiments of the present invention in which an exclusive property or privilege is claimed, are as follows:

The method of determining the amount of beta-lipoprotein present in serum which comprises adding to said serum a beta-lipoprotein antiserum which is capable of precipitating all of the beta-lipoprotein in human serums that contain from 300 to 1500 mg. of beta-lipoprotein per 100 ml. of blood serum, drawing the resultant mixture into a capillary tube for a prescribed distance, sealing said tube, centrifuging the capillary tube, measuring the length of the column of precipitate, and relating the height of the precipitate to a control standard.

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