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PROSTATIC ACID PHOSPHATASE DETERMINATION

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ABSTRACT OF THE DISCLOSURE

Sodium and other alkali metal salts and alkaline earth metal salts of thymolphthalein monophosphate are disclosed as substrates which exhibit a high order of specificity in the quantitative detection of prostatic acid phosphatase.

FIELD OF INVENTION

This invention relates to the detection and quantitative determination of enzymes in biological fluids. It relates more especially to the detection and quantitative determination of prostatic acid phosphatase in blood serum and in other biological fluids.

BACKGROUND OF INVENTION

The detection and quantitative determination of prostatic acid phosphatase in biological fluids is useful in the clinical diagnosis of prostatic carcinoma, a known symptom of which is an increase in the content of prostatic acid phosphatase in blood, urine and other biological fluids.

There are several substrates which are known to be responsive to the catalytic activity of prostatic acid phosphatase, those most generally recognized in this regard being phenol-phosphate, phenolphthalein monophosphate; alphanaphthyl-phosphate, p-nitro-phenol-phosphate, and beta-glycerophosphate. However, there are acid phosphates other than prostatic acid phosphatase which may occur in biological fluids. Those which generally are present in significant amounts in blood are erythrocytes from blood cells and platelet acid phosphatase. If any of the substrates hereinabove mentioned is employed in an attempted diagnosis for prostatic acid phosphatase difficulties are encountered because of activity induced by other acid phosphatases which, if present, result in a false-positive reaction. Of the foregoing substrates those having the greatest specificity for prostatic acid phosphatase are beta-glycerophosphate and alpha-naphthylphosphate, but even these are deficient in specificity and while such deficiency to substantial extent may be lessened by resort to the technique of comparative results with the aid of known inhibitors such as tartrate or formalin, such techniques are relatively cumbersome. Moreover, the test procedure using beta-glycerophosphate is a matter of considerable difficulty.

There has been need for a substrate which is sufficiently specific for prostatic acid phosphatase to enable it to be used with acceptable diagnostic accuracy in the detection and quantitative determination of prostatic acid phosphatase and the use which involves relatively simple test procedure.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a substrate which has greater specificity for prostatic acid phosphatase than substrates heretofore known. It is a further object of this invention to provide a reliable diagnostic test procedure for the detection and determination of prostatic acid phosphatase which is simple and which may be carried out with conventional and readily available equipment.

According to this invention, the aforesaid objects have been obtained by the utilization of the sodium salt of thymolphthalein monophosphate as the substrate for prostatic acid phosphatase. While the sodium salt is preferred, other salts of thymolphthalein monophosphate may be employed, namely the salts of other alkali metals than sodium, e.g., potassium and salts of the alkaline earth metals. It is to be understood herein and in the claims that reference to the salts of the alkaline earth metals includes magnesium salts. Salts of other alkaline earth metals also may be employed, i.e., calcium and barium.

Magnesium thymolphthalein monophosphate has heretofore been proposed as a substrate for the detection and quantitative determination of alkaline phosphatase in blood serum. Elevated values of alkaline phosphatase occur in increased osteoblastic activity, impairment of liver function, pregnancy and biliary obstruction. Lowered values are observed in hypophosphatasia and in malnutrition. Alkaline phosphatase determination is ordinarily carried out with the substrate, e.g., magnesium thymolphthalein monophosphate, dissolved in a solution buffered at a pH of 10. The pending application of Alejo V. Roy, Ser. No. 43,983 filed June 5, 1970, and now abandoned discloses a method whereby alkali metal salts of thymolphthalein as exemplified by sodium thymolphthalein can be produced by a method whereby the previously known magnesium thymolphthalein monophosphate may successfully be converted to the sodium salt. The alkali metal salts by reason of their greater solubility at the alkaline pH at which an alkaline phosphatase determination is made affords enhanced effectiveness for such determination as compared with the use therein of the magnesium salt.

It has now been discovered that the sodium salt of thymolphthalein monophosphate possesses specificity as a substrate for prostatic acid phosphatase. Moreover, it may be employed in a determination procedure that is simple to perform. Quantitative readings merely require comparison of color in relation to similarly tested known standards which may, if desired, be indicated on a chart or curve. While it is preferable to employ the sodium salt, the other alkali metal and alkaline earth metal salts likewise may be availed of, the lesser solubility of the alkaline earth metal salts as compared with alkali metal salts being less of a limitation than with an alkaline phosphatase determination because of greater solubility of the alkaline earth metal salts at the acid pH which characterizes acid phosphatase determination as compared with their solubility at an alkaline pH.

The technique involved in the practice of this invention is simple. A measured quantity of serum or other biological fluid is added to a solution of the substrate buffered at an acid pH. Incubation proceeds at a given temperature for a predetermined period of time. During incubation free thymolphthalein is released and at the end of the incubation period the solution is rendered alkaline, thereby stopping further reaction and fully developing the color of the thymolphthalein which will remain stable for at least two hours. The test is read against a blank and the absorbance obtained is compared with absorbances obtained with pure solutions of thymolphthalein in different amounts. The results are expressed in International Units (I.U.), i.e., as micromoles of substrate converted per minute per liter of biological fluid. It is of advantage in the practice of this invention not only that the technique is simple but also that it employs stable reagents and lends itself to standardization by a primary standard.

The method likewise is of advantage in that it follows zero kinetics over a wide range of enzyme concentrations and against incubation time. When tested with several dilutions of prostatic cancer sera and a prostatic extract,

activities obtained were proportional to enzyme concentrations up to approximately 110 I.U., which is approximately 300 times the normal upper limit. When incubated for periods of time ranging from 10 to 120 minutes, departure from linearity did not occur until an activity of 110 I.U. was obtained.

A most important advantage of the method of this invention is its good specificity for prostactic acid phosphatase, the method being less affected than other substrates by non-prostatic acid phosphatase isozymes that are responsible for false-positive reactions for prostatic acid phosphatase when other known substrates are employed.

More specifically, the activity or affinity of erythrocytic acid phosphatase with respect to phenol-phosphate is approximately 88 times that of its activity with respect to sodium thymolphthalein monophosphate. This is of major diagnostic significance due to the fact that erythocytic acid phosphatase usually is contained in blood serum in addition to any prostatic acid phosphatase and its reactivity with phenol-phosphate is such as to produce a positive reaction that interferes with or obscures the detection of prostatic acid phosphatase. On the other hand, when the sodium salt of thymolphthalein monophosphate is used as the substrate with serum containing prostatic acid phosphatase together with erythrocytic acid phosphatase, the activity thereon of the erythrocytic acid phosphatase is so slight in relation to the activity thereon of prostatic acid phosphatase that a positive reaction is quantitatively indicative of the presence of the prostatic acid phosphatase in the serum sample to the extent that the indicated positive reaction is in excess of the normal values for the method that are obtained with serum obtained from males who are free of kidney and liver diseases as well as malignancies. The normal value for the method has been determined to be of the order of 0.04 and 0.37 I.U.

The specificity of the sodium salt of thymolphthalein in comparison with other known substrates as indicated by their relative activities responsive to erythrocytic acid phosphatase follows the same general pattern. Thus, phenolphthalein monophosphate and p-nitro-phenol-phosphate are 500 times more reactive with erythrocytic acid phosphatase than the sodium salt of thymolphthalein monophosphate while alpha-naphthyl-phosphate is three times more active. While these activities responsive to erythrocytic acid phosphatase which interfere with the detection of a positive reaction responsive to prostatic acid phosphatase may be minimized by tartrate or formalin inhibition procedures, the method of this invention has the practical advantage of greater simplicity in that such inhibition procedures are not required.

Another acid phosphatase isozyme that commonly is encountered in blood serum in addition to any prostatic acid phosphatase that may be present is platelet acid phosphatase. Here, also, the sodium salt of thymolphthalein monophosphate has distinct advantages in relation to other substrates as regards specificity in the detection of prostatic acid phosphatase. Thus phenol-phosphate and phenolphthalein monophosphate are above five times more active toward platelet acid phosphatase than the sodium salt of thymolphthalein monophosphate. Alpha-naphthyl-phosphate has about the same activity. p-nitro-phenol-phosphate is about sixteen times more active and beta-glycerophosphate is about four times more active. Except for phenolphthalein monophosphate, neither tartrate nor formalin will eliminate these interferences.

In normal blood sera the only interfering acid phosphatases that are encountered in significant amount are the erythrocytic acid phosphatases and platelet acid phosphatase. Accordingly, to the extent that there is positive reactivity above the normal reactivity of blood serum the presence of prostatic acid phosphatase is indicated. It is not necessary to do further testing using an inhibitor such as tartrate or formalin.

The sodium salt of thymolphthalein monophosphate may be employed in the detection of prostatic acid phosphatase in other body fluids such as urine, although the degree of specificity in relation to possible interferences is not as great as in the use of the method of this invention with blood sera.

While much of the foregoing description has been exemplified by the employment of the sodium salt of thymolphthalein monophosphate which is regarded as preferable, the specificity afforded by its use likewise may be realized when employing the salts of other alkali metals and salts of the alkaline earth metals.

DETAILED DESCRIPTION

The method is carried out at an acid pH. While the pH is not critical, it is preferably between about 5.5 and about 6.4 and normally is at a pH of 6 at which maximum activity occurs. These pH values are measured at 25° C. Any compatible buffer may be employed, although that which normally is used is a conventional citrate or acetate buffer. Reactivity starts immediately after zero incubation time and is linear up to about 35 minutes. In normal procedure the incubation is for a period of 30 minutes at 37° C. The molarity of the new substrate is not critical in that activity in terms of International Units increases until an optimum occurs at about 2.2 mmol per liter, after which activity falls off gradually. The preferred range of molarity is of the order of 1.5 to 3 mmol per liter.

The method of this invention may be illustrated by the following example of preferred practice. There is added to a 0.1 M citrate buffer solution (citric acid plus sodium citrate) sufficient sodium thymolphthalein monophosphate to provide a concentrate of 2.2 mmols per liter, the pH of the solution being 6 at 25° C. 1.0 ml. of the buffered substrate is incubated with 0.2 ml. of serum for 30 minutes at 37° C. If prostatic acid phosphatase is present the substrate is acted upon to release free thymolphthalein, the rate of release being greater for greater amounts of prostatic acid phosphatase present in the serum sample. At the end of the incubation period 5 ml. of a reagent containing 0.05 mol each of sodium carbonate and sodium hydroxide per liter is added, thereby stopping the enzymatic reaction and at the same time fully developing the color of the released thymolphthalein. The absorbance obtained is evaluated preferably by measurement with a spectrophotometer at 590 nm. in comparison with absorbances obtained with pure solutions of thymolphthalein which, for convenience, may have been recorded on a chart or curve. Alternatively, a colorimeter may be used. The test is read against a serum blank and any activity in excess of normal is, as aforesaid, expressed in International Units.

In practice, an admixture of the substrate and buffer in the dry state may be prepackaged in a vial to which a given quantity of distilled water may be added to produce a solution having predetermined desired molarities for the substrate and for the buffer in a convenient amount for such number of tests as may be desired.

We claim:

1. In the method for the determination of prostatic acid phosphatase enzyme in a biological fluid wherein a substrate is incubated with the enzyme in an aqueous medium that is free of inhibitor for prostatic acid phosphatase and that is at an acid pH and the amount of substrate converted during a specific period of time is measured, the improvement which comprises incubating an aqueous solution of said biological fluid containing as the sole substrate an alkali metal or alkaline earth metal salt of thymolphthalein monophosphate, measuring the amount of substrate which is converted after incubation and comparing said amount with the normal amount of substrate which is converted when similarly measured employing a fluid from a male who is free of kidney or liver disease or malignancy, thereby determining any amount that is in excess of said normal amount.

2. A method according to claim 1 wherein said salt of thymolphthalein monophosphate is an alkali metal salt.

3. A method according to claim 2 wherein said salt of thymolphthalein monophosphate is the sodium salt.

4. A method according to claim 1 wherein at the end of the incubation period the reaction mass is rendered alkaline thereby stopping the enzymatic reaction and developing the color of any released thymolphthalein and wherein the quantity of released thymolphthalein is measured colorimetrically.

References Cited

Coleman ("II"), "Clin. Chem.," *12*(8): 529 (1966).
Roche, in "The Enzymes," Sumner and Myrbäck, Editors, Vol. 1, part 1, pp. 486–8 (1950).
Gutman et al., "J. Biol. Chem.," 136: 201–209 (1940).
Coleman, "Clin. Chim. Acta," *13*: 401–403 (1966).
Huggins et al., "J. Biol. Chem.," *159*: 399–410 (1945).

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