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(54) A METHOD FOR THE DETERMINATION OF ACID PHOSPHATASE

(71) We, F. HOFFMANN-LA ROCHE & CO., AKTIENGESELLSCHAFT, a Swiss Company of 124—184 Grenzacherstrasse, Basle, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a method for the determination of acid phosphatase activity. More particularly, the invention is concerned with an improved method for the determination of the activity of acid phosphatase of human origin using a reagent for activating the acid phosphatase, with said reagent per se and with a reagent kit containing said reagent.

Acid phosphatase is an enzyme which splits phospho-monoesters in the acid pH range. Acid phosphatase of human origin is found in various organs (e.g. in the prostate gland, the liver and the spleen) and in blood cells, and is transferred to the serum when cells are damaged. The determination of acid phosphatase in body fluids, in particular in serum or plasma, thus plays an important part in the diagnosis of diseases of the organs concerned.

The activity of acid phosphatase in serum or plasma increases considerably if a carcinoma is present in the prostate gland and in the case of hypertrophy of the prostate gland and the determination of prostate gland phosphatase activity in these body fluids enables the diagnosis of these specific diseases to be made.

According to the present invention there is provided a method for the determination of acid phosphatase activity in a body fluid of human origin which method comprises subjecting a solution containing, as a substrate for the phosphatase, 50 μmols/litre to 50 mmols/litre of a phospho-monoester, 50 to 300 mmols/litre of a buffer substance to adjust the pH value to between 4.5 and 6.5 and 10 to 300 mmols/litre of an activator consisting of at least one straight-chain alcohol containing 4 to 6 carbon atoms to the enzymatic phosphatase action of the body fluid under investigation at an incubation temperature between 20°C and 45°C, stopping the reaction and measuring the amount of substrate hydrolysis to an acid and an alcohol.

The activity of acid phosphatase in body fluids of human origin, which is relatively low, is considerably increased by the presence of a straight-chain alcohol containing 4 to 6 carbon atoms, and the addition of such an alcohol facilitates determination of the activity of the phosphatase.

The phospho-monoester used as the substrate in the enzymatic reaction can be represented by the general formula

$$R—O—PO_3H_2$$

in which R represents any desired organic group which yields an alcohol ROH on hydrolysis of the substrate.

R preferably represents a chromophoric group (e.g. the 4-nitrophenyl, thymolphthalyl, phenolphthalyl or 2-chloro-4-nitrophenyl group) or a fluorophoric group (e.g. the 1-naphthyl group). R can also represent the adenosinyl, glyceryl or phenyl group.

The substrate is preferably employed at an optimum concentration which is different for each individual substrate and is also influenced by the pH value. The

optimum substrate concentration which effects the maximum enzyme activity possible at a given pH value can be determined from a substrate activity curve.

The substrate activity curve for a given substrate is obtained by measuring the enzyme activity at various substrate concentrations under otherwise identical conditions.

The optimum substrate concentration for the acid phosphatase is between 50 μmols/liters and 50 mmols/litres, and the optimum concentration is preferably between 60 μmols/litres and 25 mmols/liters. Thus, at a pH value of 5.5 the optimum substrate concentration is approximately 25 mmols/litres for β-glycerine phosphate, 2.5 mmols/liters for 4-nitrophenyl phosphate and 100 μmols/litres for phenolphthalein diphosphate.

According to the present method, the pH value is adjusted to between 4.5 and 6.5, preferably between 5 and 6, using a buffer substance. Examples of suitable buffer substances are citrate buffer and acetate buffer. The buffer substance concentration is between 50 mmols/litre and 300 mmols/litre and is preferably 100 mmols/litre.

According to the present method, the activator used consists of at least one a straight-chain alcohol containing 4 to 6 carbon atoms, preferably n-butanol, n-pentanol or 1,5-pentanediol. 1-Pentanol and 2-pentanol are particularly preferred.

The concentration of the activator is 10 mmols/litre to 300 mmols/litre, preferably 100 mmols/litre to 200 mmols/litre.

The activator can consist of one or more straight-chain alcohols containing 4 to 6 carbon atoms, but preferably consists of a single, straight-chain alcohol of this type.

A sample of the body fluid under investigation is mixed with the test solution and the enzymatic reaction is then carried out by incubating the solution at a temperature between 20°C and 45°C, preferably between 30°C and 37°C. The incubation period will usually be 5 to 60 minutes, but the reaction is preferably stopped after 30 minutes by adding a suitable substance (e.g. sodium hydroxide solution of trisodium phosphate solution).

By choosing a suitable substrate (e.g. 2-chloro-4-nitrophenyl phosphate) the enzymatic reaction can also be measured kinetically.

The substrate conversion is a measure of the enzyme activity. It is measured by determining the decrease in the phospho-monoester concentration or the increase in the concentration of the alcohol ROH liberated. Since the activation of acid phosphatase is effected by a transphosphorylation reaction, i.e. a phosphate transfer from the phospho-monoester used to the alcohol used as the activator, the amount of free phosphate after the enzymatic reaction does not correspond to the substrate conversion and accordingly cannot be taken as a measure thereof.

The type of measurement depends on the substrate used. If 4-nitro-phenyl phosphate, thymolphthalyl phosphate, phenolphthalyl phosphate or naphthyl phosphate is used as the substrate, the increase in the concentration of the organic radical liberated can be determined directly photometrically or fluorimetrically after suitably stopping the enzymatic reaction.

If other substrates (e.g. β-glyceryl phosphate, phenyl phosphate or adenosyl monophosphate) are used, the measurement of the substrate conversion can be carried out by chemically or enzymatically converting the alcohol ROH liberated into a substance, the concentration of which can be determined photometrically. For example, if phenyl phosphate is used, the phenol (ROH) liberated can be converted into a blue dyestuff using a phenol reagent [Folin-Ciocalteus, Merck 9001].

The present method is suitable for both manual and automatic determinations of acid phosphatase activity. Examples of substances on which the determination can be carried out are serum, plasma, blood, fluid and urine.

Additives suitable for diagnostic purposes [e.g. detergents, such as Brij-35 (Registered Trade Mark) (polyoxyethylene lauryl ether), or stabilisers, such as magnesium ions] can be added to the phosopho-monomers, buffers and activators.

The reagents are preferably packed in a reagent kit. A reagent kit used in a method according to the present invention comprises, in one or more containers,

a) 0.005 to 5 mmols of a phospho-monoester,
b) 5—30 mmols of a buffer substance to adjust the pH value to between 4.5 and 6.5 and
c) 1 to 30 mmols of a straight-chain alcohol containing 4 to 6 carbon atoms as an activator,

these quantities relating to a decilitre of the respective reagent.

The reagent kit can additionally comprise, in a container, a control solution or standard solution and/or, in a container, a reagent for stopping the reaction and/or, in a container a reagent for the conversion of the organic radical liberated and/or, in a container, the additives mentioned hereinbefore which are suitable for diagnostic purposes (e.g. detergents and stabilizers).

However, it is not necessary to store the reagents and additives in separate containers. The present invention is also concerned with a reagent kit in which some of the components necessary for carrying out the method of the invention are stored together in the same container. For example the buffer substance and the activator can be stored together in dissolved form in a single container.

With the exception of the activator which is always in liquid form, the various reagents and additives can be packed both in the liquid and in the solid form (e.g. as a powder, granules, tablets or a lyophilisate).

The following Examples illustrate the present invention:

Example 1.

0.1 ml of acid prostate gland phosphatase solution (isolated by the method described by Lam et al., Clin. 19, 483, 1973) is mixed with 2.0 ml of a buffer substrate solution consisting of 0.1 mol/litre of citrate buffer (pH 5.25), 5 g/litres of Brij-35, 5 mmols/litre of 4-nitro-phenyl phosphate and various concentrations of 1-pentanol and the mixture is incubated at 37°C for 30 minutes. The reaction is stopped by adding 1.0 ml of 1-N sodium hydroxide solution and the 4-nitro-phenolate liberated is determined photometrically at a wave-length of 405 nm.

The results are given in Table I in % of the prostate gland phosphatase activity at a 1-pentanol concentration of zero.

TABLE I

1-Pentanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	137%
100 mmols/litre	173%
150 mmols/litre	189%
200 mmols/litre	139%

Example 2.

The method described in Example 1 hereinbefore is repeated using 2-pentanol in place of 1-pentanol. The results are given analogously in Table II.

TABLE II

2-Pentanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	138%
100 mmols/litre	172%
150 mmols/litre	190%
200 mmols/litre	139%

Example 3.

The method described in Example 1 hereinbefore is repeated using 1,5-pentanediol in place of 1-pentanol. The results are given analogously in Table III.

TABLE III

1,5-Pentanediol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	120%
100 mmols/litre	131%
150 mmols/litre	140%
200 mmols/litre	148%

Example 4.

The method described in Example 1 hereinbefore is repeated using 1-butanol in place of 1-pentanol. The results are given analogously in Table IV.

TABLE IV

1-Butanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	119%
100 mmols/litre	131%
150 mmols/litre	131%
200 mmols/litre	116%

Example 5.

The method described in Example 1 hereinbefore is repeated using 1-hexanol in place of 1-pentanol. The results are given analogously in Table V.

TABLE V

1-Hexanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
25 mmols/litre	107%
50 mmols/litre	114%
100 mmols/litre	120%

Example 6.

0.05 ml of prostate gland phosphatase solution is mixed with 2.0 ml of a substrate buffer solution consisting of 0.1 mol/litre of acetate buffer (pH 5.5), 62 µmols/litres of phenolphthalein diphosphate and various concentrations of 1-pentanol and the mixture is incubated at 37°C for 15 minutes. In order to stop the reaction and to develop the colour, 2.0 ml of 0.4 mol/litre sodium phosphate buffer (pH 10) are admixed and the colour intensity is measured at a wave-length of 546 nm.

The results are given in Table VI in % of the prostate gland phosphatase activity at a 1-pentanol concentration of zero.

TABLE VI

1-Pentanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	154%
100 mmols/litre	174%
150 mmols/litre	177%
200 mmols/litre	131%

Example 7.

0.1 ml of erythrocyte phosphatase is mixed with 2.0 ml of a substrate buffer solution consisting of 0.1 mol/litre of acetate buffer (pH 5.5), 5 mmols/litre of 4-nitrophenyl phosphate, 5 g/litres of Brij-35 and various concentrations of 1-pentanol. After 30 minutes incubation at 37°C, the reaction is stopped by adding 1.0 ml of 1-N sodium hydroxide solution and the 4-nitrophenolate liberated is determined photometrically at a wavelength of 405 nm.

The results are given in Table VII in % of the erythrocyte phosphatase activity at a 1-pentanol concentration of zero.

TABLE VII

1-Pentanol concentration in the test solution	Activity of the erythrocyte phosphatase
0 mmol/litre	100%
50 mmols/litre	132%
100 mmols/litre	153%
150 mmols/litre	162%
200 mmols/litre	136%

Example 8.

0.1 ml of serum is mixed with 0.5 ml of a substrate buffer solution consisting of 0.1 mol/litre of citrate buffer (pH 5.5) and 5 mmols/litres of 4-nitrophenyl phosphate. After 30 minutes incubation at 37°C, the reaction is stopped by adding 2.5 ml of 0.1-N sodium hydroxide solution and the 4-nitrophenolate liberated is determined photometrically at a wavelength of 405 nm. This determination of the

activity of acid phosphatase is carried out once in the absence of 1-pentanol and once in the presence of 150 mmols/litres of 1-pentanol.
The results are given in Table VIII.

TABLE VIII

Sample	Activity without 1-pentanol (U/l)	Activity with 150 mmols/litre of 1-pentanol (U/l)
Serum 1	1.12	2.10
Serum 2	16.63	24.55
Serum 3	18.41	31.53
Serum 4	20.65	33.59

Example 9.

0.1 ml of prostate gland phosphatase solution is mixed with 2.0 ml of a substrate buffer solution consisting of 0.1 mol/litre of acetate buffer (pH 5.5) and various concentrations of phenyl phosphate. After 5 minutes incubation at 37°C, 0.5 ml of phenol reagent [Folin-Ciocalteus] and 1.0 ml of 20% by weight strength sodium carbonate solution are pipetted in and the mixture is incubated for a further 10 minutes at 37°C. The intensity of the blue dye, which corresponds to the concentration of phenol liberated, is measured at a wavelength of 578 nm. These determinations are carried out once in the absence of 1-pentanol and once in the presence of 150 mmols/litre of 1-pentanol.

The results are given in Table IX.

TABLE IX

Phenyl phosphate concentration in the test solution	$\Delta E_{578\ nm}$/5 Min. in the absence of 1-pentanol	$\Delta E_{578\ nm}$/5 min. in the presence of 150 mmols/litres of 1-pentanol
0.078 mmol/litre	0.105	0.169
0.155 mmol/litre	0.150	0.280
0.312 mmol/litre	0.198	0.390
0.625 mmol/litre	0.245	0.490
1.25 mmols/litre	0.275	0.550
2.50 mmols/litre	0.280	0.590
5.00 mmols/litre	0.280	0.590

Example 10.

0.05 ml of purified prostate gland phosphatase solution is mixed with 1.0 ml of a substrate buffer solution consisting of 0.1 mol-litre of acetate buffer (pH 5.5), 0.5 mmol/litre of phenolphthalein monophosphate and various concentrations of 1-pentanol. After 10 minutes incubation at 37°C, the reaction is stopped by adding 2.0 ml of a 0.4 mol/litre sodium phosphate buffer solution (pH 10) and the phenolphthalein liberated is determined photometrically at a wavelength of 546 nm.

The results are given in Table X and are expressed as a percentage of the prostate gland phosphatase activity observed at zero 1-pentanol concentration.

TABLE X

1-Pentanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	125%
100 mmols/litre	135%
150 mmols/litre	140%

Example 11.

0.05 ml of purified prostate gland phosphatase solution is mixed with 1.0 ml of a substrate buffer solution consisting of 0.1 mol/litre of citrate buffer (pH 5.75), 1.2 mmols/litres of thymolphthalein phosphate, 5 g/litres of Brij-35 and various concentrations of 1-pentanol. After 10 minutes incubation at 37°C, the reaction is stopped by adding 2.0 ml of a 0.2 mol/litre bicarbonate buffer solution (pH 10) and the thymolphthalein liberated is determined photometrically at a wavelength of 578 nm.

The results are given in Table XI in % of the prostate gland phosphatase activity at a 1-pentanol concentration of zero.

TABLE XI

1-Pentanol concentration in the test solution	Activity of the prostate gland phosphatase
0 mmol/litre	100%
50 mmols/litre	117%
100 mmols/litre	125%
150 mmols/litre	140%

WHAT WE CLAIM IS:—

1. A method for the determination of acid phosphatase activity in a body fluid of human origin which method comprises subjecting a solution containing, as a substrate for the phosphatase, 50 μmols/litre to 50 mmols/litre of a phospho-monoester, 50 to 300 mmols/litre of a buffer substance to adjust the pH value to between 4.5 and 6.5 and 10 to 300 mmols/litre of an activator consisting of at least one straight-chain alcohol containing 4 to 6 carbon atoms to the enzymatic phosphatase action of the body fluid under investigation at an incubation temperature between 20°C and 45°C stopping the reaction and measuring the amount of substrate hydrolysis to an acid and an alcohol.

2. A method according to claim 1, wherein the acid phosphatase of human origin is prostate gland phosphatase.

3. A method according to claim 1, wherein the acid phosphatase of human origin is erythrocyte phosphatase.

4. A method according to any one of claims 1 to 3 inclusive, wherein 4-nitrophenyl phosphate is used as the phospho-monoester.

5. A method according to any one of claims 1 to 3 inclusive, wherein thymolphthalyl phosphate is used as the phospho-monoester.

6. A method according to any one of claims 1 to 3 inclusive, wherein phenolphthalyl phosphate is used as the phospho-monoester.

7. A method according to any one of claims 1 to 3 inclusive, wherein 1-naphthyl phosphate is used as the phospho-monoester.

8. A method according to any one of claims 1 to 3 inclusive, wherein β-glyceryl phosphate is used as the phospho-monoester.

9. A method according to any one of claims 1 to 3 inclusive, wherein adenosyl monophosphate is used as the phospho-monoester.

10. A method according to any one of claims 1 to 3 inclusive, wherein phenyl phosphate is used as the phospho-monoester.

11. A method according to any one of claims 1 to 3 inclusive, wherein 2-chloro-4-nitrophenyl phosphate is used as the phospho-monoester.

12. A method according to any one of claims 1 to 11 inclusive, wherein a buffer substance is used to adjust the pH value to about 5.5.

13. A method according to claim 12, wherein a citrate buffer is used as the buffer substance.

14. A method according to claim 12, wherein an acetate buffer is used as the buffer substance.

15. A method according to any one of claims 1 to 14 inclusive, wherein the activator used consists of n-butanol.

16. A method according to any one of claims 1 to 14 inclusive, wherein the activator used consists of 1-pentanol.

17. A method according to any one of claims 1 to 14 inclusive, wherein the activator used consists of 2-pentanol.

18. A method according to any one of claims 1 to 14 inclusive, wherein the activator used consists of 1,5-pentanediol.

19. A method according to any one of claims 1 to 6 inclusive and claims 11 to 18 inclusive, wherein the substrate conversion is determined by direct photometric measurement of the alcohol liberated.

20. A method according to any one of claims 1 to 3 inclusive, claim 7 and claims 12 to 18 inclusive, wherein the substrate conversion is determined by direct fluorimetric measurement of the alcohol liberated.

21. A method according to any one of claims 1 to 3 inclusive, claims 8 to 10 inclusive and claims 12 to 18 inclusive, wherein the substrate conversion is determined by chemically or enzymatically converting the alcohol liberated into a chromophoric or fluorochromic substance which can be measured photometrically.

22. A reagent kit for the determination of acid phosphatase of human origin, comprising a reagent which contains 50 μmols/litre to 50 mmols/litre of a phospho-monoester, 50 to 300 mmols/litre of a buffer substance to adjust the pH value to between 4.5 and 6.5, and 10 to 300 mmols/litre of an activator consisting of at least one straight-chain alcohol containing 4 to 6 carbon atoms.

23. A reagent according to claim 22, in which the phospho-monoester is 4-nitrophenyl phosphate.

24. A reagent according to claim 22, in which the phospho-monoester is thymolphthalyl phosphate.

25. A reagent according to claim 22, in which the phospho-monoester is phenolphthalyl phosphate.

26. A reagent according to claim 22, in which the phospho-monoester is naphthyl phosphate.

27. A reagent according to claim 22, in which the phospho-monoester is β-glyceryl phosphate.

28. A reagent according to claim 22, in which the phospho-monoester is adenosyl mono phosphate.

29. A reagent according to claim 22, in which the phospho-monoester is phenyl phosphate.

30. A reagent according to claim 22, in which the phospho-monoester is 2-chloro-4-nitrophenyl phosphate.

31. A reagent according to any one of claims 22 to 30 inclusive, which reagent contains a buffer substance to adjust the pH value to about 5.5.

32. A reagent according to claim 31, in which the buffer substance is a citrate buffer.

33. A reagent according to claim 31, in which the buffer substance is an acetate buffer.

34. A reagent according to any one of claims 22 to 33 inclusive, which reagent contains n-butanol.

35. A reagent according to any one of claims 22 to 33 inclusive, which reagent contains 1-pentanol.

36. A reagent according to any one of claims 22 to 33 inclusive, which reagent contains 2-pentanol.

37. A reagent according to any one of claims 22 to 33 inclusive, which reagent contains 1,5-pentanediol.

38. A reagent according to any one of claims 22 to 37 inclusive, which reagent additionally contains a detergent.

39. A reagent according to any one of claims 22 to 37 inclusive, which reagent additionally contains a stabiliser.

40. A reagent kit, when used for the determination of acid phosphatase activity by a method according to claim 1, which reagent kit comprises, in one or more containers,

a) 0.005—5 mmols of a phospho-monoester,

b) 5—30 mmols of a buffer substance to adjust the pH value to between 4.5 and 6.5 and

c) 1—30 mmols of a straight-chain alcohol containing 4 to 6 carbon atoms as the activator,

these quantities relating to a decilitre of the respective reagent.

41. A reagent kit according to claim 40, additionally comprising, in a container, a control solution or standard solution.

42. A reagent kit according to one of claims 40 to 41, additionally comprising, in a container, a reagent for stopping the enzymatic reaction.

43. A reagent kit according to any one of claims 40 to 42 inclusive, additionally comprising, in a container, a reagent suitable for conversion of the alcohol liberated from the phospho-monoester by the phosphatase.

44. A reagent kit according to any one of claims 40 to 43 inclusive, additionally comprising, in a container, a detergent.

45. A reagent kit according to any one of claims 40 to 44 inclusive, additionally comprising, in a container, a stabiliser.

46. A method according to claim 1, substantially as herein described.

47. A method of determining acid phosphatase activity as claimed in any of claims 1 to 21, the method being substantially as herein described in any of the Examples.

48. A reagent according to claim 22, substantially as herein described.

49. A reagent kit according to claim 40, substantially as herein described.

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