

PATENT SPECIFICATION

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(54) IMPROVEMENTS IN OR RELATING TO THE DETERMINATION OF ISOENZYMES

(71) We, KOMMANDITGESELLSCHAFT SCHWARZHAUPT, a Company of the Federal Republic of Germany, of 5 Köln 1, Sachsenring 37—47, West Germany, 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:—

This invention relates to a method of determining isoenzymes 4 and 5 of lactate dehydrogenase (LDH) isoenzymes by converting lactate and nicotinamide-adenine-dinucleotide (NAD⁺) to pyruvate.

As is known, lactate dehydrogenase, usually called LDH, occurs in five isoenzyme-forms. In the determination of the activity of individual isoenzymes, it is important to maintain various optimum substrate and pH conditions, which are not yet completely known for individual isoenzymes.

The prior art method of determining all the isoenzymes has been either to separate them by electrophoresis (see for example "Die Isoenzyme der Lactatdehydrogenase", Reihe Biochemie und Klinik, by S. L. Kowalewski, published by G. Thieme Verlag, Stuttgart, 1972, page 29) or to keep all the LDH—1 in solution, so that the LDH—2 to LDH—5 remain in chemical combination and LDH—1 (the most frequently occurring substance) is determined. A review of present standard methods can be found for example in "Enzymatische Analyse" Volume I, Verlag Chemie, Weinheim 1970, page 557. In the prior art, an optimum pH of between 8.3 and 8.9 is recommended for the conversion of lactate to pyruvate. In all known methods of determination, the pH region used is basic or, at most, approximately neutral, in order to determine the isoenzymes as completely as possible.

It has been disclosed by Lamprecht *et al* on (Cardiology 56; 371—375 (1971/72) and Fortschritte der klinischen Chemie, Enzyme und Hormone, Verlag der Wiener medizinischen Akademie 1972, 277—283) that individual isoenzymes can be converted into one

another by a change in pH, that is a change in configuration occurs.

An object of the invention is to eliminate the complicated separation processes and the constantly occurring inaccuracies in the total determination, in the case of the known method, and to ensure more optimum determination of the total of the LDH, particularly the isoenzyme 4 and/or 5 but also the isoenzyme 3.

The invention also aims to provide a combination of reagents and indicate special applications of the method.

According to the first aspect of the present invention there is provided a method of simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase (LDH) by utilising the reaction of lactate and nicotinamide-adenine-dinucleotide whereby lactate is converted to pyruvate, wherein the conversion is brought about at a pH in the range of from 6 to 6.5.

According to the second aspect of the present invention there is provided a reagent for simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase by converting lactate and nicotinamide-adenine-dinucleotide into pyruvate, containing lactate, nicotinamide-adenine-dinucleotide, a tetrazolium salt (for demonstrating the enzymatic reduction of NAD) and a buffer (optionally on a carrier, the reagent having a pH adjusted such that the pH when a sample is present is in the range of from 6 to 6.5.

It has been found that, in the known conversion of lactate and NAD⁺ to pyruvate, a reduction in pH below the neutral point results in optimum determination of the total LDH isoenzymes, more particularly of isoenzymes 4 and 5. It is particularly noteworthy that measurements have to be made at an acid pH in order to determine LDH 4 and 5 completely. Accordingly, the total quantity of determined LDH isoenzymes reaches an optimum value when measurements are made in the acid region and LDH 4 or 5 can be completely determined, either alone or mixed

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together, or mixed with other LDH isoenzymes.

Accordingly, the invention provides a method of simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase (LDH) isoenzymes, by converting lactate and nicotinamide-adenine-dinucleotide (NAD^+) to pyruvate. The conversion is carried out at a pH which is specific to the buffer system used, for example at a pH of 6.3 to 6.4 for a triethanolamine-NaOH buffer. A small amount of liquid is sufficient for the conversion.

It has also been found that optimum results in determining a number of isoenzymes together are also dependent on the buffer. A pH of approximately 6.3 to 6.4 is particularly suitable for simultaneously determining LDH 4 and 5, which may occur for example in vaginal fluids.

Preferably, the conversion medium is provided with a large buffer capacity. This has the advantage that the pH does not go below or above the optimum value during the conversion, even when the body fluids investigated have a pH considerably different from 6.

The conversion is generally brought about in conventional manner at a temperature of about 37°C . The quantities of coenzymes are adjusted so as to be optimum and adequate for all the enzymes present. The quantities can be determined by a few routine tests, in accordance with the following information.

The determination of LDH is very important, particularly during the investigation of body fluids. In healthy people, the isoenzymes are mainly retained in the tissues, but in cases of abnormal tissue growth, leukaemia or other diseases, considerable concentrations occur in the serum or other body fluids. More particularly, isoenzymes appear in the vaginal fluid as a result of pathological changes in the lower female genital tract.

The method of determination according to the invention can be applied to all body fluids. The pH of the specimen under test is adjusted, depending on its natural pH value. In all cases, the actual conversion should occur at a pH of 6.3 to 6.4, preferably approximately 6.3. Corresponding adjustments should be made in the composition of the reagent used for the determination.

In the standard method (c.f. *Enzymatische Analyse*, Volume I, op.cit.), an optimum pH between 8.3 and 8.9 is recommended, but the invention departs deliberately from this pH region. The reason for recommending the aforementioned basic pH region is that, during the reaction

a proton is produced in stoichiometric proportion to the amount of lactate converted. In order to displace the LDH equilibrium to the right, the operation is performed in the alkaline state.

According to the invention, on the other hand, for example during determination in the serum or plasma, the specimen under test is buffered to approximately pH 6.3 to 6.4 generally with triethanolamine-NaOH and the test combination or test reagent used is adjusted to the same value. Briefly, for the purpose of rough calculation, the pH is adjusted to between 6.0 and 6.5 and buffered so that the pH during the actual determination is between 6.3 and 6.4. In the case of serum albumin, glutathione should be added in known manner.

Lactate dehydrogenase isoenzymes can be determined in optimum manner at pH 6.3 to 6.4 if care is taken that the equilibrium of the reaction



is displaced to the right by subsequent reactions. This can be done by adding phenazine methosulphate, which continuously re-oxidises the coenzyme NADH. Measurements between pH 6.0 and 6.5 provide for the first time the possibility of determining all the LDH 5. This is particularly important since the presence of LDH 5 is a sign of carcinomas and can thus, if accurately and sensitively measured, provide a means for early detection of carcinomas.

In the case of some body fluids, however, the aforementioned conditions are different, since the natural pH of the fluids differs appreciably from 6.3 to 6.4. This applies particularly to the vaginal fluid, which has a pH of approximately 4. In this case, the test combination must be designed to provide an optimum pH during the conversion, that is must have a higher pH, so that it can be brought to approximately 6 to 6.5 by the pH of the vaginal fluid.

Accordingly, according to another feature, the invention relates to a test combination comprising lactate, nicotinamide-adenine-dinucleotide (NAD^+) and a tetrazolium salt for showing up the enzymatic NAD reaction, which is buffered to a pH such that when the actual conversion occurs in the body fluid under test, the pH is kept between 6.3 and 6.4 depending on the buffer, for example triethanolamine-NaOH buffer or other adjustable acid buffers. If a dry preparation is used, it must have the aforementioned pH after dissolving. A pH of approximately 6.0 to 6.5 is a suitable order of magnitude.

In the special case where a test is made

LDH



on a vaginal fluid without the original pH being altered, it is particularly advantageous to make the determination with triethanolamine-NaOH buffer. When the specimens under test have been adjusted to pH 6.3 to 6.4 the test combination should be adjusted to the same value.

According to another feature, therefore, the invention relates to the application of the method and test combination according to the invention to the special case where vaginal fluid is tested in the vagina by means of a

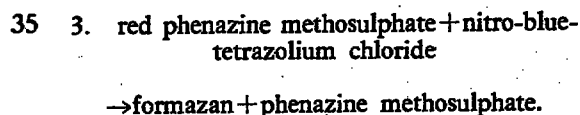
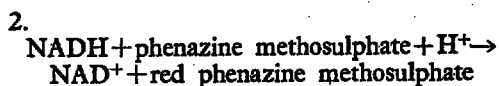
test material for detecting any pathological change in the lower female genital tract, the test combination being disposed on suitable carriers, for example on a tampon or test strip, and inserted into the vagina so as to bring about the reaction with the LDM isoenzymes in the vagina, at a pH in the range of from 6.0 to 6.5, more particularly 6.3 to 6.4.

The test combination preferably comprises a solution having the following compositions:

	Amount preferably used in the test	Lower and upper limiting region
Triethanolamine-NaOH buffer, pH 7.0	5.0mM	1.0-150mM
Na-Lactate (sodium salt of D,L-lactic acid)	67.5mM	20-350mM
Phenazine methosulphate (PMS)	0.1mM	0.01-1.0mM
Nicotinamide-Adenine-Dinucleotide (NAD ⁺)	1.5mM	0.1-10 mM
Nitro-blue-tetrazolium chloride (NBT)	0.3mM	0.01-1.5mM

This test combination is sheltered from light and applied to a suitable carrier, more particularly a tampon, and kept away from the light until used.

The presence of LDH in the vaginal fluid is indicated if the tampon becomes blue after approximately 5 to 10 minutes. The following reactions occur:—



Since the second and third reactions associated with the lactate conversion remove the reduced coenzyme when it is formed, and since the first reaction, that is the equilibrium of lactate conversion, is thus displaced even more to the right, when the test substance is buffered to pH 7.0 and the tampon is inserted the actual conversion occurs at a pH of about 6, more particularly 6.3 to 6.4, resulting in optimum determination of the isoenzymes, more particularly LDH-4 and LDH-5, which appear as a result of abnormal changes in the vaginal fluid.

In the case of test combinations used inside the vagina, the buffer should be stable and compatible with tissue. Triethanolamine-NaOH buffer is particularly suitable.

The test combination can be applied in any suitable manner to the carrier, more particularly a tampon. As is known, test combinations of the aforementioned kind must be kept away from light when used and the carrier bearing the test combination must be kept in a place sheltered from light until used. The following are two advantageous methods of introducing the test combination into a tampon: Injecting about 2 ml of the preferred test combination or impregnating about 3/4 cm of one end of a tampon with about 2 ml of test combination and subsequently drying, more particularly freeze-drying, the tampon. Alternatively, the test combination can be applied, for example only to a pull-out cord, which can easily be separated from the tampon later if required. The test combination may also advantageously be applied to a cotton strip or cord which is inserted into the tampon.

Of course, when the invention is applied to the rapid determination of a pathological change in the lower female genital tract by insertion into the vagina, the amount of vaginal fluid present is unknown and there may be variations from one case to another in the quantity and the pH of the fluid. Accordingly, the test combination must be provided with a high buffer capacity so that the pH is maintained in the range of from

6 to 6.5, more particularly at approximately 6.3 to 6.4, during the actual determination at the site of the reaction. Since, in practice, the cord is often insufficient, it is advantageous in this case to introduce a larger quantity of buffer capacity of the test combination in the buffer substance into the tampon, so as to ensure adequate buffering in at least that part of the cord near the tampon.

Carriers of the aforementioned kind provided with the test combination do not need to be used by the doctor; any woman can apply them herself. If the tampon turns blue after 15 to 30 minutes in the vagina, it indicates the risk of a pathological change and is a warning to visit the doctor.

If, however, the tampon or cord has to be stored, for example for use by the doctor, or if the patient wishes to send it to the doctor, the tampon has to be treated after use, that is de-odorised, disinfected and colour-stabilized so that it can be stored for relatively long periods. It has also been found that the subsequent treatment can consist in immersing the used tampon in a 20% solution of Paraloid (that is a spontaneously cross-linking acrylic resin) in toluene ("Paraloid" is made by Merck and the word "Paraloid" is a Trade Mark).

Whereas the prior-art methods of LDH determination are exclusively laboratory methods and have the previously-mentioned disadvantages, the invention provides a rapid method of determination which is sufficiently quantitative for certain applications when used by an untrained person, for example in the case of vaginal fluid. This is particularly important for patients suffering from tumours. As disclosed in the literature, 26 enzymes have hitherto been tested in a large number of tumour patients. LDH has the highest diagnostic sensitivity; an increase of between 40 and 90% has been found in the serum (not the excreta). When a tumour is detected, routine determination of the LDH in the serum has been found useful for monitoring the progress of the disease and the treatment. Of course, an increase in LDH is not specific to a tumour, but it indicates a serious disease and, in conjunction with other features, may advantageously be used to confirm the diagnosis of a suspected tumour.

The following Examples further illustrate the present invention.

Example 1.

The aforementioned preferred test combination was applied to tampons and used in large-scale *in situ* tests on about 100 healthy women and on patients suffering from Carcinoma colli uteri, Carcinoma corporis and Carcinoma.

For best results, the tampon is kept in for between 15 and 30 minutes. After it has been taken out, the following colours can be immediately detected:

No colour	negative
Pink	still negative
Light blue with violet tinge	+
Blue	++
Dark blue	+++

A blue (++) or dark blue (+++) colour always indicates a Corcinoma, which always agrees with the pathological findings. Almost without exception, a light blue colour confirms the diagnosis "metamorphosis region". Hitherto, the colour pink has occurred only in healthy women (including pregnant women up to the 15th day).

The method of determination *in vitro*, for example tests on serum or plasma, is conventional. The only change required is the adjustment of the pH to a specific value of for example 6 to 6.5, depending on the buffer, in contrast to the strongly basic pH hitherto used. A detailed description is therefore unnecessary. In order to make absolute determinations, the colour change can be calibrated using a standard LDH sample. The colour of the calibration sample can be kept unchanged for some time, by the same process as is used to preserve the tampon.

Example 2.

The immersion method

Conventional hygienic tampons, for example "o.b." manufactured by Messrs. Dr. Carl Hahn, are tightly wrapped once or twice round the middle by a Tesa strip in the form of a belt 1 cm wide. The tampon cover is opened at the end remote from the pull-out thread, thus exposing the cylindrical surface of the tampon. The curved bottom is broken off conventional 18 ml test-tubes (DIN size, made by Messrs. Schott, Mains), forming glass tubes into which the tampon is introduced so that the open end is flush with the end of the tube. The pull-out thread is secured by adhesive plaster or a Tesa strip or the like to the other end of the tube. The end of the tube containing the opened tampon is immersed in the solution specified in 1). 2.0 ml is absorbed by each tampon. The manipulation of the solution, and all the operations described here in connection with the test combination solution must be performed in a dark chamber in monochromatic light (red photographic light or "red light").

The thus-prepared tampons and glass tubes are freeze-dried (lyophilized) in the dark. The dried tampons are taken out of the tubes, for example on the pull-out threads, wrapped in opaque paper or foil or coloured paper in darkness or in a dark chamber in photographic light, and stored away from the light.

Example 3.

The injection method

The following operations are performed in the dark chamber. 2.0 ml of the test combina-

tion solution in 1) is injected into the tampon, using a 2 ml syringe. At the end of the tampon remote from the pull-out thread, the injection needle is stuck through the cover into the tampon and guided, slowly injecting, in the longitudinal direction up to the other end of the tampon, which is thus uniformly moistened. The tampon is then freeze-dried and wrapped up and stored as in Example 2.

In principle, the reagent mixture can be applied to any absorptive carrier. Transparent film, for example cellulose acetate film used for electrophoresis or the product "Parafilm M" made by American Chemcompany, have been found very suitable for determining the vaginal fluid or for use in the vagina. The foils turn bluish at the place where they come into contact with LDH. The advantage of films of this kind is that they are mechanically stronger than, for example filter paper and can also be made completely transparent for stabilisation or storage, so that the colour intensity is completely unchanged. The word "Parafilm" is a Trade Mark.

The following transparency baths can be used for cellulose acetate film:

- a) Methanol:glacial acetic acid in the ratio 85:15,
- b) Isobutanol:dioxane in the ratio 1:1 to 3:7
- c) Methyl ethyl ketone:dioxane in the ratio 3:2
- d) Glacial acetic acid:dioxane in the ratio 3:2
- e) Methanol glacial acetic acid:glycerol in the ratio 87:12:1.

The Parafilm film may advantageously be briefly immersed in 7.5% glacial acetic acid solution.

Oil, for example Whitemore-Oil 120 or Ondino-Oil 17 (Shell), can also be used for treatment. (The words "Whitemore" and "Ondino" are Trade Marks.)

The films, which have an area of 1.5 to 2.0 cm², may advantageously be used by the specialist during a colposcopic examination to confirm a suspected change in the mucous membrane in the vagina. A colour change similar to that of the tampon always occurs at the place where there is an accumulation of tumour cells.

WHAT WE CLAIM IS:—

1. A method of simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase (LDH) utilising the reaction of lactate and nicotinamide - adenine - dinucleotide whereby lactate is converted to pyruvate, wherein the conversion is brought about at a pH in the range of from 6 to 6.5.

2. A method according to Claim 1, wherein the conversion is brought about at a pH in the range of from 6.3 to 6.4.

3. A method according to Claim 1 or 2, wherein the buffer system used is triethanolamine-NaOH.

4. A method according to any one of the preceding claims, wherein the body fluid under examination and the test reagent used for the examination are both adjusted to a pH in the range of from 6.3 to 6.4.

5. A method according to any one of Claims 1 to 3, wherein the buffer system used has a pH such that, if the natural pH of the body fluid under investigation deviates from 6 to 6.5 during the conversion of lactate to pyruvate, the pH is adjusted to a value in the required range.

6. A reagent for simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase, containing lactate, nicotinamide-adenine-dinucleotide, a tetrazolium salt and a buffer, optionally on a carrier, the reagent having a pH adjusted such that the pH when a sample is present is in the range of from 6 to 6.5.

7. A reagent according to Claim 6, wherein the pH is adjusted to a pH in the range of from 6.3 to 6.4.

8. A reagent according to Claim 6 or 7, wherein the system is adjusted to pH 7.0 for testing vaginal fluid.

9. A reagent according to any one of Claims 6 to 8, wherein the buffer in the system is triethanolamine-NaOH.

10. A reagent according to Claim 9, which contains from 1.0 to 150 mM of triethanolamine buffer, pH 7.0, from 20 to 350 mM of Na-lactate (the sodium salt of DL lactic acid), from 0.01 to 1.0 mM of phenazine methosulphate (PMS), from 0.1 to 10 mM of nicotinamide-adenine-dinucleotide (NAD⁺), and from 0.01 to 1.5 mM of nitro-blue-tetrazolium chloride (NBT).

11. A reagent according to Claim 9, which contains about 5.0 mM of triethanolamine buffer, pH 7.0, about 67.5 mM of Na-lactate, about 0.1 mM of phenazine methosulphate (PMS), about 1.5 mM of nicotinamide-adenine-dinucleotide (NAD⁺), and about 0.3 mM of nitro - blue - tetrazolium chloride (NBT).

12. A diagnostic means for determining a pathological change in the lower female genital tract, which comprises a carrier bearing a reagent according to any one of Claims 6 to 11.

13. A diagnostic means according to Claim 12, wherein the carrier is a tampon.

14. A method in accordance with Claim 1, substantially as described in foregoing Example 1.

15. A method in accordance with Claim 1, substantially as described in foregoing Example 3.

16. A reagent in accordance with Claim 6, substantially as hereinbefore described.



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tion solution in 1) is injected into the tampon, using a 2 ml syringe. At the end of the tampon remote from the pull-out thread, the injection needle is stuck through the cover into the tampon and guided, slowly injecting, in the longitudinal direction up to the other end of the tampon, which is thus uniformly moistened. The tampon is then freeze-dried and wrapped up and stored as in Example 2.

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1. A method of simultaneously determining isoenzymes 4 and 5 of lactate dehydrogenase (LDH) utilising the reaction of lactate and nicotinamide - adenine - dinucleotide whereby lactate is converted to pyruvate, wherein the conversion is brought about at a pH in the range of from 6 to 6.5.

2. A method according to Claim 1, wherein the conversion is brought about at a pH in the range of from 6.3 to 6.4.

3. A method according to Claim 1 or 2, wherein the buffer system used is triethanolamine-NaOH.

4. A method according to any one of the preceding claims, wherein the body fluid under examination and the test reagent used for the examination are both adjusted to a pH in the range of from 6.3 to 6.4.

5. A method according to any one of Claims 1 to 3, wherein the buffer system used has a pH such that, if the natural pH of the body fluid under investigation deviates from 6 to 6.5 during the conversion of lactate to pyruvate, the pH is adjusted to a value in the required range.

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FORRESTER, KETLEY & CO.,
Chartered Patent Agents,
Forrester House,
52 Bounds Green Road,
London, N11 2EY
— and also at—
Rutland House,
148 Edmund Street,
Birmingham, B3 2LD
— and —
Scottish Provident Building,
29 St. Vincent Place,
Glasgow, G1 2DT.
Agents for the Applicants.

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