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A Rapid Screening Test for Lactic Aciduria

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Summary: A rapid test for the determination of lactate in urine is described, which differentiates adequately between normal and elevated urine lactate levels. The method depends on the enzymatic decolourization of the blue dye dichlorophenolindophenol by lactate in the presence of the enzyme *L*-lactate cytochrome c oxidoreductase (EC 1.1.2.3). Interference by vitamin C is prevented by pre-treatment with charcoal.

The test is simple and inexpensive, and a compact testing unit, which is stable and convenient for most laboratories, can be assembled in a glass capillary.

Ein schneller Screening-Test für Milchsäure im Harn

Zusammenfassung: Ein schneller Test für die Bestimmung von Lactat im Harn wird beschrieben. Der Test ist zur Unterscheidung normaler und erhöhter Lactatkonzentrationen im Harn geeignet. Die Methode beruht auf der enzymatischen Entfärbung des blauen Farbstoffs Dichlorphenolindophenol durch Lactat in Gegenwart von *L*-Lactat: Cytochrom c Oxidoreductase (EC 1.1.2.3). Störungen durch Ascorbinsäure werden durch Vorbehandlung mit Aktivkohle vermieden.

Der Test ist einfach, billig und eine kompakte Testeinheit kann in einer Glaskapillare untergebracht werden. Sie ist stabil und geeignet für die meisten Laboratorien.

Introduction

A number of screening methods have been developed for the rapid indication of the presence or absence of certain metabolic abnormalities. Examples include the gas chromatographic procedures for volatile fatty acids (1) and for amino acids (2), the simple spot test for reducing sugars (3) and the commercially available test strips for glucose, protein etc. The detection of increased amounts of lactate in blood or urine, however, requires in many cases a rather time-consuming quantitative determination (4–7). A rapid method has been decribed by *Wieland & Jagow-Westermann* (8) using *L*-lactate cytochrome c oxidoreductase (or yeast lactate dehydrogenase, Y-LDH, EC 1.1.2.3). These methods require a spectrophotometer and are therefore less suitable for rapid screening for lactaciduria. For this reason, a rapid screening test for lactate in urine has been developed. The test is based on the following reaction:

$$\text{dichlorophenolindophenol (DCIP)} + \text{lactate} \xrightarrow{\text{Y-LDH}} \text{reduced DCIP} + \text{pyruvate.}$$

In this reaction, one mol of dichlorophenolindophenol is reduced by one mol of lactate. The reaction is catalyzed by the enzyme *L*-lactate cytochrome c oxidoreductase and it can be followed visually because the oxidized form of the dye is blue, while the reduced form is colourless. Excess lactate completely decolourizes the dye in the reaction mixture. Ascorbic acid, which is oxidized by the same dye non-enzymatically, could give rise to a false positive result if the vitamin concentration

exceeded 0.11 mmol/l. Therefore urine samples must be pretreated with charcoal, a procedure which oxidizes the ascorbic acid to dehydroascorbic acid leaving the lactate unaffected.

Materials and Methods

Lactate was determined enzymatically as described by *Hohorst* (4), and ascorbic acid by the photometric indophenol method as described by *Roe* (9). Protein concentrations were determined according to *Lowry* et al (8). The activity of *L*-lactate: cytochrome c oxidoreductase was expressed in units. One unit of enzyme reduces one μmol of ferricyanide per minute at 25 °C. Specific activity was expressed in units/mg protein. The activity was determined in 0.1 mmol/l K-phosphate buffer, pH 7.2, 1.0 mmol/l ferricyanide, 20.0 mmol/l lithium *L* (+) lactate, following the absorbance decrease at 420 nm (absorption maximum of ferricyanide). Determination of the absorption coefficient, ϵ_{420}, gave a value of 1.042×10^3 cm²/mol.

Preparation of L-lactate: cytochrome c oxidoreductase

The preparation procedure used was a combination of the method of *Morton & Shepley* (10) and the method of *Spyridakis* et al (12) (both type I ferrocytochrome b_2).

As starting material 800 g fresh, compressed baker's yeast was used. This was lyophilized and finely powdered. It was then extracted and fractionated with acetone as was described by *Spyridakis* et al (12) except that the autolysis was performed for 20 h at 4 °C. The pink precipitate was extracted for 1 h at – 2 °C to 0 °C with 0.15 mol/l sodium lactate in 0.1 mmol/l EDTA, 1 mmol/l $MgSO_4$, pH 6.8, containing 150 ml/l acetone; this was preferable to dialysis of the pink, sticky precipitate obtained after acetone precipitation, which results in severe loss of activity. The extract was then treated as described by *Morton & Shepley* (11) until the pyrophosphate extract was obtained; the latter was not dialyzed but saturated with $(NH_4)_2SO_4$ to 0.7. This solution was stored overnight at – 20 °C. After centrifugation (50 min, 11,000 *g*, 0 °C), the crude enzyme was dissolved in 10 ml of 0.1 mol/l Tris-HCl buffer, pH 8.0 and chromatographed on a column of DEAE-Sephadex, A-25 (diameter 2.5 cm, length 30 cm) at 4 °C. At this stage of purification the enzyme has a sufficient specific activity for the preparation of test capillaries.

The enzyme solution, saturated with $(NH_4)_2SO_4$ to 0.7, can be stored at – 20 °C and concentrated by centrifugation.

Reaction mixture

The reaction mixture contained 0.4 mmol/l dichlorophenolindophenol (oxidized blue form), 15 U/ml *L*-lactate: cytochrome c oxidoreductase of specific activity of 10–20 U/mg protein, 1 mmol/l EDTA and 0.15 mol/l potassium phosphate, pH 7.2. A solution of 2.4 mmol/l dichlorophenolindophenol was prepared in distilled water. To this solution were added 10 μl 1 mol/l NaOH. Pure oxygen was then bubbled through the solution. The concentration of dichlorophenolindophenol was checked by measuring the absorbance at 600 nm. (ϵ_{600} = 20.1×10^3 cm²/mol). This solution was diluted with 5 volumes of 0.18 mol/l K-phosphate buffer, pH = 7.2, and 1.2 mmol/l EDTA, to obtain the desired concentration. Finally the enzyme was dissolved in this mixture.

Preparation of test capillaries

A glass capillary of 1.5 mm inner diameter (e. g. a 100 μl disposable micropipet) was filled with 40 μl of the reaction mixture. The contents of the glass capillary were immediately frozen and lyophilized for about 17 hours. The dry capillaries were tested with standard lactate solutions of 0.3 mmol/l, 0.5 mmol and 0.8 mmol/l as described for urine testing. The capillaries filled with the lyophilized reaction mixture were stored dry, in the dark, at – 20 °C.

Sample pre-treatment

Urine was pre-treated with charcoal in a ratio of 0.4 g per 5 ml urine. The charcoal was removed by centrifugation or filtration.

Test procedure

To test a charcoal-treated urine for pathological amounts of lactate, an amount of urine equal to the original volume of reagent mixture before freeze-drying, is taken up by the capillary and mixed carefully. A decolourization within 2 min indicates that the lactate concentration exceeds 0.4 mmol/l.

Results

Dichlorophenolindophenol solution

The dye dichlorophenolindophenol can exist in 3 different forms in solution: the colourless reduced form and the red or blue oxidized forms. The interconversion between these forms is pH-dependent. A titration curve is given in figure 1. The blue form has an absorption maximum at 600 nm, with an absorption coefficient of $\epsilon = 20.1 \times 10^3$ cm²/mol, which was determined after alkalinization and careful oxidation of the solution (13). In contrast to what was expected, it was virtually impossible to obtain 95% of the dye in the blue form by dissolving solid dichlorophenolindophenol in buffer pH 7.2. By preparing a fully oxidized alkalinized, rather concentrated dichlorophenolindophenol solution in distilled water and diluting this solution with buffer, the desired concentration of blue dichlorophenolindophenol was obtained as illustrated in table 1.

Specific activity of L-lactate: cytochrome c oxidoreductase

It proved to be essential to use an enzyme preparation with a specific activity of 10–20 U/mg protein; at a higher specific activity not enough supporting material was retained in the capillary to maintain the dry reaction mixture inside, and when the specific activity was lower it was difficult to introduce the urine sample into the capillary.

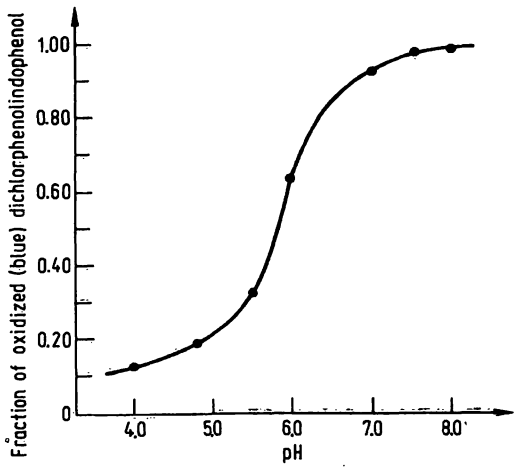

Fig. 1. Fraction of blue oxidized form of dichlorphenolindophenol at different pH values.

Tab. 1. The effect of alkalinization and oxygenation on the absorbance of blue dichlorophenolindophenol (2.5 mmol/l). The concentrations are calculated from the absorption at 600 nm of a 100-fold diluted solution, using $\epsilon_{600} = 20.1 \times 10^3$ cm²/mol.

	A_{600} of 100 x diluted solution	Calculated concentration in mmol/l
A – dichlorophenolindophenol, dissolved in distilled water	0.248	1.24
B – 5 ml sol. A + 10 µl 10 mol/l NaOH	0.463	2.30
C – Sol. B after oxidation with pure oxygen	0.510	2.54
D – Sol. C, 6 x diluted with 0.18 mol/l phosphate, pH 7.2	0.084	0.41

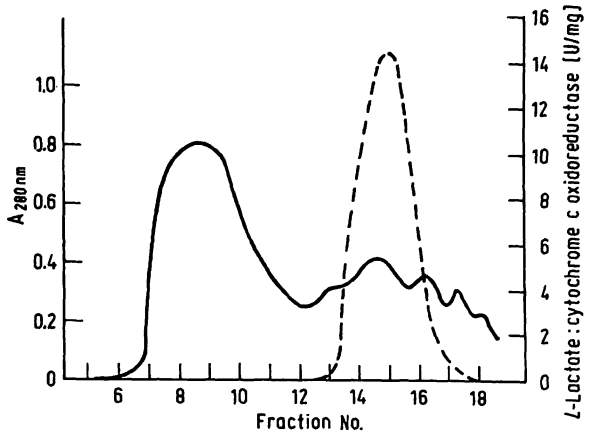

Fig. 2. Chromatographic purification of *L*-lactate: cytochrome c oxidoreductase on a column of DEAE-Sephadex A-25 (2.5 X 30 cm). Elution was accomplished with 200 ml 0.1 mmol/l Tris-HCl buffer, pH 8.0 at a flow rate of 25 ml/h.
Purification factor: 10 times, activity loss: 10 to 40%.
The enzyme-containing fractions were adjusted to 0.7 saturation $(NH_4)_2SO_4$, immediately after elution.
—— absorbance at 280 nm
--- dehydrogenase activity in U per mg protein

Enzyme purification

In table 2 the results are given of the purification procedure. Even if after the first step a specific activity of only 1.5 is obtained, purification by the subsequent chromatographic procedure increases the specific activity to 15.0, which was sufficient for the preparation of test capillaries. The elution profile is given in figure 2. Determinations of the K_m for lactate and dichlorophenolindophenol according to *Eadie-Hofstee* (14) yielded values of 0.40 mmol/l and 0.014 mmol/l respectively (c. f. ref. 11).

Tab. 2. Purification of *L*-lactate: cytochrome c oxidoreductase
a) At several steps of the isolation procedure the mean yield and specific activity is given with standard deviations calculated from 10 enzyme preparations, using different yeast batches of 800 g wet weight. For experimental details: see Methods.

	Total activity in U	Specific activity in U/mg protein
Crude extract	4400 ± 1600	0.5 ± 0.3
Precipitate, 0.34 acetone	3630 ± 1270	5.0 ± 2.7
Pyrophosphate extract	2540 ± 1100	6.2 ± 3.2
Precipitate 0.7 $(NH_4)_2SO_4$	2110 ± 1610	11.1 ± 8.9

b) Examples of purification by DEAE-Sephadex chromatography and recovery of *L*-lactate: cytochrome c oxidoreductase.

Sample applied to the column		Eluted enzyme	
Total activity in units	Specific activity	Recovered activity in percentage of applied units	Specific activity
571	3.2	55	15.4
300	1.2	80	15.5
312	2.3	88	17.7

Calibration line

In figure 3 the decolourization time is plotted against the lactate concentration. Samples with a lactate concentration below 0.4 mmol/l will not decolourize, owing to the effect of the dye concentration in the reaction mixture and the stoichiometry of the relation.

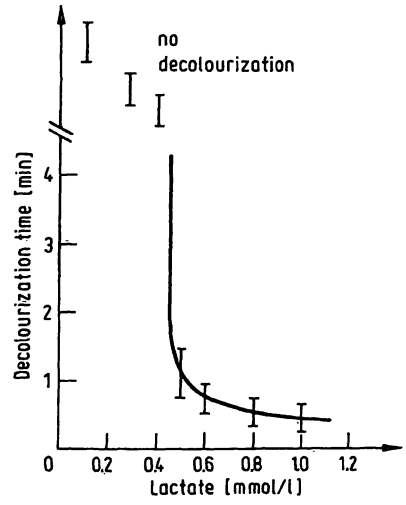

Fig. 3. Standard line. The decolourization time of test capillaries and urines with different lactate concentrations. Each urine sample was tested with 10 test capillaries. The variation in the decolourization times is given in the figure. At a lactate concentration of 0.4 mmol/l, 1 capillary decolourized at 4 minutes, the other 9 retained a light blue colour. A difference exists in the colour depth of capillaries with 0.1 mmol/l and 0.4 mmol/l lactate. Test capillaries were prepared with a reaction mixture containing 0.4 mmol/l dichlorophenolindophenol and 17.4 U/ml *L*-lactate: cytochrome c oxidoreductase with specific activity of 15.5 U/mg protein.

The decolourization time is concentration-dependent for the range 0.5—0.8 mmol/l lactate. Higher lactate concentrations yield a minimum time. This time is dependent on the activity of the enzyme in the capillary. This activity is lower than the original activity, because a slight inactivation occurs during the freeze-drying procedure. At an original enzyme concentration of 15 U/ml, the minimum time is between 30 and 90 seconds. The decolourized capillaries in the lower lactate concentration range become light blue again after about half an hour standing at room temperature, owing to the reoxidation of reduced dichlorophenol-indophenol.

Precision

With test capillaries from one batch (that is, capillaries filled with the same reaction mixture and dried together) good reproducibility was obtained (fig. 3). A variation of about 20% in decolourization time is of little importance for a semi-quantitative test which is based on colour change within a certain time. To determine this time, a standard line has to be made with each batch of test capillaries.

Specificity

L-lactate: cytochrome c oxidoreductase has a rather high degree of substrate specificity. In addition to lactate, some α-hydroxymonocarboxylic acids (such as α-hydroxybutyrate and α-hydroxycaproate) act as substrates, although with a lower velocity (15); these compounds, however, are usually not found in urine. A compound that may be present in urine is β-hydroxybutyrate. This metabolite, however, does not influence the lactate test (tab. 3). In addition to the enzyme specificity, the specificity of the test is also determined by nonenzymatic reduction of the dye. Non-enzymatic reduction is caused by ascorbic acid. Since the concentration of vitamin C in urine varies with the diet, experiments were conducted to show that amounts up to 6.2 mmol/l were fully oxidized by the charcoal pretreatment, leaving lactate concentrations unaffected (tab. 4). In addition, the charcoal pre-treatment also removed coloured pigments which may be present in urine and which may interfere with the visually inspected colour change.

Tab. 3. Effect of β-hydroxybutyrate on the decolourization times of test capillaries with urines of different lactate concentration. Test capillaries were prepared with a reaction mixture containing 0.6 mmol/l dichlorophenol-indophenol, 12 U/ml *L*-lactate: cytochrome c oxidoreductase.

Urinary concentrations of lactate (mmol/l)	and β-hydroxybutyrate (mmol/l)	Decolourization time (s)
–	10.0	no decol. after 5 min
0.5	–	no decol. after 5 min
0.5	10.0	no decol. after 5 min
0.6	–	light blue after 5 min
0.6	100	light blue after 5 min
0.8	–	110, 120
0.8	10.0	120, 105, 120
1.0	–	70
1.0	10.0	50
10.0	–	50, 35
10.0	10.0	40, 50

Tab. 4. The effect of charcoal treatment of urines with added lactate and ascorbic acid. Lactate and ascorbic acid were added to urine samples and the concentrations of these acids were determined before and after charcoal treatment as described under Materials and Methods.

Urine sample	Before charcoal treatment		After charcoal treatment	
	Lactate (mmol/l)	Ascorbic acid (mmol/l)	Lactate (mmol/l)	Ascorbic acid (mmol/l)
1	0.09	0.02	0.09	< 0.02
1	0.37	1.21	0.35	< 0.02
1	0.97	2.38	0.98	< 0.02
2	0.43	3.90	0.38	< 0.02
2	0.39	4.65	0.38	< 0.02
2	0.48	6.20	0.52	< 0.02

pH of the sample

The reaction mixture in the capillary is sufficiently buffered to neutralize the lowest urinary pH (between 4.4 and 4.5 (16), as is shown in figure 4.

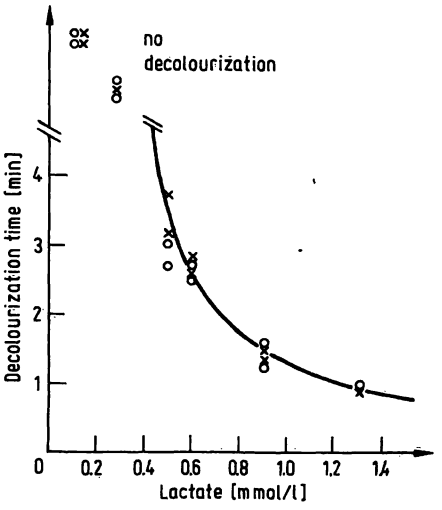

Fig. 4. Decolourization times of urines with different lactate concentrations at pH 6.7 (0) and the decolourization times of the same urines adjusted to pH 4.2 (x).

Stability

When stored dry, at −20 °C, the test capillaries are stable for at least 6 months (tab. 5).

Tab. 5. Stability of four different series of test capillaries after storage at – 20 °C for several months.

Period stored (month)	dichlorophenolindophenol concentration in reaction mixture (mmol/l)	Lactate concentration in test solution (mmol/l)	Original decol. time (s) ± S. D. (n = 10)	Decol. time after storage, (s), average of 3 determinations
5	0.4	0.4	No decol. after 5 min	No decol. after 5 min
		0.8	75 ± 10	80
7.5	0.4	0.4	No decol. after 5 min	No decol. after 5 min
		0.6	85 ± 10	90
6	0.2	0.2	No decol. after 5 min	No decol. after 5 min
		0.4	40 ± 5	35
6	0.5	0.5	No decol. after 5 min	No decol. after 5 min
		0.8	90 ± 10	110
		1.0	85 ± 10	70

Discussion

Lactic acid accumulation may be a cause of metabolic acidosis, resulting from a variety of pathological conditions. It may occur when the oxygen supply to the tissues is impaired (17), in association with several metabolic disorders such as diabetes (18), glycogen storage disease (19), congenital lactic acidosis (20) and in patients with neoplastic proliferative disorders (21). Increased lactate excretion will also be found in patients with impaired renal function (22). When an indication is needed as to whether or not pathological amounts of lactate are excreted, the test as described here has several advantages over the different methods of quantitative assay as described by *Hohorst* (4), modified by *Leroux* (7), *Mann & Shute* (5), *Jones-Owen & Lechocki* (6) and *Wieland & Jagow-Westermann* (8).

First, the test takes only 3 minutes, while the quantitative determination takes at least 15 minutes. Furthermore, no equipment such as a gaschromatograph (6) an Auto-Analyser (5) or a spectrophotometer (4, 8) is required, and finally the test is inexpensive.

Given the known volume of urine taken up by the capillary, the amount of dichlorophenolindophenol in the test capillary determines whether or not complete decolourization will take place. The amount of dichlorophenolindophenol has been chosen so that complete decolourization occurs when the urinary lactate concentration exceeds 0.4 mmol/l. The concentration of lactate in urine resulting in complete decolourization can be chosen at will by varying the amount of dichlorophenolindophenol in the test capillary.

The concentration of 0.4 mmol/l has been selected as a result of previous studies on normal and pathological lactate excretion in children (22). It was found that the lactate concentration in a random urine sample is an acceptable measure for lactate excretion. A concentration exceeding 0.4 mmol/l should be considered as pathological, due to lactacidemia or a disturbed renal function. Therefore, while a positive test may suggest the existence of lactic acidosis, such a diagnosis must be confirmed by a quantitative blood lactate determination.

The test can be constructed for limiting lactate concentrations other than 0.4 mmol/l. For instance, by using two test capillaries with different dichlorophenolindophenol concentrations, it is possible to obtain more information about lactate excretion within a very short time. The same test principle can be applied using test tubes containing freeze-dried reaction mixtures, instead of capillaries. This has the advantage that the chances of losing some reaction mixture during the freeze-drying procedure are minimized, but it has the disadvantage that pipettes have to be used for quantification of the sample and that more reaction mixture is required for a test.

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