PHYSICO – CHEMICAL METHODS

DETERMINATION OF Fe^{2+} IONS CONCENTRATION IN SERUM USING ANALYTICAL CURVE

Name, group No: Date:

Principle

The solution of bathophenanthroline forms a stable red-coloured complex with Fe^{2+} ions. This complex is suitable for spectrophotometric determination at its absorption maximum (535 nm).

Using the measurement of absorbances of several different solutions of Fe^{2+} with known concentrations we can create an analytical curve (dependence of A_{535} on concentration). Concentration of unknown sample can be read from this curve or can be calculated.

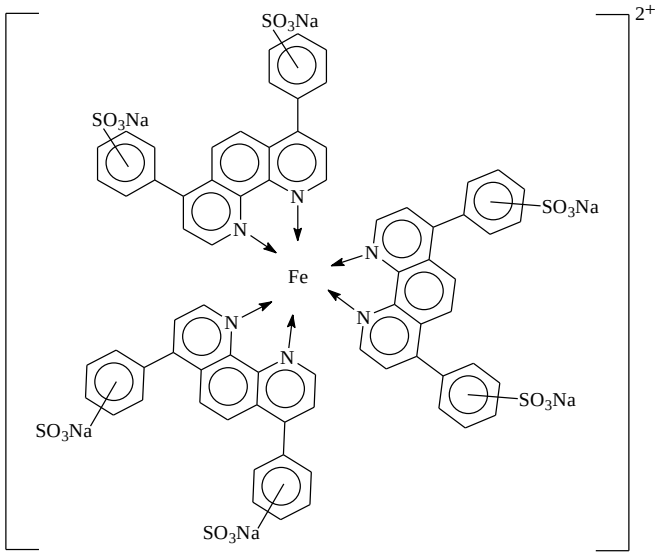


Figure: Structure of bathophenanthroline

Reagents and accessories

bathophenanthroline (4,7-diphenyl -1,10-phenanthroline-3,6-disulfonic acid) (0.46 mmol/l),
natrium acetate CH_3COONa (2 mol/l),
stock standard solution of Fe^{2+} ions (18 μmol/l)

Accessories and equipment

spectrophotometer

Experimental procedure

A standard solution of ferrous salt of known concentration (c = 18 μmol.l^{-1}) is diluted with water according to the working table, thus preparing solutions of different, but known, concentrations of Fe^{2+} ions, in order to construct an analytical curve, A = f(c).

The concentrations of ferrous ions in solutions prepared by diluting the stock standard Fe^{2+} solution (c = 18 μmol.l^{-1}) are calculated from the dilution balance equation (see solutions):

$$\mathbf{c_1.V_1 = c_2.V_2}$$

c_1 = 18 μmol.l^{-1} V_1 = 0,5; 1,0; 1,5 or 2,0 ml

c_2 = x V_2 = 2,0 ml

Test tube No	**1**	**2**	**3**	**4**	**serum sample**	**reference sample**
Standard solution Fe^{2+} (ml) (17.9 μmol/l)	0.5	1.0	1.5	2.0		
H_2O (ml)	1.5	1.0	0.5	-	-	2.0
Serum sample (ml)	-	-	-	-	2.0	-
Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5
A_{535} is measured against reference sample within 5-60 min						
A_{535}						-
c (Fe^{2+}) (μmol/l)				18		-

Evaluation

1. Construction of analytical curve using absorbances and concentrations in test tubes No. 1 – No. 4

2. Read the concentration of Fe^{2+} in serum from curve. (see *Fig.*)

3. Calculate the concentration of Fe^{2+} ions in serum using one of the known standard solutions:

$$c_{sam} = \frac{A_{sam}}{A_{st}} . c_{st}$$

where A_{sam} is absorbance of sample, A_{st} is absorbance of standard solution, c_{sam} is concentration of the sample and c_{st} is concentration of the standard

4. Compare the determined concentration with physiological values:
fS (Fe^{2+}) males = 9,6 – 30,2 μmol.l^{-1}
fS (Fe^{2+}) females = 8,9 – 27,3 μmol.l^{-1}

fS (Fe^{2+}) children = 9 – 30 $\mu mol.l^{-1}$

Calculations and conclusion

SOLUTIONS

PREPARATION OF 250 ml OF NaCl SOLUTION WITH A CONCENTRATION 0.15 mol.l^{-1}

Name, group No: Date:

Principle

Solution with concentration expressed as the ratio of the amount of substance concentration to the total volume of the solution is being prepared. The following formula is used:

$$c = \frac{n}{V} \qquad n = \frac{m}{Mr} \qquad \boldsymbol{m = c\,.V\,.Mr}$$

The solution will be prepared in the 250 ml volumetric flask.

Reagents

NaCl p.a. (Mr = 58)

Accessories and equipment

volumetric flask (250 ml), funnel, watch glass, scale

Experimental procedure

The mass of NaCl necessary to prepare the solution is calculated and the amount is weighed on the scale. This amount is transferred through the funnel into the volumetric flask and the watch glass is carefully washed with the distilled water into the volumetric flask. NaCl is dissolved in distilled water and the flask is filled with water to the graduation mark. The flask is equipped with a label stating composition and concentration of the compound and the date of preparation.

Calculations and conclusion:

SOLUTIONS

PREPARATION OF 100 ml OF 75 AND 100 mmol.l^{-1} NaCl SOLUTIONS BY DILUTION OF 150 mmol.l^{-1} NaCl SOLUTION

Name, group No: Date:

Principle

If the composition of solution is given as amount-of-substance concentration, the following balance equation- holds for calculation of changes in solution composition by addition of a solvent:

$$c_1 \,.\, V_1 = c_2 \,.\, V_2$$

The equation expresses that when diluting a solution with a solvent, the volume of the solution and its concentration change, while the substance amount remains the same. The definition of substance concentration implies:

$$n_1 = c_1 \,.\, V_1 \qquad n_2 = c_2 \,.\, V_2$$

From the definition of amount of substance concentration it follows:

$$n_1 = n_2$$

$$c_1 \,.\, V_1 = c_2 \,.\, V_2$$

E. g., to prepare 100 ml of a 75 mmol.l^{-1} solution NaCl by diluting physiological saline (150 mmol.l^{-1}) it holds that:

c_1 = 150 mmol.l $^{-1}$ c_2 = 75 mmol.l^{-1}

V_1 = ? ml V_2 =100 ml

$$V_1 = \frac{c_2 \,.\, V_2}{c_1} = \frac{75 \,.\, 100}{150} = 50 \text{ ml}$$

In calculation of the amount of 150 mmol/l NaCl required to prepare 100 ml 100 mmol/l NaCl is proceed in the same way.

Reagents and accessories

NaCl solution, c = 0.15 mol.l^{-1},
a set of 100 ml volumetric flasks, pipettes, graduated cylinder, funnel, wash bottle

Experimental procedure

Calculated volumes of physiological NaCl solution necessary for preparation of the diluted solutions are measured with a graduated cylinder, transmitted into 100 ml volumetric flasks, the flasks are filled with distilled water to the mark, closed with a stopper and well mixed.

Flasks are identified with a label of composition and concentration of the compound, and date of preparation.

Calculations and conclusion

SOLUTIONS

EXAMINATION OF HYPOTONIC HEMOLYSIS (OSMOTIC FRAGILITY) OF ERYTHROCYTES

Name, group No: Date:

Principle

In hypotonic environment, erythrocytes undergo hemolysis. Osmotic resistance (or, alternatively, osmotic fragility) is examined by monitoring of erythrocytes hemolysis in hypotonic environment.

The maximal osmotic resistance is determined at the concentration of NaCl, where the beginning of hemolysis is observed (staining of the supernatant over erythrocyte sediment is slightly pink or clear due to the small amount of released hemoglobin).

Minimal osmotic resistance is measured at that concentration of NaCl, where hemolysis is maximal (erythrocytes are completely hemolyzed, the solution has red color and no sediment is observed at the bottom of the test tube, similarly to the control tube with distilled water).

Examination of osmotic resistance (fragility) is of diagnostic value. It is used for diagnosis as well as for differentiation of hemolytic disease.

In clinical practice, as well as in research, osmotic fragility is the more frequently used term. Not to be confused of these two terms, one has to recognize that maximal osmotic resistance indicates minimal osmotic fragility, and minimal osmotic resistance indicates maximal osmotic fragility. As it turns out, the more osmotically resistant (more stable against hemolysis) the erythrocyte is, the smaller its osmotic fragility (sensitivity to hemolysis) is.

Reagents and accessories

Suspension of washed erythrocytes in an isotonic NaCl solution (20%, v/v), NaCl solutions with concentrations 0.075, 0.1 and 0.15 $mol.l^{-1}$ prepared in previous practical, test tubes of the same size, glass and automatic pipettes, aluminium foil, centrifuge, spectrophotometer

Experimental procedure

20% suspension of erythrocytes preparation: Erythrocytes are isolated from blood by centrifugation (200rpm for 10 min). After sucking off the plasma erythrocytes are three times washed with isotonic NaCl solution and subsequently centrifuged at the same conditions. Sediment with erythrocytes is resuspended at five times their volume of physiological solution.

Osmotic fragility examination: Prepared NaCl solutions in different concentrations are pipetted into a set of centrifuge tubes, according to the table:

Test tube No	**1**	**2**	**3**	**4**
erythrocyte suspension (ml)	0.1	0.1	0.1	0.1
0.15 mol/l NaCl (ml)	5	-	-	-
0.1 mol/l NaCl (ml)	-	5	-	-
0.075 mol/l NaCl (ml)	-	-	5	-
distilled water (ml)	-	-	-	5
1. incubation 15 min/ 37°C 2. centrifugation 5 min/ 2500 r.p.m. 3. measurement of A_{540} in supernatant against water				
A_{540}				
% of hemolysis				100

The mixtures in tubes are carefully mixed (cover the test tubes with aluminium foils and turn them upside down), incubated in a water bath at 37 °C for 15 minutes. After centrifugation (5 min at 2500 rpm) supernatant is transferred into a cuvette and absorbance is measured against water at 540 nm. The values are recorded into the table.

Evaluation

After centrifugation we observe that the volume of sediment (erythrocytes) in test tubes is inversely proportional to NaCl concentration. In the test tubes No. 2, 3, 4 (hypotonic medium) hemolysis is observed (supernatant is red colored due to hemoglobin presence). The solution in the test tube with physiological NaCl solution is isotonic with the inner environment of erythrocytes and hemolysis is not observed (clear supernatant).

Hemolysis is evaluated quantitatively by calculating percentage of hemolysis at different NaCl concentrations. The A_{540} value in the tube with water (tube No. 4) is taken as 100 **%,** because in water hemolysis is complete. The bar graph is constructed.

In a healthy person: *hemolysis begins in a NaCl solution with a concentration of 0.078-0.086 mol/l, complete hemolysis occurs in NaCl solution with a concentration of 0.052-0.057 mol/l*

Increased osmotic fragility *is associated with congenital or acquired spherocytosis (a disease in which erythrocytes have a congenital defect in membrane proteins that causes the movement of water and sodium into erythrocytes, while erythrocytes lose their biconcave shape and acquire a round shape).*

We speak of ***reduced osmotic fragility*** *when complete hemolysis of erythrocytes in a NaCl solution with a concentration of 0.052-0.057 mol/l did not occur, i.e. they are more resistant to a hypotonic solution. Decreased osmotic fragility is associated with chronic liver disease, iron*

deficiency anemia, thalassemia, hyponatremia (serum Na+ concentration <130 mmol/l) or sickle cell anemia after splenectomy (removal of the spleen).

Conclusion:

Compare behavior of erythrocytes (percentage of hemolysis) in solutions of NaCl with different concentrations.

SACCHARIDES

DETECTION OF THE PRESENCE OF REDUCING SACCHARIDES IN THE URINE

Name, group No: Date:

Principle

Evidence for the presence of reducing saccharides in urine is based on their ability to oxidize to aldonic acids, thereby reducing other substances. First, we perform a Fehling's test (a) in the urine, which only determines the presence of reducing carbohydrates (qualitative determination). The principle of this reaction is the reduction of Cu^{2+} ions, present in Fehling's reagent, to Cu^{+} with the formation of a yellow-red to red precipitate of copper oxide (Cu_2O).

In order to determine whether the carbohydrate (or other reducing substance) in the urine positive for Fehling test is glucose, we use a semi-quantitative determination using the diagnostic strip GlukoPHAN (b). These diagnostic strips are used for quick and specific determination of the presence of glucose in urine, as well as indicative determination of glucose concentration in urine. Their indicator zone contains *glucose oxidase* and *peroxidase* enzymes with a special chromogenic system that is oxidized to a green product in the presence of glucose.

a) Fehling's test

Reagents and equipment

Fehling's I and Fehling's II reagents, fresh urine, glass pipettes, boiling water bath

Experimental procedure

2 ml of Fehling's reagent (Fehling I and Fehling II in a 1:1 ratio) is prepared in a test tube. An equal volume of examined urine is added and the reaction mixture is heated for 2 minutes in the boiling water bath. Reaction is positive, if an orange-red (ginger) precipitate of cuprous oxide forms. Sensitivity is about 10 mmo1.l^{-1} Reducing tests have to be performed in a fresh, non-turbid urine. In case of present proteins, urine has to be deproteinated before the test.

b) Detection and semiquantitative determination of glucose with diagnostic strips "GLUCOPHAN"

Reagents and equipment

Fresh urine (that one, which was positive in previous test),

diagnostic strips Glucophan (stored in a well closed bottle, in a dry and cool place)

Experimental procedure

The strip is immersed into the examined urine and immediately taken out. After three minutes the test is evaluated by comparing intensity of the color of indicating zone that has been formed with the scale on the case. Color frames on the scale correspond approximately to concentrations of glucose given in the table:

Evaluation:

In the case of a positive Fehling reaction, confirm whether the reducing substance is glucose by a specific test using GlukoPHAN strips. Write the results in the prepared table.

	Urine 1	**Urine 2**
Fehling test		
GlukoPHAN Glucose (mmol/l)		

LIPIDS

DETERMINATION OF CONCENTRATION OF SERUM LIPIDS

Name, group No: Date:

Principle

After hydrolysis with concentrated sulfuric acid blood serum lipids (including non-esterified fatty acids) react with vanillin and phosphoric acid, forming a red-colored product. Its intensity is proportional to the amount of total lipids in serum.

Reagents

Bio – La Test kit (vanillin, c = 10 mmol.l^{-1}, phosphoric acid, c = 11.5 mol.l^{-1}), H_2SO_4, concentrated, serum, standard solution of total lipids, c = 8 g.l^{-1}

!!!Attention! The reagent and sulfuric acid are strong caustics!!!

Accessories and equipment

water bath, spectrophotometer, burner

Experimental procedure

Determination of total serum lipids concentration is performed according to the table:

Test tube	**serum sample**	**standard solution**	**blank**
Serum (ml)	0.02	-	-
Standard lipid solution (ml)	-	0.02	-
H_2SO_4 (ml)	1.5	1.5	-
Mixture is stirred and heated in thin – walled tubes for 10 minutes in a boiling water bath. The tube contents are cooled down under a stream of cold water and from the hydrolysate (in the case of reference – solution of H_2SO_4) following amounts are pipetted into dry test tubes			
Hydrolysate (ml)	0.10	0.10	-
H_2SO_4 (ml)	-	-	0.10
Reagent (ml)	1.5	1.5	1.5
The tube contents are stirred, let stand for 10 – 15 min at room temperature and absorbances of the sample and standard are measured at 530 nm against blank			
A_{530}			-

Total lipids ($g.l^{-1}$)		**8**	

Evaluation

Concentration of total serum lipids is calculated from sample (A_{sam}) and standard (A_{st}) absorbances according to the formula:

$$c_{sam} = \frac{A_{sam}}{A_{st}} \cdot 8 \text{ (g/l)}$$

Compare the calculated concentration of total lipids with physiological values.

fs total lipids: 4 – 8 g/l
(f = fasted, s = serum)

Calculations and conclusion

AMINOACIDS, PROTEINS

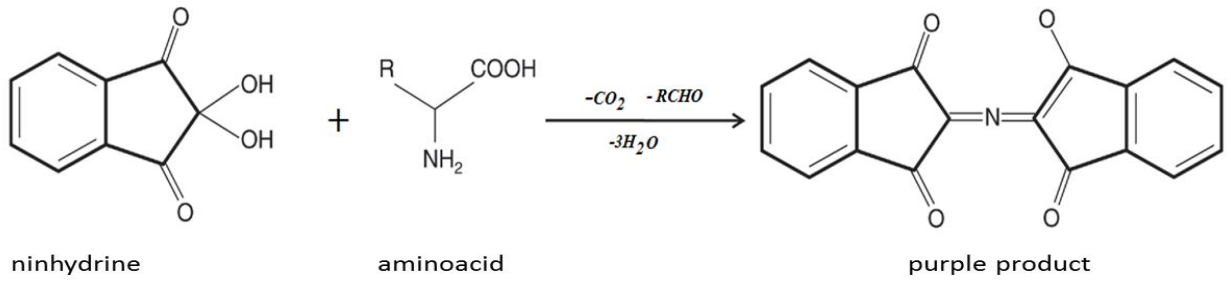
THIN – LAYER chromatography SEPARATION OF AMINO ACIDS

Name, group No: Date:

Principle

The principle of TLC (thin – layer chomatography) method is distribution of the components of the sample between the mobile and the solid phase (stationary) of the thin layer based on the different adsorption forces of the components of the sample. The stationary phase is formed by a thin layer of fine adsorbent which is either intersperse or, preferably, fixed on a base (aluminum foil, a glass plate). The mobile phase is formed by a mixture of organic solvents. The components of the sample are separated according to their different affinities to the adsorbent. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. E.g., if as the stationary phase the polar one is used and we separate two compounds that differ in polarity, the more polar compound has a stronger interaction with stationary phase and the less polar compound moves higher up the plate.

Detection of separated components is carried out by spraying an appropriate visualizing agent (ninhydrine), which gives a positive reaction with free alpha – amino acids. Each component is characterized by their retention factor.



ninhydrine aminoacid purple product

Reagents

solutions of amino acids: leucine, lysine, glycine, glutamate, their mixture and unknown samples of amino acids (w = 1%),
detection spray [solution of ninhydrine in acetone (w = 0,4)],
developing system [n-butanol – acetic acid - water (4:1:5)]

Accessories and equipment

silufol plate, chromatographic chamber

Procedure

Samples of the known amino acids (leucine, lysine, glycine, and glutamate), their mixture and samples of unknown amino acids are applied in the form of small spots (about 10-20 ml) on the thin layer silufol plate. The plate is dried out, placed and closed in a chamber saturated with vapors of a developing system. The separation proceeds until the front of the mobile phase reaches a distance of about 1-2 cm from the top of the plate. The plate is taken out, the front of

the mobile phase is marked by the pencil and the plate is dried out in an oven at 100 ° C. Detection of the samples is carried out by spraying with ninhydrine solution to form a purple complex which is detectable as color spots. Retrace the outline of the stains and mark the center of each spot.

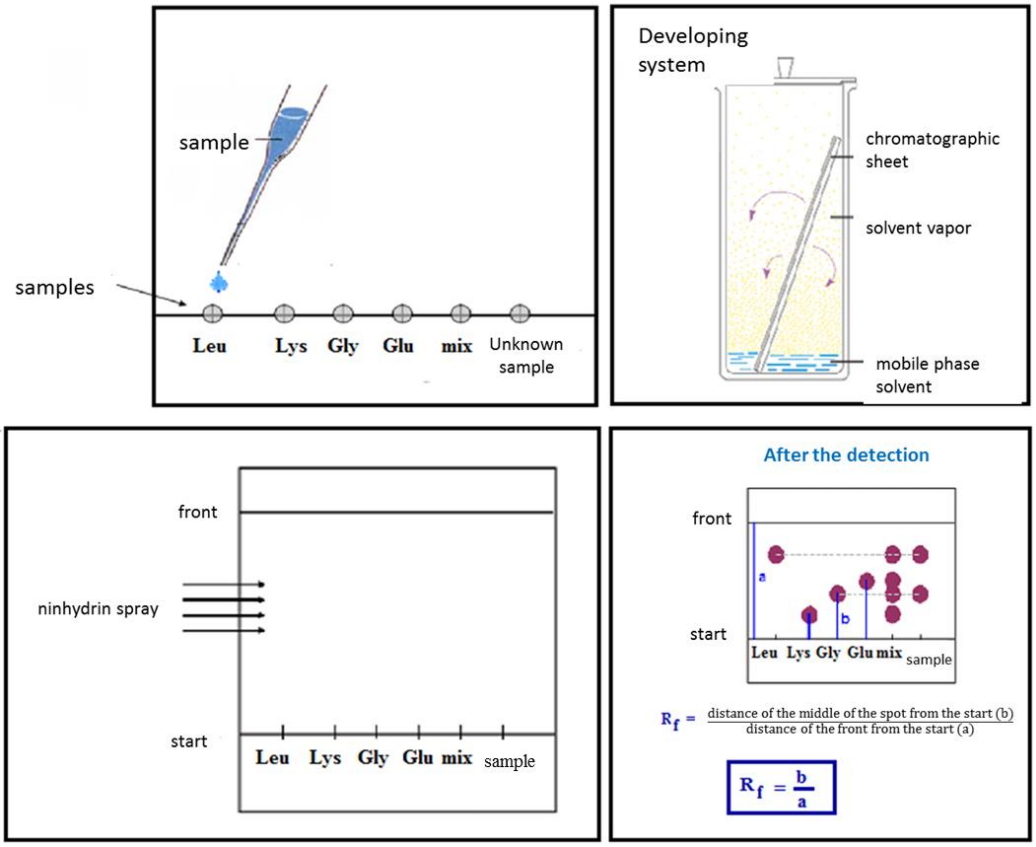


Figure: Illustration of thin layer chromatography procedure and evaluation

Evaluation

From the position of the center of the spot of amino acid calculate the value of retention factor R_f according to the formula

$$\mathbf{R_f} = \frac{\boldsymbol{b}}{\boldsymbol{a}}$$

where (b) is the distance of the center of the spot from the start (cm), and (a) is the distance of the front of solvent from the start (cm).
Write the values of retention factors to table:

Amino acid	R_f value	R_f Values			
		Sample No 1	Sample No 2	Sample No 3	Sample No 4
Leucine					
Lysine					
Glycine					
Glutamate					

The identity of R_f of known amino acids (standards) with R_f for the unknown samples determines the presence of the corresponding amino acids.

Calculations and conclusion

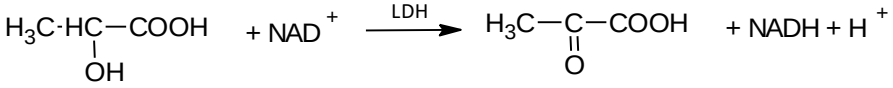
ENZYMES

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITY. DETERMINATION OF MICHAELIS CONSTANT (K_M) OF THE LACTATE DEHYDROGENASE (LDH)

Name, group No: Date:

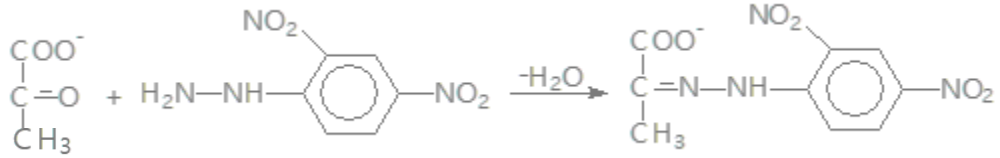
Principle

Lactate dehydrogenase is enzyme which catalyzes reaction of lactic acid oxidation utilizing NAD^+ as coenzyme:



lactic acid pyruvic acid

At different substrate (lactate) concentrations the enzymatic reaction proceeds at different rates. The rate is directly proportional to the amount of created product (pyruvate). In alkaline environment pyruvate gives with 2,4-dinitrophenylhydrazine (DNPH) a brown – orange pyruvate hydrazone, which is suitable for spectrophotometric determination.



From the values of measured absorbance at 505 nm the graphical dependence of enzymatic reaction rate on substrate concentration is constructed, and the value of Michaelis constant K_M is determined either approximately according to Michaelis and Menten, or exactly according to Lineweaver and Bürk.

Reagents

Solution of 2,4 - dinitrophenylhydrazine (DNPH) (w = 0.02 %), lactate solution (c = 3 mmol.l^{-1}), NaOH solution (c = 0.1 mol.l^{-1}), Tris-HCl buffer solution (c = 0.05 mol.l^{-1}) pH 8.5 –for dilution of lactate and for use in a reference solution.

Accessories and equipment

glass and automatic pipettes, spectrophotometer, water bath

Experimental procedure

Different concentrations of lactate are prepared as follows:

Test tube No.	**1**	**2**	**3**	**4**
Concentrated lactate c = 3 mmol/l (ml)	2	1.5	1	0.5
Buffer solution, pH 8.5 (ml)	-	0.5	1.0	1.5
Lactate concentration (mmol/l)	*3.0*	*2.2*	*1.5*	*0.75*

Enzymatic reaction at the different lactate concentration will be set according table:

Test tube No.	**1**	**2**	**3**	**4**	**5**
Lactate (ml) - different concentrations	0.4 *(3 mM)*	0.4 *(2.2 mM)*	0.4 *(1.5 mM)*	0.4 *(0.75mM)*	0.4* *(buffer)*
LDH (ml)in 30s intervals	0.2	0.2	0.2	0.2	0.2
Resulting substrate concentration	*2*	*1.5*	*1.0*	*0.5*	-
Incubate for 5 min at 37°C					
DNPH (ml) in 30s intervals	0.5	0.5	0.5	0.5	0.5
Stir and let stand for 10min at room temperature					
NaOH (ml)	5	5	5	5	5
Stir and let stand for 5 min at room temperature. Measure absorbance at 505 nm against the reference solution (tube No 5)					
A_{505}					-

*buffer solution, pH 8.5

Evaluation

Absorbance values are proportional to the amount of generated product of the enzymatic reaction –pyruvate; therefore reaction rate can be plotted directly as absorbance values. The results are recorded into the table:

Test tube No.	**1**	**2**	**3**	**4**
Resulting concentration [S] in the reaction mixture (mmol/l)	2	1.5	1.0	0.5
Reaction rate „v“ (A_{505})				
1/[S]	0.5	0.67	1.0	2.0
1/v (1/A)				

The tabulated values are used to create a plot for determination of K_M of LDH on a millimeter paper:

1. According to Michaelis-Menten for approximate K_M determination:

$$\mathbf{v = f\ ([S])}$$

2. According to Lineweaver-Bürk for precise determination of K_M:

$$\frac{\mathbf{1}}{\boldsymbol{v}} = \mathbf{f}\ \left(\frac{\mathbf{1}}{[\boldsymbol{S}]}\right)$$

From the values plotted in the value of K_M is calculated:

$$-\frac{\mathbf{1}}{\boldsymbol{K_M}} = -\boldsymbol{x} \quad \text{(mmol/l)}$$

$$\boldsymbol{K_M} = \frac{\mathbf{1}}{\boldsymbol{x}} \quad \text{(mmol/l)}$$

Conclusion:

Compare the K_M values obtained by both ways and explain, which one is more exact and why.