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CHAPTER II

LITERATURE REVIEW

Methomyl

Methomyl is a highly hazardous (class IB) carbamate insecticide which is used extensively in Thailand. It is often cited as an agent which may cause pesticide poisoning in Thailand (Swaddiwudhipong W. et al., 1988, 1989). Its mode of action is the inhibition of cholinesterase enzyme which is rapidly reversible. Previous studies showed that the cholinesterase inhibition level was often used as a marker of methomyl intoxication (Driskell, Groce, and Hill, 1991; Ekins and Geller, 1994; Gupta, Goak, and Kadel, 1994; Lifshitz et al., 1994; Miyazaki et al., 1989; Saiyed et al., 1992; Tanaka et al., 1987; Tsatsakis et al., 1996). In recent years, the possible involvement of some other enzymes in organisms exposed to methomyl were reported. The study of Saiyed et al. (1992) stated that the activity of total LDH significantly increased in spraymen coupled with electrocardiogram change after using methomyl for 5 days in the field. Another study of Gupta et al. (1994) also indicated that the total LDH activity and the activities of all LDH isoenzymes including LDH-4 were significantly elevated at various time intervals in the diaphragm and serum of the rats treated with a single dose of 5 mg/kg of methomyl.

Recently, Sinhaseni et al. (1995) conducted a monitoring survey of pesticide poisoning case reports in the tangerine orchards. Pesticide poisoning reported cases at Nongseu Hospital, Patumthani Province revealed that for the period January-March 1993, there were 18 illnesses due to pesticides. Thirteen out of pesticides involved with methomyl were recorded to be related to single use of 90% soluble powder formulation of methomyl (3 cases) and multiple uses of the same formulation of methomyl with other insecticides (10 cases) (Sinhaseni, 1994). From the unpublished further laboratory investigation (Personal communication, 1995), total LDH activity

and LDH-4 increases were found in two cases reported to be poisoned by methomyl. This is an interesting observation since such isoenzyme change is rare and has never been reported in any pesticide poisoning cases before.

1. Structure and properties

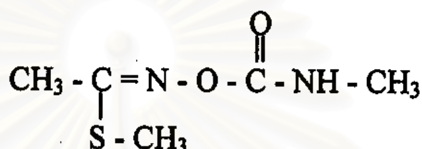


Figure 2. Chemical structure of methomyl.

Methomyl is a carbamate insecticide used on a wide range of crops worldwide. It is listed in Class IB (highly hazardous) in the WHO Recommended Classification of Pesticides by Hazard and Guideline to Classification (1994-1995) on the basis of its rat acute oral LD_{50} value of 17 mg/kg body weight (WHO, 1994).

The main formulations are water-soluble powder (25-90% methomyl) and water-miscible liquid (12.5-29% methomyl). These products are diluted with water and applied by ground or aerial spray equipment. The major sources of human exposure are during the preparation and application of these products. Typical active ingredient rates are 0.15-1.0 kg/ha or up to 2 kg/ha for some purposes (International Programme on Chemical Safety [IPCS], 1996).

Table 2. Physical and chemical properties of methomyl.

Methomyl (S-methyl-N-[(methyl-carbamoyl)oxy]thio-acetimidate; $C_5H_{10}N_2O_2S$ MW 162.2)	
Physical state	crystalline solid
Color	white
Odor	slightly sulfurous
Melting point	77°C
Vapor pressure	0.72 mPa (at 25°C)
Octanol-water partition coefficient (K_{ow})	1.24
Water solubility	54.7 g/liter
Stability	Stable at temperature up to 140°C, sunlight and stable in sterile water at pH7 (at 25°C), but unstable at higher pH and temperature
Half-life	30 days at pH9 and 25°C

2. Kinetics and metabolism in laboratory animals

The absorption, metabolism and excretion of methomyl after oral administration to rats are very rapid. The methomyl are undetectable in the tissues or excretory products within a few hours of dosing. The highest concentration of methomyl was found in blood (representing about 2% of the original dose) and 0.4%, 0.6% and < 0.1% were found in liver, gastrointestinal tract and other individual tissue respectively (Hawkins et al., 1991). Most of the dose (about 80%) is metabolized and eliminated within 24 hours with an estimated half-life of 5 hours (Harvey, Jelinek, and Sherman, 1973; Hawkins et al., 1991). There are three proposed metabolic pathways in mammals (as shown in figure 3). Its major metabolites, in urine (the mercapturic acid derivative) and in expired air (CO_2 and acetonitrile), are also eliminated rapidly (Harvey et al., 1973; Hawkins et al., 1991; Hawkins et al., 1992; Huhtanen and Dorough, 1976). There is no evidence indicating the accumulation in tissues.

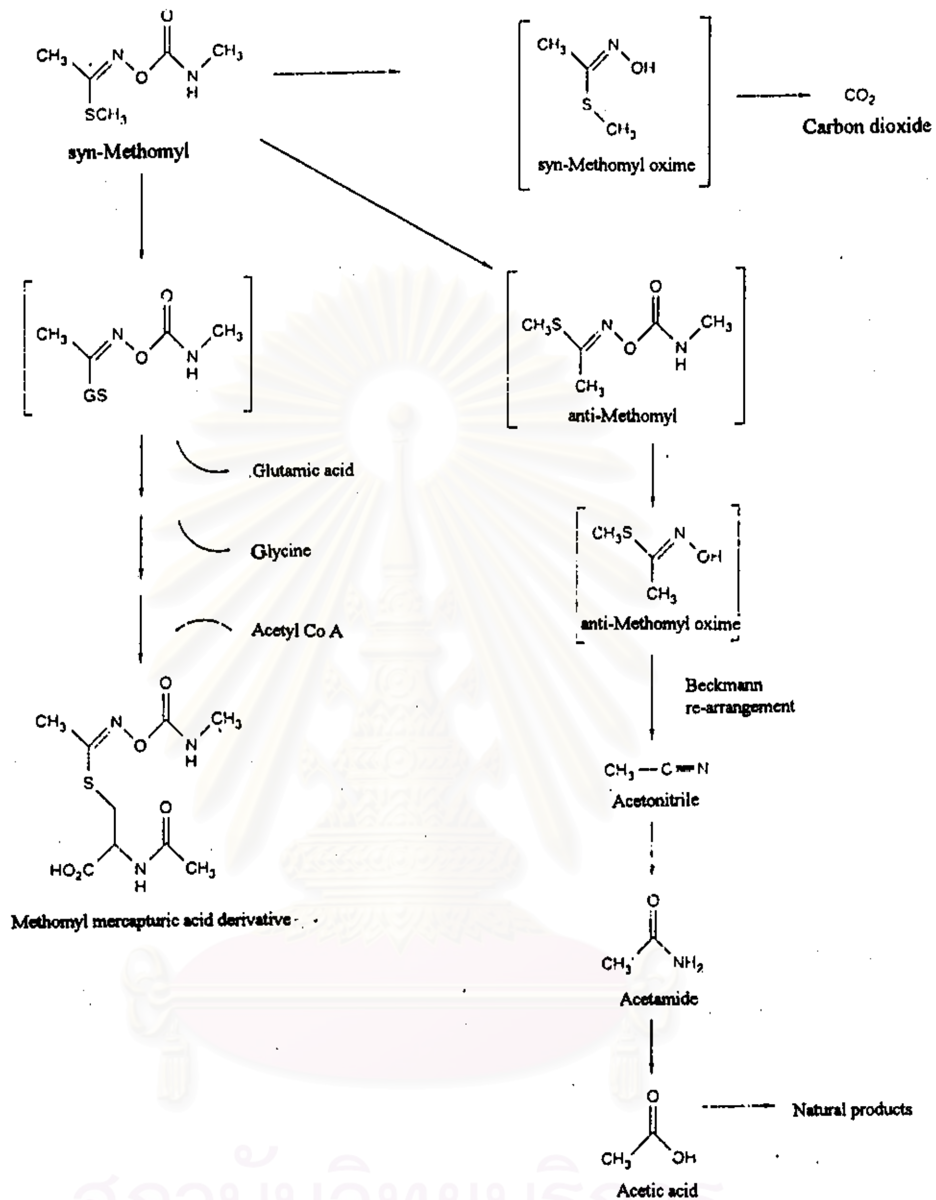


Figure 3. The proposed metabolic pathways of methomyl in mammals (IPCS, 1996).

1) Displacement of the S-methyl moiety by glutathione and enzymatic transformation to give the mercapturic acid derivative.

2) Hydrolysis of methomyl to give S-methyl-N-hydroxythioacetimidate (MHTA) which is rapidly broken down to carbon dioxide.

3) Conversion of the syn-isomer of methomyl (the insecticidal form) to its anti-isomer which then undergoes a hydrolysis, rearrangement and elimination reaction to form acetonitrile. Acetonitrile may undergo further reactions to give the products incorporated into natural body constituents such as fatty acids, neutral lipids and glycerol.

3. Mechanism of toxicity

Methomyl is a carbamate insecticide with a well-known mechanism of action via inhibition of the enzyme acetylcholinesterase. The acute toxic action of methomyl is characterized by typical signs of anticholinesterase action such as lacrimation, profused salivation, tremor and pupil constriction. Previous studies with methomyl showed that the onset of toxic action is rapid. The toxic effect is rapidly reversible because of the fast spontaneous reactivation of the carbamylated enzyme, which is facilitated by the rapid clearance of the compound from the body. Surviving animals quickly show signs of recovery, often within hours. Data from accidental and suicidal human poisoning cases revealed that the characteristic and level of acute methomyl toxicity in humans were similar to that found in laboratory animals (Driskell et al., 1991; Ekins and Geller, 1994; Gupta et al., 1994; Lifshitz et al., 1994; Miyazaki et al., 1989; Tanaka et al., 1987; Tsatsakis et al., 1996).

Therefore, when measuring the cholinesterase activities during toxicity studies or diagnosis, it is crucial to analyze samples in the minimum possible time after sampling and to use an appropriate assay methods which requires the minimum of sample dilution and shortest assay time. Failure to allow for the rapid reversibility of the action can lead to underestimation of enzyme inhibition or misinterpretation.

4. Toxicological Studies

According to the acute toxicological studies, methomyl is very toxic by the oral and inhalation routes. However, its acute dermal toxicity is low (table 3). This may be due to its rapid degradation during penetration through skin. The toxic actions are the typical signs of cholinesterase inhibition as mentioned above. At lethal doses the animals died within hours. The reversibility of toxic signs is rapid. The survivors recover from the effects within several hours and fully recover within days as well as the reversal of blood and brain cholinesterase inhibition. In histopathological examination, there was no compound-related change found in subjected organs.

Table 3. Acute toxicity of technical grade methomyl in laboratory animals (Morrow, 1972; Panepinto, 1991; Sarver, 1991a; Sarver, 1991b; Sherman, 1966; Sherman, 1968; Trivits, 1979).

Oral LD_{50} (rat)	17-45 mg/kg body weight
4-h Inhalation LC_{50} (rat)	0.26 mg/liter (in aerosol form)
Dermal LD_{50} (rabbit)	>2000 mg/kg body weight (intact skin)
	>1000 mg/kg body weight (abraded skin)

According to short-term and long-term studies, acute toxic signs and blood cholinesterase inhibition were rarely seen in dietary studies. Most of adverse effects seen at the higher dietary levels were the reduction of body weight gain in rodents and decreased red blood cell indices in rodents and dogs. In older studies, the compound-related histopathological changes were reported, for example, moderate erythroid hyperplasia in the bone marrow of male rats fed diets containing 250 mg/kg methomyl for 13 weeks. (Busey, 1966; Paynter, 1966), the increase in the incidence and severity of extra medullary haematopoiesis in the spleen and the vacuolization of epithelial cells and hypertrophy of the proximal convoluted tubules of rats fed 400 mg/kg methomyl in diet for 12 months (Kaplan and Sherman, 1977).

There was no evidence of treatment-related increases in tumor incidences in 2-year studies on rats and mice, indicating that methomyl is not likely to be carcinogenic in man (Kaplan, 1981; Kaplan and Sherman, 1977; Quarles et al., 1979; Serota et al., 1981).

Methomyl did not produce embryotoxic or teratogenic effects in rats or rabbits. It did not affect fertility, gestation or lactation indices. It was not genotoxic and did not show delayed neurotoxicity after single or repeated administration (Christian, Hoberman, and Fuessner, 1983; Rogers et al., 1978). However, the recent study of Amer, Fahmy, and Donya (1996) showed that methomyl caused chromosomal aberrations in mouse spleen cells within 24 hours after an intraperitoneal injection of 1 mg/kg of methomyl.

Lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase; E.C.1.1.1.27.)

Total LDH activity and alteration of LDH isoenzymes pattern are routinely used as a diagnostic tool in the hospital. The total LDH activity remarkably elevates in many diseases such as acute myocardial infarction, blood disorders, acute renal disease, pulmonary infarction, malignancies and skeletal muscle disorders. Total LDH activity is nonspecific to damaged tissues, whereas the determination of LDH isoenzymes lead to more specific clue to the specific organ injured. (Calbreath, 1992; Moss and Henderson, 1994; Rider and Taylor, 1980; Zimmerman and Henry, 1974). The most important use of LDH isoenzymes is in the diagnosis of myocardial damage which has been validated in 2,029 patients (Kairisto et al., 1994). Other previous studies have indicated favorably results (Bhayana and Henderson, 1995; Lee and Goldman, 1986; Levinson and Hobbs, 1994).

1. Roles in carbohydrate metabolism

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the reversible reaction between lactate and pyruvate, using NAD^+/NADH as coenzyme (figure 4).

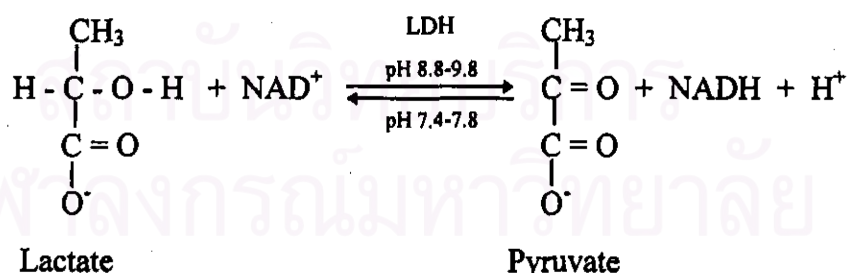


Figure 4. The reversible reaction between lactate and pyruvate catalyzed by LDH (Moss and Henderson, 1994).

In living organisms, LDH plays important roles in the Embden-Meyerhof metabolic pathway of glycolysis in carbohydrate metabolism (figure 5). It maintains balance between the anabolism and catabolism of carbohydrate as follows (Everse and Kaplan, 1973):

a) Anaerobic condition (pyruvate → lactate)

- LDH promotes the breakdown of glucose to lactate.
- LDH facilitates the production of ATP.
- LDH promotes gluconeogenesis and/or glycogen synthesis from lactate.

b) Aerobic condition (lactate → pyruvate)

- LDH enables lactate to enter Kreb's cycle and be used as a fuel for ATP and NADH generation.

2. Total lactate dehydrogenase

2.1 Tissue distribution

LDH is present in virtually all cells of the body and is invariably found only in the cytosol. Normally, cell membranes are impermeable to any enzymes and hence enzyme activities in the serum are very low compared with those in cells. If the cell activity is impaired or destroyed as a result of any causes such as infections, chemicals, etc., the enzymes will then leak into the blood circulation. Since the LDH concentrations in tissues are some 500-fold greater than those found in serum under normal circumstance, even small amount of tissue damage can significantly increase the observed serum level of LDH. Nevertheless, there are some pathological conditions which may cause a decrease in the serum enzyme level. The proposed mechanism for abnormal serum enzyme levels are summarized in table 4 including abnormal synthesis or release, impaired excretion, increased tissue sources of enzyme, enzyme inhibition, and lack of cofactors.

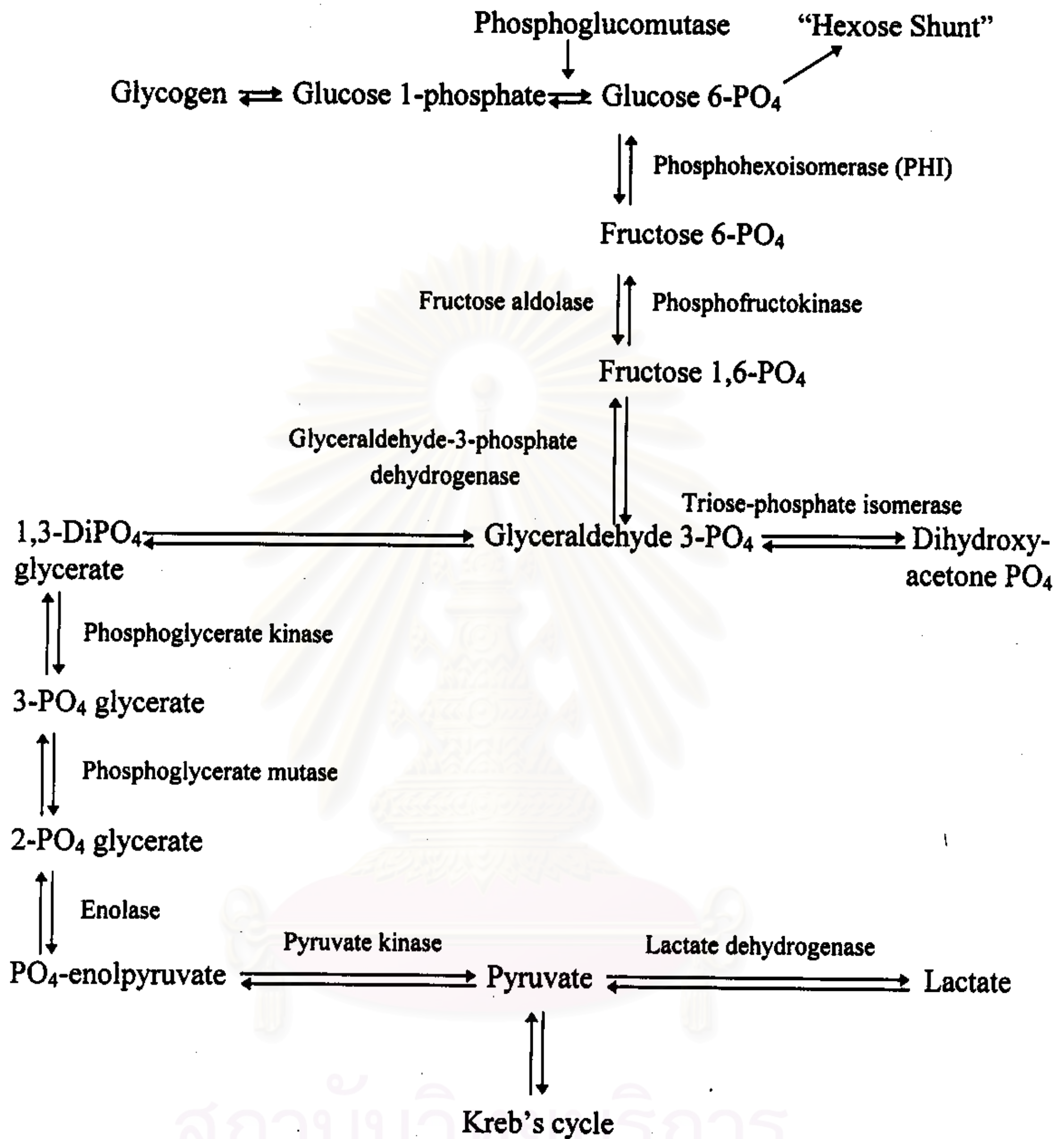


Figure 5. Schematic diagram of glycolytic pathway of carbohydrate metabolism (Zimmerman and Henry, 1974).

Table 4. Proposed mechanism for abnormal serum enzyme levels (adapted from Moss and Henderson, 1994).

Mechanism	Example	Examples of enzymes
I. Increased serum levels		
A. Increase release		
1. Necrosis	Myocardial infarction	LDH, CPK, GOT
	Acute hepatitis	LDH, GOT, GPT, ALP, aldolase
	Acute pancreatitis	Amylase, lipase, trypsin
2. Increased permeability of cell membranes without necrosis	Progressive muscular dystrophy, delirum tremens, dermatomyositis	CPK, aldolase, LDH, GOT, GPT
B. Increased tissue source of enzymes or increased release from tissue or both	Neoplastic disease, granulocytic leukemia	LDH, aldolase, glucuronidase
	Megaloblastic anemia	LDH, aldolase
	Osteoblastic lesions	ALP, ATPase
	Peptic ulcer	Pepsinogen
C. Impaired excretion of enzyme	Uremia	Amylase
II. Decreased serum levels		
A. Decreased formation		
1. Genetic	Hypophosphatasia	ALP
	Acholinesterasemia	Pseudocholinesterase
2. Acquired	Hepatitis	Pseudocholinesterase
	Starvation	Amylase
B. Enzyme inhibition	Insecticide poisoning	Pseudocholinesterase
C. Lack of cofactors	Pregnancy, Cirrhosis	GOT

2.2 Clinical significance of total LDH

In the hospital, total LDH is routinely used as a diagnostic tool. It is remarkably elevated in many diseases, especially in acute myocardial infarction and blood disorders. In other diseases such as acute renal disease, pulmonary infarction, malignancies and skeletal muscle disorders, the total LDH increase moderately (table 5). Unfortunately, the increase in serum concentration of total LDH can result from release of the enzyme from a variety of possible tissue sources. Such tissue nonspecificity of total LDH activity limits the usefulness of determination in diagnosis. However, the definitive testing for the tissue origin of elevated LDH activity may be accomplished with isoenzyme assessment (Calbreath, 1992; Moss and Henderson, 1994; Rider and Taylor, 1980; Zimmerman and Henry, 1974).

3. Lactate dehydrogenase isoenzymes

3.1 Classification of LDH isoenzymes and their tissue distributions

In general, LDH in most tissues and serum can be separated into five isoenzymes by the electrophoretic technique. Each isoenzyme is designated by a number according to its electrophoretic mobility. The fraction with the greatest mobility (anodic) is called LDH-1, the one with the least anodic mobility is called LDH-5, and the other three are designated as LDH-2, LDH-3, and LDH-4, respectively (figure 6).

The five isoenzymes have the same molecular weight of 135,000 Da, but differ in their kinetic properties (V_{max} and K_m). The LDH molecule consists of four polypeptide chains (or subunits). There are two types of these subunits, designated H (heart) and M (muscle). These tetramers are the result of a random combinations of monomers H and M (table 6).

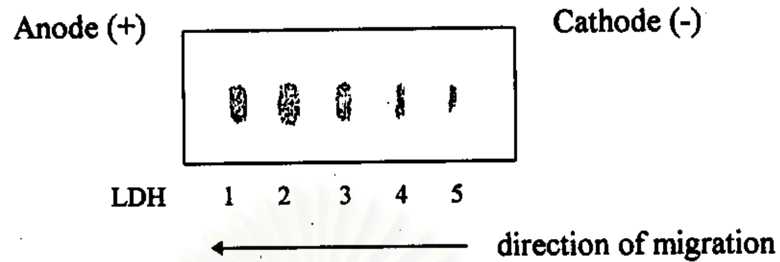
Table 5. Conditions affecting the total LDH activity in human serum (adapted from Calbreath, 1992; Everse and Kaplan, 1973; Moss and Henderson, 1994; Walmsley and White, 1994; Zimmerman and Henry, 1974).

Marked elevation (5 or more times normal)	
Megaloblastic anemia	Hepatitis
Systemic shock and hypoxia	Renal infarction
Widespread carcinomatosis, especially hepatic metastases	
Moderate elevation (3 to 5 times normal)	
Myocardial infarction	Pulmonary infarction
Hemolytic conditions	Leukemias
Infectious mononucleosis	Delirium tremens
Muscular dystrophy	
Slight elevation (up to 3 times normal)	
Most liver diseases	Nephrotic syndrome
Hypothyroidism	Cholangitis

Table 6. Nomenclature, composition, and tissue source of LDH isoenzymes found in human serum by electrophoretic techniques (adapted from Anderson and Cockayne, 1993; Wilkinson, 1965; Zimmerman and Henry, 1974).

Nomenclature of isoenzymes	Composition proportion of monomers in each isoenzyme	Tissues rich in the isoenzymes
LDH-1	HHHH	Heart, brain, erythrocytes
LDH-2	HHHM	Heart, brain, erythrocytes
LDH-3	HHMM	Brain, kidney
LDH-4	HMMM	Liver, skeletal muscle, kidney
LDH-5	MMMM	Liver, skeletal muscle, ileum
LDH-X	XXXX	Post-pubertal testis, spermatozoa

A.



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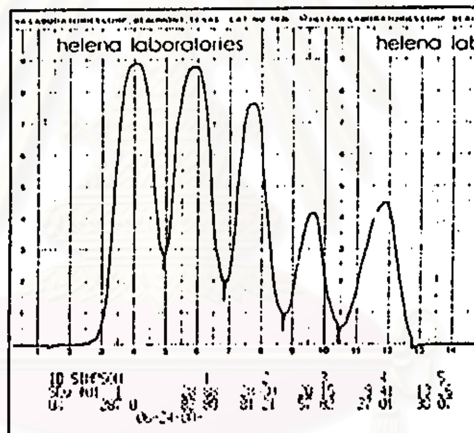

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Figure 6. A. The five LDH isoenzymes separated by electrophoretic technique
B. The LDH isoenzymes pattern in the normal serum of human (Helena Laboratories, 1995).

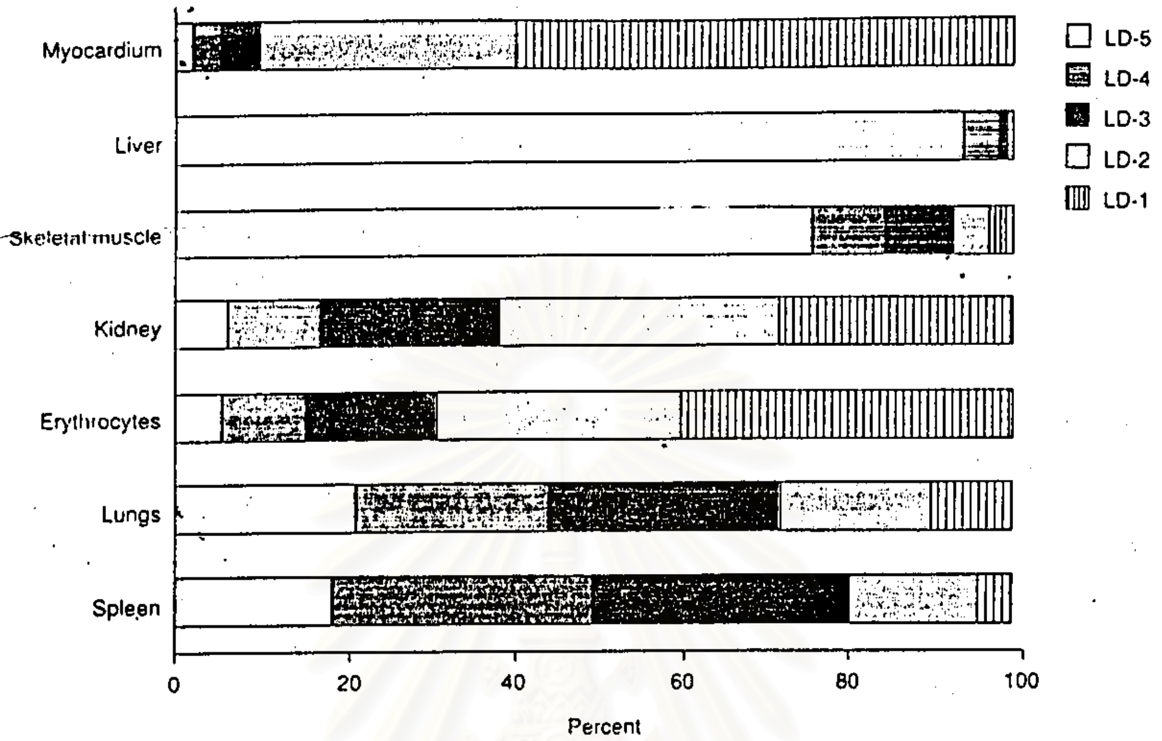

Figure 7. The distribution of LDH isoenzymes in various tissues of human. (Anderson and Cockayne, 1993).

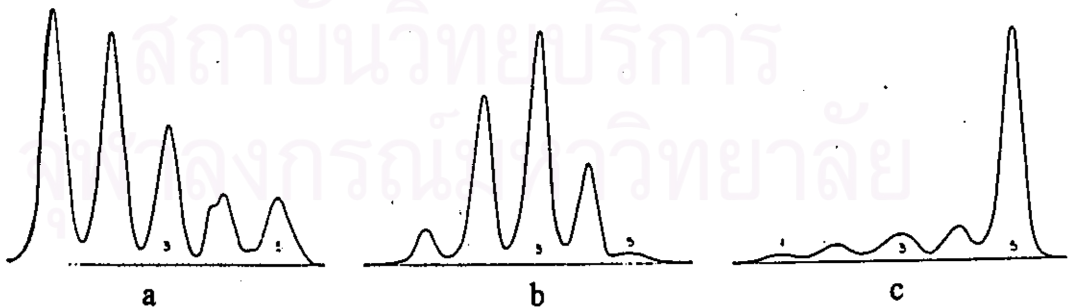

Figure 8. Tissue LDH isoenzyme patterns obtained with a thin-layer agarose gel electrophoretic system. Pattern a, myocardial tissue; pattern b, platelets; pattern c, liver tissue (Moss and Henderson, 1994).

In different tissues and different species, LDH show different isoenzyme compositions, but are very similar in their structures and activities (figure 7 and 8). In human, the cardiac muscle, kidneys, and erythrocytes consist mainly of the faster-moving isoenzyme LDH-1 and LDH-2, whereas in liver and skeletal muscle, the more cathodal LDH-4 and LDH-5 isoenzyme predominate. Isoenzymes of intermediate mobility account for the LDH activity of many tissues, for instance, endocrine gland, spleen, lungs, lymph nodes, platelets, and nongravid uterine muscle. Rarely, another type of LDH isoenzyme is found between LDH-3 and LDH-4, namely LDH-X, consisting of four identical "X" subunits (table 6). It normally occurs in post-pubertal testis and spermatozoa and appears to be related to spermatogenesis (Anderson and Cockayne, 1993; Egbunike et al., 1986; Orlando et al., 1988; Skude, Eyben, and Kristiansen, 1984; Wilkinson, 1965).

3.2 Clinical significance of LDH isoenzymes

The combination of total LDH determination and isoenzymes pattern in the serum is extremely useful in the diagnosis of specific tissue damage, particularly in heart, skeletal muscle and liver disorders in humans (Table 7 and figure 9). There are many reports in the literature concerning the usage of total LDH and LDH isoenzymes as a marker in many diseases such as various forms of malignancies, but they must have further studies for conclusion (Castaldo et al., 1991).

The validated use of LDH isoenzymes is in the diagnosis of myocardial damage that was validated in previous studies (Bhayana and Henderson, 1995; Lee and Goldman, 1986; Kairisto et al., 1994; Levinson and Hobbs, 1994). Following acute myocardial infarction, a rise in total LDH occurs between 8 and 12 hours after the onset of pain. Peak activity usually reaches between 18 and 24 hours and the activity returns to normal by the fifth to sixth days, but may remain elevated for a week to 10 days. The greater diagnostic specificity of LDH measurement can be obtained by quantitating the LDH-1 fraction or by performing LDH isoenzyme

electrophoresis and examining the LDH-1:LDH-2 ratio. In normal human serum, LDH-2 is found in the highest concentration and the ratio of LDH-1:LDH-2 is therefore less than one. After myocardial infarction, there is substantial increase in LDH-1 so that the LDH-1:LDH-2 ratio is greater than one, called "flipped pattern" (Figure 9). Within the first 48 hours after myocardial infarction, approximately 80% of patients show the reversal of LDH-1:LDH-2 ratio which correlate well with other indicators, with 94% specificity to this disease. If the flipped pattern is seen in conjunction with a significant amount of CK-MB, there is more than 95% probability that the patient has experienced a myocardial infarct. However, the reversal is short-lived (2 or 3 days) and can occur in hemolysis or kidney infarction, therefore these diagnostic possibilities must be ruled out before the reversal is attributed to myocardial infarction. (Anderson and Cockayne, 1993; Calbreath, 1992; Moss and Henderson, 1994).

Although the LDH isoenzymes alteration is not sensitive enough to indicate a reinfarction shortly after the initial event, but it may be suitable for patients reporting several days after the onset of symptoms since the time course of elevation and decrease of the serum LDH isoenzyme appears to be slower than other enzymes and isoenzymes (figure 10) (Collinson et al., 1988; Galbraith et al., 1990; Moss and Henderson, 1994).

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Table 7. Relative degree of increase of total LDH and abnormality of isoenzyme pattern in certain disease states (adapted from Anderson and Cockayne, 1993; Zimmerman and Henry, 1974).

Disease	Relative degree of increase of total LDH activity	Isoenzyme fraction most abnormal				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Myocardial infarction	++	x	x			
Pulmonary infarction*	+				x	x
Congestive heart failure	+				x	x
Viral hepatitis	+				x	x
Toxic hepatitis	+				x	x
Cirrhosis	+				x	x
Leukemia, granulocytic	++		x	x		
Pancreatitis	+		x	x		
Carcinomatosis (extensive)	+++		x	x		
Megaloblastic anemia	++++	x	x			
Hemolytic anemia	+	x	x			
Muscular dystrophy**	+	x	x			

* In pulmonary infarction, LDH-3 may be elevated.
** In muscular dystrophy, LDH-1 and LDH-2 are elevated only in a relative sense because LDH-4 and LDH-5 are depressed.

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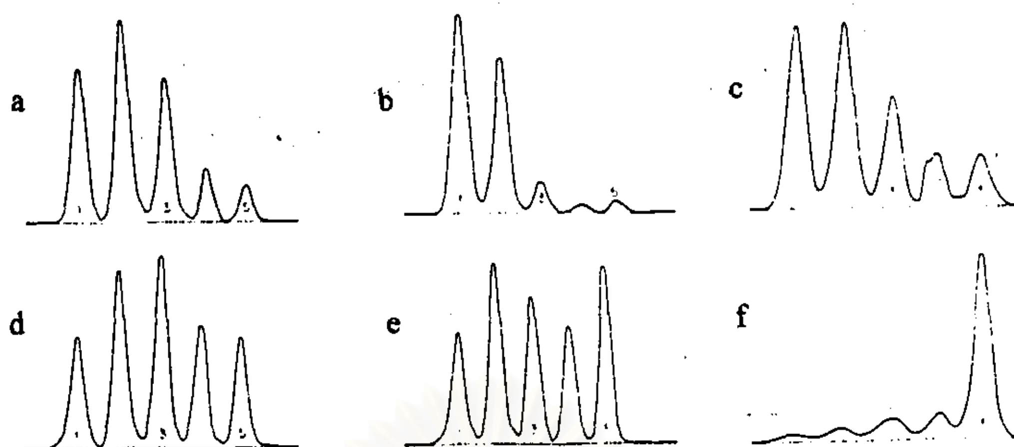

Figure 9. Serum LDH isoenzyme patterns obtained with a thin-layer agarose gel electrophoretic system (Moss and Henderson, 1994).

Pattern a, normal serum. Pattern b, acute myocardial infarction with flipped LDH-1. Pattern c, acute myocardial infarction without flipped LDH-1. Pattern d, infectious mononucleosis (involving platelets or lymphatic tissue). Pattern e, congestive cardiac failure showing elevated LDH-5 as a result of hepatic anoxia. Pattern f, acute circulatory shock showing the result of very severe hepatic anoxia.

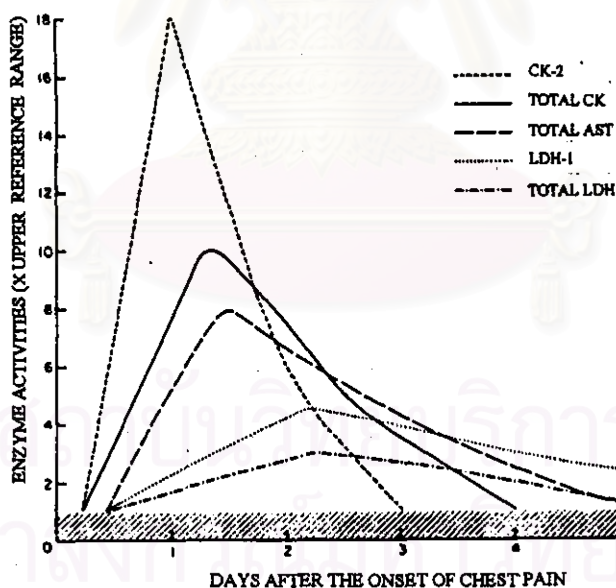

Figure 10. Typical pattern of changes in serum enzyme activities following an uncomplicated myocardial infarction. These patterns and the magnitude of the peaks can vary quite markedly among patients, depending on the location and size of the infarct and the enzyme assay methodology in use. The *hatched area* indicates the reference interval of all enzymes (Moss and Henderson, 1994).

3.3 LDH isoenzymes in rats and other species

As mentioned above, the pattern of LDH isoenzymes are different from tissues to tissues within an organism, and they are also varied from species to species (figure 11 and table 8, 9). Although LDH isoenzymes are well-understood and are widely used as diagnostic tool in human disease, there are few reports concerning LDH isoenzymes in the serum (or plasma) and tissues of different animal species (Preus, Karsten, and Bhargava, 1989; Wilkinson, 1965; Yasuda et al., 1990). Moreover, the methods employed differ regarding the carrier used for electrophoretic separation, or the substrate used for staining, resulting in different detection limits for these isoenzymes.

LDH isoenzymes have been occasionally used as tools for indicating the target organs of toxicity in laboratory animals also, for example, serum LDH-1 and LDH-2 increased in mercury-exposed rats indicating the kidney toxicity (Ringoir, 1970), and serum LDH-1 and LDH-2 increased in the case of cardiac damage after isoproterenol application in rats (Preus et al., 1988).

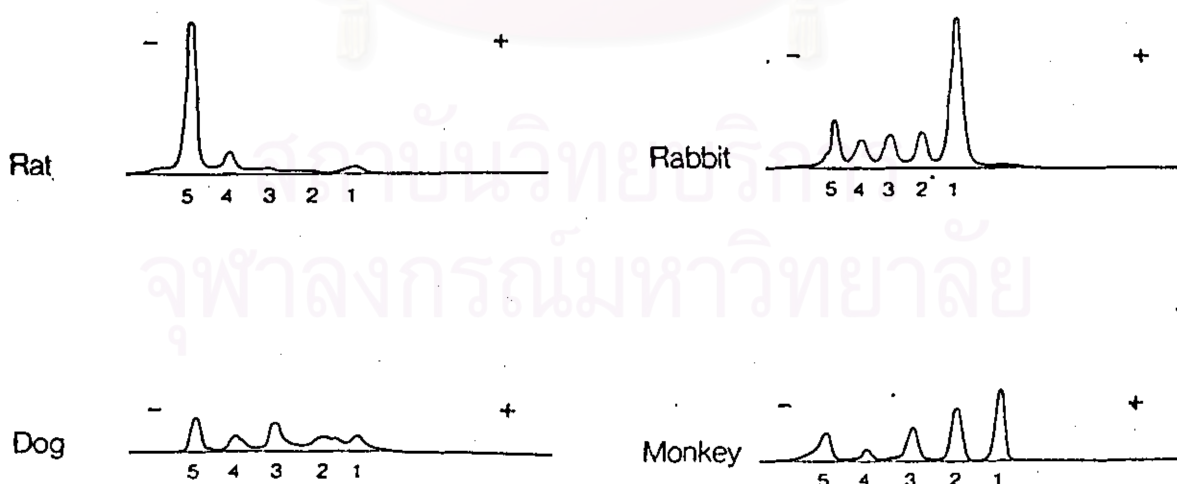

Figure 11. Typical LDH isoenzyme pattern in the serum of various normal animal species (Preus et al., 1989).

Table 8. Total LDH and LDH isoenzyme activities in the serum of various normal animal species (mean ± SD, range; n = 6) (adapted from Preus et al., 1989).

Animal species	Total LDH (U/l)	Relative distribution of LDH isoenzymes (%)				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Rat	239 ± 132 (128-467)	6 ± 2 (3-8)	3 ± 1 (1-5)	4 ± 2 (2-6)	10 ± 4 (3-13)	78 ± 8 (71-89)
Rabbit	231 ± 76 (162-361)	50 ± 9 (37-60)	13 ± 2 (9-15)	13 ± 2 (11-16)	10 ± 3 (6-13)	15 ± 6 (8-24)
Dog	54 ± 22 (38-98)	11 ± 3 (6-16)	23 ± 4 (17-28)	30 ± 4 (26-36)	14 ± 2 (13-18)	22 ± 3 (17-26)

Table 9. Tissue distribution of LDH isoenzymes in rats (adapted from Wilkinson, 1965; Yasuda et al., 1990).

Tissues	Relative distribution of LDH isoenzymes (%)				
	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Heart	50	30	12	6	2
Brain	40	20	15	20	5
Kidney	20	15	10	20	35
Spleen	1	5	9	17	68
Erythrocyte	0.2	< 0.1	< 0.1	<0.1	99
Skeletal muscle	1	1	3	5	90
Liver	1	1	3	5-10	85-90

4. Sample collection

Serum is the specimen of choice. Plasma from blood samples collected in heparin or EDTA may be used, but some anticoagulants such as oxalate can inhibit the enzyme. Hemolysis must be avoided and the serum or plasma should be promptly separated to avoid releasing LDH-1 from the human erythrocytes or LDH-5 from the rat erythrocytes.

Ideally, the enzyme is relatively stable at room temperature (25°C). However, the serum (or plasma) should be analyzed as soon as possible after collection or within 24 hours because different storage condition have varying affects on the isoenzymes. If the samples must be kept for a longer period of time, refrigeration at 4°C is preferable.

In the case of myocardial infarction, a minimum of three specimens should be collected. The first sample should be collected immediately upon admission of the patient to the hospital. The second specimen should be collected 6-13 hours later, and the third one collected 24-37 hours after admission (Calbreath, 1992; Helena Laboratories, 1995; Moss and Henderson, 1994; Wilkinson, 1965).

Certain chemicals can cause reduction of LDH activity such as ascorbic acid, urea, benzene, clofibrate and fluoride. On the other hand, anabolic steroids, certain anesthetic agents, aspirin, carbenicillin, clindamycin, codeine, dicumarol, ethanol, levodopa, morphine, nitrofurantoin can produce the elevation of LDH activity (Siest and Galteau, 1988).

5. Measurement of total LDH activity and LDH isoenzymes (Anderson and Cockayne, 1993; Calbreath, 1992; Helena, 1995; Moss and Henderson, 1994).

5.1 Total LDH activity

The major assay approaches for the determination of LDH in serum or other body fluids are based on the detection of $NADH/NAD^+$ in the reaction at 340 nm by spectrophotometer. The reaction can proceed either in the forward (lactate → pyruvate) or reverse (pyruvate → lactate) direction, at pH 8.3-8.9 and pH 7.1-7.4, respectively. Another approach applies *p*-nitrophenylhydrazine to form a colored complex with pyruvate, but this method is imprecise and is not employed in many laboratories.

1) The Wacker method. This method utilizes the forward reaction with formation of NADH from NAD^+. The NADH concentration can be detected directly by spectrophotometer at 340 nm or indirectly by colorimetric method. A mixture of phenazine methosulfate and a tetrazolium salt reacts with the NADH to produce a blue purple color. The absorbance of this product is proportional to the amount of NADH present. This reaction is widely used for the detection of LDH isoenzymes fractionated by electrophoresis. This method has following advantages: substrate inhibition by lactate is less than that produced by pyruvate and the reaction has a more prolonged reaction linearity after a 20-second incubation time.

2) The Wroblewski-LaDue method. This method employs the reverse reaction, so NADH is consumed during the course of the reaction. If kinetic measurement of activity is carried out, a decrease in 340-nm absorbance with time is observed. Although the reverse reaction is up to three times faster than the forward reaction which permits smaller sample volumes and shorter reaction times, but the initial high NADH concentration can cause some difficulties in accurate measurement, especially the presence of inhibitors in the NADH preparations. However, This method has many advantages, for example, it uses less expensive assay formulation

because of the much lower concentration of reactants and has greater change in absorbance with time that allows more precise measurements including the greater stability of the prepared reagents.

Both methods must be run over short periods due to the presence of product inhibition. This is not a significant factor if kinetic assays are performed, since the rate curve can be monitored. The reaction rate is temperature dependent, therefore the tight control of temperature is very important in achieving accurate assays. The selection of buffer type and pH requires some compromises. Since there are five isoenzymes of LDH, there is some variations in activity for a given serum sample, which depends on the isoenzyme concentrations.

5.2 LDH isoenzymes

1) Electrophoresis. The most commonly used method for analysis of LDH isoenzymes is electrophoris performed on agarose gel, acrylamide gel or cellulose acetate. After separation, a reaction mixture containing buffer, lactate, and NAD^+ is applied to the gel, which is then incubated at either 30°C or 37°C for a period (30 minutes or more). The relative amount of each LDH isoenzyme can be determined by densitometry scanning of the gels and detecting the amount of fluorescence generated by NADH. Additionally, the colorimetric reaction may also be used by adding a with a tetrazolium salt and phenazine methosulfate to the substrate mixture and then a colored formazan is formed in any area of the gel where LDH activity is found. The relative percentages of the LDH isoenzyme fractions are determined (and reported along with the total activity of the sample).

The use of electrophoresis allows identification and quantitation of each of the five fractions, but it is expensive, time-consuming (a single run may require 2 hours or more) and is rather complex, depending on the apparatus and materials employed. Nevertheless, electrophoresis provides more useful information than any of the other methods for separation and quantitation of LDH isoenzymes.

2) Ion-exchange chromatography. Separation systems have been developed which use a minicolumn to isolate LDH-1 and LDH-2. A sample is applied to a diethylaminoethyl (DEAE) type of ion-exchange column, which has antibody to the M subunit bound to it. By use of a series of three buffer solution, the undesired fractions (LDH-3, LDH-4, and LDH-5) are first eluted, followed by LDH-2 and finally LDH-1. After removal, the fractions are then quantitated by a standard LDH method.

This method is considered a more sensitive and more accurate method for determination of early elevation in LDH-1 and quantitating LDH-1:LDH-2 ratio after an acute myocardial infarction (96% sensitive and 97% specific to this disease when a decision threshold of 0.76) (Moss and Henderson, 1994). However, it has certain significant shortcomings such as the large sample volume required (often as much as 0.5 mL) and the loss of much useful information regarding damage to other tissues, particularly lung and liver, by using only the first two LDH isoenzyme fractions.

3) Immunoprecipitation. Antibody to the M subunit of LDH is added to a serum sample and then any isoenzymes in the sample containing an M-subunit bind to it. Therefore, only LDH-1 show enzyme activity when the treated sample is assayed for total LDH activity. By comparing the total activity which and without antibody present in the sample, the relative percentage of LDH-1 can easily be determined.

This method is reasonably quick and has high diagnostic specificity (94%) of myocardial infarction, so it may be substituted for electrophoresis or chromatography in suspicious cases. However, it cannot ruled out the other conditions that cause LDH-1 elevation.

6. Reference ranges

Normal LDH and LDH isoenzymes values depend on the assay technique employed.

6.1 Total LDH activity

For the reverse reaction method, the reference ranges of total LDH activity in the normal human serum are followed:

95-200 U/L	at 30°C	(Calbreath, 1992)
90-320 U/L	at 37°C	(Anderson and Cockayne, 1993)
200-380 U/L	at 37°C	(Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974)

If the assay is run in the forward direction, the reference range of total LDH activity is lower as follows:

35-90 U/L	at 30°C	(Calbreath, 1992)
100-225 U/L	at 37°C	(Anderson and Cockayne, 1993)

There is apparently no difference in the normal values for this enzyme between men and women and no significant age-related differences.

6.2 LDH isoenzymes

Reference ranges of LDH isoenzymes are studied as shown in table 10. These ranges are also due to the carrier used for electrophoretic separation, buffer, the substrate used for staining and other conditions.

Table 10. Reference ranges of LDH isoenzymes in the human serum.

Isoenzyme	cellulose acetate		agarose gel	
	(Helena Laboratories, 1995)	(McKenzie and Henderson, 1983)	(Calbreath, 1992)	(McKenzie and Henderson, 1983)
LDH-1	24.6-29.8%	22-36%	14-26%	27-35%
LDH-2	31.8-35.8%	35-46%	29-39%	34-44%
LDH-3	20.0-24.8%	13-26%	20-26%	16-22%
LDH-4	6.8-10.2%	3-10%	8-16%	4-8%
LDH-5	6.5-9.7%	2-9%	6-16%	3-7%

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