18BCU511A **CLINCAL BIOCHEMISTRY PRACTICAL** **Semester IV**
3H-1C

COURSE OBJECTIVE

To teach students on phlebotomy, serum and/or plasma collection.

To impart skills to assess various biomolecules to diagnose the functioning of vital orgams.

COURSE OUTCOME

Students acquire the skills to perform phlebotomy and to estimate biomarkers to assess the vital organ functions.

1. Collection of blood and storage
2. Separation and storage of serum
3. Estimation of blood glucose by glucose oxidase peroxidase method.
4. Estimation of triglycerides.
5. Estimation of bilirubin (direct and indirect).
6. Quantitative determination of serum creatinine and urea.
7. Estimation of creatine kinase.

REFERENCES

Mukherjee, K.L., (2010). Medical Laboratory Technology - a Procedure Manual for Routine Diagnostic Tests Vol. I Tata Mc Graw–Hill Publishing Company Limited (New Delhi). ISBN:9780070076594 / ISBN:9780070076631

Mukherjee, K.L., (2010). Medical Laboratory Technology - a Procedure Manual for Routine Diagnostic Tests. VoI. II, Tata Mc Graw – Hill Publishing Company Ltd. (New Delhi), ISBN: 9780070076648.

Baynes, J.W., and Dominiczak, M.H., (2005). Medical Biochemistry, 2nd ed., Elsevier Mosby Ltd. (Philadelphia), ISBN:0-7234-3341-0.

Rao, B.S., and Deshpande, V., (2005). Experimental Biochemistry: A Student Companion IK International Pvt. Ltd. (New Delhi), ISBN:81-88237-41-8.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2016 onwards)
DEPARTMENT OF BIOCHEMISTRTY

SUBJECT : CLINICAL BIOCHEMISTRY PRACTICAL -A

SEMESTER : V

SUBJECT CODE: 18BCU511A **CLASS : III B. Sc. BC**

EXPERIMENTAL DETAILS
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
1	3	Collection of blood and storage	Mukherjee, K.L., (2010). Medical Laboratory Technology - a Procedure Manual for Routine Diagnostic Tests Vol. I Tata Mc Graw–Hill Publishing Company Limited (New Delhi). ISBN:9780070076594 / ISBN:9780070076631
2	3	Separation and storage of serum	
3	3	Estimation of blood glucose by glucose oxidase peroxidase method.	
4	3	Estimation of triglycerides.	
5	3	Estimation of bilirubin (direct and indirect).	
6	3	Quantitative determination of serum creatinine and urea.	
7	3	Estimation of creatine kinase.	
Total No Of Hours Planned For Practical = 36			

REFERENCES

Mukherjee, K.L., (2010). Medical Laboratory Technology - a Procedure Manual for Routine Diagnostic Tests Vol. I Tata Mc Graw–Hill Publishing Company Limited (New Delhi).

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Signature of the Staff

PRACTICAL SYLLABUS

1. Collection of blood and storage
2. Separation and storage of serum
3. Estimation of blood glucose by glucose oxidase peroxidase method.
4. Estimation of triglycerides.
5. Estimation of bilirubin (direct and indirect).
6. Quantitative determination of serum creatinine and urea.
7. Estimation of creatine kinase.

EXPERIMENT NO: 1 **DATE:**

COLLECTION OF BLOOD AND STORAGE

Aim: To collect the blood sample and its storage.

Reagents Required:

Collect all the equipment needed for the procedure and place it within safe and easy reach on a tray or trolley, ensuring that all the items are clearly visible. The equipment required includes: • A supply of sample tubes within the expiration date, which should be stored dry and upright in a rack, blood can be collected in ¬ Sterile glass or plastic tubes with rubber caps (the choice of tube will depend on what is agreed within the Centre ¬ Vacuum-extraction blood tubes; or ¬ Glass tubes with screw caps • Never combine tubes, holders or needles from different manufacturers. • A sterile glass or bleeding pack if large quantities of blood are to be collected. • Well fitting, non-sterile gloves. • An assortment of blood-sampling devices, of different sizes • A tourniquet • Alcohol hand rub • Antiseptic swabs for skin disinfection • Gauze or cotton-wool ball to be applied over puncture site • Laboratory sample labels • Writing equipment • Laboratory forms • Leak proof transportation bags and containers • A puncture resistant sharps container

Procedure:

• Wash hands with soap and water, and dry with single use towels. • Extend the subject's arm and inspect the antecubital fossa or forearm. • Ask the subject to form a fist so the veins are more prominent. • Locate a vein of a good size that is visible, straight and clear. The median cubital vein lies between muscles and is usually the most easy to puncture. Under the basilica vein runs an artery and a nerve, so puncturing here runs the risk of damaging the nerve or artery

and is usually more painful. Do not insert the needle where veins are diverting, because this increases the chance of haematoma; Appendix 1: Venipuncture • The vein should be visible without applying the tourniquet. Locating the vein will help in determining the correct size of needle. • Apply the tourniquet about 4-5 finger widths above the venepuncture site and reexamine the vein. • Clean hands with alcohol rub – use 3ml of alcohol rub on the palm of the hand, and rub into fingertips, back of hands and all over the hands until dry. • After performing hand hygiene, put on well-fitting, non-sterile gloves. • Clean the site with an antiseptic swab for 30 seconds and allow to dry completely. • Apply firm but gentle pressure. Start from the centre of the venepuncture site and work downward and outwards to cover an area of 2cm or more. • Allow the area to dry. Failure to allow enough contact time increases the risk of contamination • Do not touch the cleaned site. If the site is touched repeat the disinfection. • Anchor the vein by holding the subject's arm and placing a thumb below the venepuncture site. • Enter the vein swiftly at a 30 degree angle or less and continue to introduce the needle along the vein at the easiest angle of entry. • When obtaining multiple tubes of blood, use evacuated tubes with a needle and tube holder. This system allows the tubes to be filled directly. If this system is not available, use a syringe or winged needle set instead. • If a syringe or winged needle set is used, best practice is to place the tube into a rack before filling the tube. To prevent needle sticks, use one hand to fill the tube or use a needle shield between the needle and the hand holding the tube. • Pierce the stopper on the tube with the needle directly above the tube using slow, steady pressure. Do not press the syringe plunger because additional pressure increases the risk of haemolysis. • Where possible, keep the tubes in a rack and move the rack towards you. Inject downwards into the appropriate coloured stopper. Do not remove the stopper as it will release the vacuum. • If the sample tube does not have a rubber stopper, inject extremely slowly into the tube as minimising the pressure and velocity used to transfer the specimen reduces the risk of haemolysis. Do not recap and remove the needle. • Before dispatch, invert the tubes containing

additives for the required number of times (as specified by the local laboratory). • Once sufficient blood has been collected, release the tourniquet before withdrawing the needle. • Withdraw the needle gently and apply gentle pressure to the site with a clean gauze or dry cotton wool ball. Ask the subject to hold the gauze or the cotton wool ball in place with the arm extended and raised. Ask the subject not to bend the arm because doing so causes a haematoma. • Discard the used needle and syringe or blood sampling device into a puncture-resistant sharps container. • Discard used items into the appropriate category of waste. • Recheck the labels on the tubes and the forms before dispatch. • Inform the subject that the procedure is over. • Check the insertion site to verify that it is not bleeding. • Notify your manager if bleeding lasts more than five minutes. • Pack samples safely in a plastic leak-proof bag with an outside compartment for the laboratory request form. Placing the requisition on the outside helps avoid contamination. • If there are multiple tubes, place them in a rack or padded holder to avoid breakage during transportation. • Perform hand hygiene again. • Record the activity either manually or electronically

Result: The blood is collected and stored safely.

EXPERIMENT NO: 2 **DATE:**

SEPARATION AND STORAGE OF SERUM

Aim: To separate the serum from the blood sample and its storage.

Principle: Blood is a liquid tissue. Suspended in the watery plasma are seven types of cells and cell fragments. -Red blood cells (RBCs) -White blood cells (WBCs) -Platelets -Five kinds of Leukocytes (lymphocytes, monocytes,neutrophils,eosinophils,basophils) -After centrifugation of blood, the blood separate into three layers.

Serum is the same as plasma except that clotting factors (such as fibrin) have been removed. -For many biochemical laboratory tests, plasma and blood serum can be used interchangeably. Serum resembles plasma in composition but lacks the coagulation factors. - It is obtained by letting a blood specimen clot prior to centrifugation.

Reagents Required:

Collect all the equipment needed for the procedure and place it within safe and easy reach on a tray or trolley, ensuring that all the items are clearly visible. The equipment required includes: • A supply of sample tubes within the expiration date, which should be stored dry and upright in a rack, blood can be collected in ¬ Sterile glass or plastic tubes with rubber caps (the choice of tube will depend on what is agreed within the Centre ¬ Vacuum-extraction blood tubes; or ¬ Glass tubes with screw caps • Never combine tubes, holders or needles from different manufacturers. • A sterile glass or bleeding pack if large quantities of blood are to be collected. • Well fitting, non-sterile gloves. • An assortment of blood-sampling devices, of different sizes • A tourniquet • Alcohol hand rub, Antiseptic swabs for skin disinfection • Gauze or cotton-wool ball to be applied over puncture site • Laboratory sample labels • Writing equipment • Laboratory forms • Leak proof transportation bags and containers • A puncture resistant sharps container

Procedure:

Procedure of Serum preparation:

1- Draw blood from patient. Select vacutainer with UNOU anticoagulant.

2- Allow to stand for 20-30min for clot formation.

3- Centrifuge the sample to speed separation and affect a greater packing of cells. Clot and cells will separate from clean serum and settle to the bottom of the vessel.

4- The supernatant is the serum which can be now collected by dropper or pipette for testing purposes or stored (-20C to - 80C) for subsequent analysis or use.

5- Serum is obtained from spontaneously coagulated whole blood by centrifugation (1000-1200 for about 10-15 minutes). It contains no clotting factors.

Result: The serum from a known of blood sample is separated.

EXPERIMENT NO: 3 **DATE:**

ESTIMATION OF BLOOD GLUCOSE BY GLUCOSE OXIDASE PEROXIDASE METHOD

Aim: To estimate the blood glucose by glucose oxidase peroxidase method

Principle: Glucose oxidase is an enzyme extracted from the growth medium of *Aspergillus niger*. Glucose oxidase catalyse the oxidation of Beta D- glucose present in the plasma to D glucono -1 ,5 - lactone with the formation of hydrogen peroxide; the lactone is then slowly hydrolysed to D-gluconic acid. The hydrogen peroxide produced is then broken down to oxygen and water by a peroxidase enzyme. Oxygen then react with an oxygen acceptor such as ortho toluidine which itself converted to a coloured compound, the amount of which can be measured colorimetrically.

Materials Required:

Collection of blood sample: About 2ml of patient's blood should be collected by venipuncture into a tube containing a mixture of ethylenediaminetetraacetic acid and sodium fluoride in the ratio of 1:2 (W/W). Five mg of the mixture is adequate for 2ml of blood. The tube should be thoroughly shaken for complete mixing.

Preparation of anticoagulant mixture: 100mg of EDTA and 20mg of sodium fluoride should be mixed and ground into a fine powder using a blender. This should preferably do in a fume hood. The mixture should be stored in a clean container.

Reagents Required:

1. 2N Sodium hydroxide (NaOH) - 8g of NaOH is dissolve and finally make up the volume to 100ml with distilled water.
2. Sodium Sulphate- Zinc sulphate reagent – Dilute 55ml of the zinc sulphate solution (10g/100ml $ZnSO_4.7H_2O$) to 1 litre with the sodium sulphate solution (93mmol/liter).
3. Phosphate buffer 0.05M pH-7.2
4. Glucose oxidase reagent: Prepare this reagent fresh by dissolving 25mg of glucose oxidase and 1% ortho - toluidine in the sodium phosphate buffer. Add a small quantity of peroxidase (2mg) and makeup to 250ml with the buffer. This solution is active for about 4 weeks if stored in a brown colored bottle at 4°C.

Procedure:

- Preparation of Test: Pipette 0.1ml of blood into 1.8ml of sodium sulphate-zinc sulphate reagent in a centrifuge tube. Add 0.1ml of 2N Sodium hydroxide, centrifuge at 3000rpm for 5 minutes and take 0.5ml of supernatant in duplicate.
- Preparation of Blank: Take 0.5ml of distilled water.
- Preparation of Standard: Prepare standard concentration of glucose (200mg/dl), use 0.5ml of a range of glucose solutions (50mg/dl, 100mg/dl, 150mg/dl and 200mg/dl) suitably diluted from standard.

  I. 50mg/dl - 125µl glucose standard + 375µl distilled water

  II. 100mg/dl - 250µl glucose standard + 250µl distilled water

  III. 150mg/dl - 375µl glucose standard + 125µl distilled water

  IV. 200mg/dl - 500µl glucose standard

- Add 5ml of the glucose oxidase reagent incubate for 1h at 37°C and read the extinction at 540nm against the reagent blank.

- If the absorbance reading of the sample is too high, dilute the supernatant which was obtained earlier, 2x with distilled water and repeat the subsequent step.

Result: The given unknown sample contains ---- mg glucose/dl.

EXPERIMENT NO: 4 **DATE:**

ESTIMATION OF TRIGLYCERIDES

Estimation of Triglycerides (Trinder, 1969)

Aim

To estimate the amount of triglycerides present in the given sample.

Principle

Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate which was again oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxide, hydrogen peroxide oxidizes phenolic chromogen to a red colored compound.

Reagents

- Reagent 1: Triglycerides enzyme reagent
- Reagent 2: Triglycerides standard 200 mg/dl

Procedure

Addition sequence	Blank	Standard	Test
Enzyme reagent	1 ml	1 ml	1 ml
Standard	-	10 µl	-
Sample	-	-	10 µl

Mixed well and incubated for 10 minutes at 37ºC. Final color developed was stable for 30 minutes and the absorbance of standard and sample against the reagent blank were measured at 505 nm.

Calculation

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 200$$

Result

The amount of triglyceride was found to be_____mg/dl.

EXPERIMENT NO: 5 **DATE:**

ESTIMATION OF BILIRUBIN (DIRECT AND INDIRECT).

Aim: To estimate the amount of bilirubin in serum.

Principle: Bilirubin in serum is coupled with diazotized Sulfanilic acid to form azobilirubin . The water soluble conjugated bilirubin (direct bilirubin) reacts easily with reagents such as diazotized sulphanilic acid. While the water insoluble unconjugated bilirubin (indirect bilirubin) requires a solubilizing reagent, such as Caffeine, in order to react with the diazotized sulphanilic acid. In this experiment, the direct bilirubin is estimated in the absence of the solubilizing agent and then further bilirubin estimation in the presence of the solubilizing agent will give the total bilirubin level. The indirect or unconjugated bilirubin is then found by difference.

Materials Required:

Method

Label 4 tubes as TT (total test), TB (total Blank), DT (direct test), DB (direct Blank).

Total bilirubin

	TB	TT
Solution-1	0.2 ml	0.2 ml
Solution-2	--	0.05 ml
Solution-3	1 ml	1 ml
Sample	0.2 ml	0.2 ml

stand for 30 min at 20-25°C.

Solution 4	1 ml	1 ml

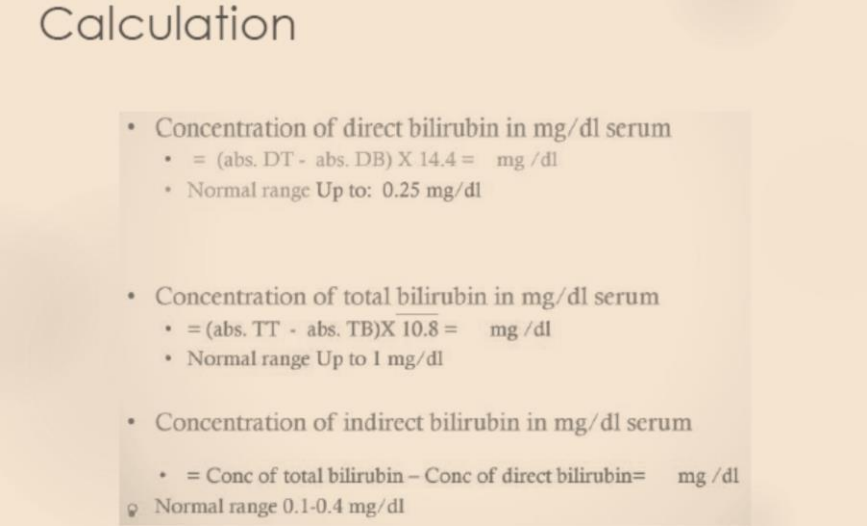
Mix and let stand for 15 min and read the absorbance at 546 nm against blank (ATB).

Direct bilirubin

	DB	DT
Solution-1	0.2 ml	0.2 ml
Solution-2	--	0.05 ml
0.9 % NaCl	2 ml	2 ml
Sample	0.2 ml	0.2 ml

Mix, let stand for 5 min. at 20-25°C. Read absorbance of test against blank (A_{DB}) for direct only at 546 nm.

Procedure:



Result: The serum from a known of blood sample is separated.

EXPERIMENT NO: 6 **DATE:**

QUANTITATIVE DETERMINATION OF SERUM CREATININE AND UREA

Aim

To estimate the amount of creatinine present in the given sample.

Principle

Creatinine reacts with picric acid to produce a colored compound creatinine alkaline picrate. The change in absorbance was proportional to the creatinine concentration.

Reagents

- Creatinine base reagent (R1)

Sodium hydroxide: 300 mM/L

Sodium phosphate: 25 mM/L

- Creatinine dye reagent (R2)

Picric acid: 8.73 mM/L

- Creatinine standard (2 mg/dL)
- Working reagent: Mix 1 volume of R1 with 1 volume of R2

Procedure

Contents added	Standard	Sample
Working reagent	1000µl	1000 µl
Standard	100 µl	-
Sample	-	100 µl
Mixed well and read the optical density at 492nm (T1) 60 seconds after the addition of samples and standard. Exactly 60 seconds after the first reading the second reading (T2) was taken		

Calculation

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{(T1-T2) of sample}}{\text{(T1-T2) of standard}} \times 2$$

Result

The amount of creatinine was found to be_____mg/dl.

DETERMINATION OF UREA IN SERUM AND URINE - DAM TSC METHOD

Aim

To estimate the amount of urea present in urine and blood

Principle

Urea directly reacts with diacetylmonoxime in the presence of thiosemicarbazide to form a red coloured product that is measured colorimetrically at 540nm.

Reagents

1. Diacetyl monoxime:

1.56g of diacetylmonoxime in 250ml of distilled water

2. Thiosemicarbazie:

41mg of thiosemicarbazied in 250 ml of distilled water (stored in brown bottle)

3. Ferric chloride reagent:

324mg ferric chloride in 10ml of 50% orthophosphoric acid (Stored in brown bottle).

4. 20% sulphuric acid
5. Stock standard urea solution:

Dissolved 100mg of urea in 100ml of distilled water. This solution is prepared in saturation solution of benzoic acid for long use. 1.0ml of this solution contains 1mg of urea

6. Working standard urea solution:

2.0ml of stock standard was diluted to 100ml with distilled water. 1.0ml of their solution

contains 20 µg of urea

Procedure

Take 1.8ml of trichloroacetic acid and 0.2ml of blood centrifuge for 10 minutes. 0.5ml of supernatant was taken for experiment into a series of test tubes take 0.5, 1.0, 1.5, 2.0 and 2.5ml of working standard urea solution. Corresponding to the values of 10, 20, 30, 40 and 50 µg respectively. The volume was made-up to 3.0ml with distilled water n all the test tubes. Added 1.0ml of diacetylmonoxime, 1.0 m of thiosemicarbazide and 3.0ml of acid reagent and heated vigorously in boiling water for 20 minutes. Along with this a blank was also conducted. Remove the tubes and cool. Reading were taken against the reagent blank at 540nm in a colorimeter.

A standard graph was drawn by plotting colorimetric reading on y axis and concentration of urea on x axis.

Normal Values

Blood – 19-30 mg/dl

Urine

Sample preparation:

1.0ml of urine was made upto 100ml with distilled water. 1.5ml of diluted urine was taken for the experiment.

Result

The amount of urea present in the given serum sample is---------- mg/dl

EXPERIMENT NO: 7 **DATE:**

ESTIMATION OF CREATINE KINASE.

Aim: To estimate creatine kinase in the serum.

Principle:

The creatine-kinase [CK] (adenosine 5'-triphosphate creatine-phosphotransferase, E.C. 2.7.3.2) catalyses the following reaction:

$$ATP^{4-} + Creatine \xleftrightarrow{Creatine-kinase} ADP^{3-} + Creatine\text{-}phosphate^{3-} + H^{+}$$

High CK enzyme activity is detectable in muscle, heart and brain. CK activity is also detectable in serum. The CK [MW.: 80,000.00 D] consist***s*** of two subunits. The subunits are signed as **CK-M** [M = muscle] and **CK-B** [B = brain]. The combination of two subunits results in the formation of three type***s*** of isoenzymes: **CK-MM**, **CK-BB** and the hybrid form of **CK-MB** are characteristic for muscle, brain and heart, respectively. CK-MB activity is characteristic for the heart.

Clinical significance - Both elevated total CK activity and elevated CK-MB activity can be detected in the serum after physical exercise, or in the serum of patients suffering myocardial infarction. CK, LDH [Lactate dehydrogenase], LDH_1 and ASAT [SGOT] enzyme activities play a crucial role in the verification of myocardial infarction through the use of laboratory methods. Myocardial infarction significantly elevates the CK activity of the serum. The probability of myocardial infarction is high, if the 6-25 % of total activity of CK of serum originates from the activity of CK-MB isoenzyme. Table 1. summarises the values of activity of CK and CK-MB enzymes of patients with myocardial infarction.

	25 °C
CK men	>80 U/l
CK women	>70 U/l
CK MB	>10 U/l

Myocardial infarction rapidly and significantly elevates the activity of CK in the serum. Elevation of activity begins about 5 hours after the onset of the infarction. CK activity of serum shows its maximal activity after 10-12 hours. To verify the myocardial infarction, blood samples should be taken at the 0^{th}, 6^{th}, 12^{th} and 24^{th} hours of the infarction. Table 2 shows the data of CK activity (IU/l serum) measured in blood samples of patients with myocardial infarction.

	MM	MB
Serum [normal]	0-90	0
Myoc. infarction	50-1000	2-250

Determination of CK activity

CK activity is determined with the help of the following coupled enzyme reactions:

$$ADP^{3-} + Creatine-phosphate^{3-} + H^{+} \xleftarrow{Creatine-kinase} ATP^{4-} + Creatine^{-}$$

$$Glucose + ATP^{4-} \xrightarrow{hexokinase} glucose-6-phosphate + ADP^{3-}$$

$$Glucose-6-phosphate + NADP^{+} \xrightarrow{glucose-6-P-dehydrogenase} 6-P-glucono-\delta-lactone + NADPH + H^{+}$$

ATP - the product of CK - is utilised by hexokinase. The reaction results in the formation of glucose-6-phosphate. Glucose-6-phosphate serves as a substrate for the next enzymatic reaction: one of its products is NADPH. The NADPH shows absorption maximum at 340 nm. The generation of reduced NADPH can be detected photometrically.

Solutions

1. 50 mM TRIS-HCl pH 7.2 containing 1 mM ADP, 20 mM Glucose, 30 mM $MgCl_2$, 5 mM Cystein, 1 mM NADP and 10 mM Creatine-phosphate
2. Hexokinase (60 U/ml)
3. Glucose-6-phosphate dehydrogenase (30 U/ml)
4. Serum [of normal and of ill patients]

Determination of enzyme activity

Fill the following solutions into cuvettes:

Solution/# of tubes	1	2	3
50 mM TRIS pH 7.2	0.90	0.90	0.90
Hexokinase	0.02	0.02	0.02
Glucose-6-P dehydrogenase	0.02	0.02	0.02
H_2O	0.10	-	-
Serum (N, H) *	-	*0.10	*0.10
Extinction (340 nm)			

Start the reaction by the addition of 0,1 ml of sera. Read the values of absorption after 1 min incubation at room temperature each minute for 3 minutes. Compare the activity values of sera of a normal patient (# = 2) to a patient with myocardial infarction (# = 3). Measure the formation of NADPH at 340 nm against blank cuvette (# = 1) containig H_2O.

Calculation -

$$\text{Serum activity } [\text{IU}/\text{l}] = \frac{\frac{\Delta \text{ extinction}}{\text{minute}} * 10,400}{6.22}$$

10,400 is a factor for the conversion of 0,1 ml serum to 1 l; 6.22 is the extinction coefficient of NADPH.

Result: The amount of creatine kinase in the serum was found to be _____