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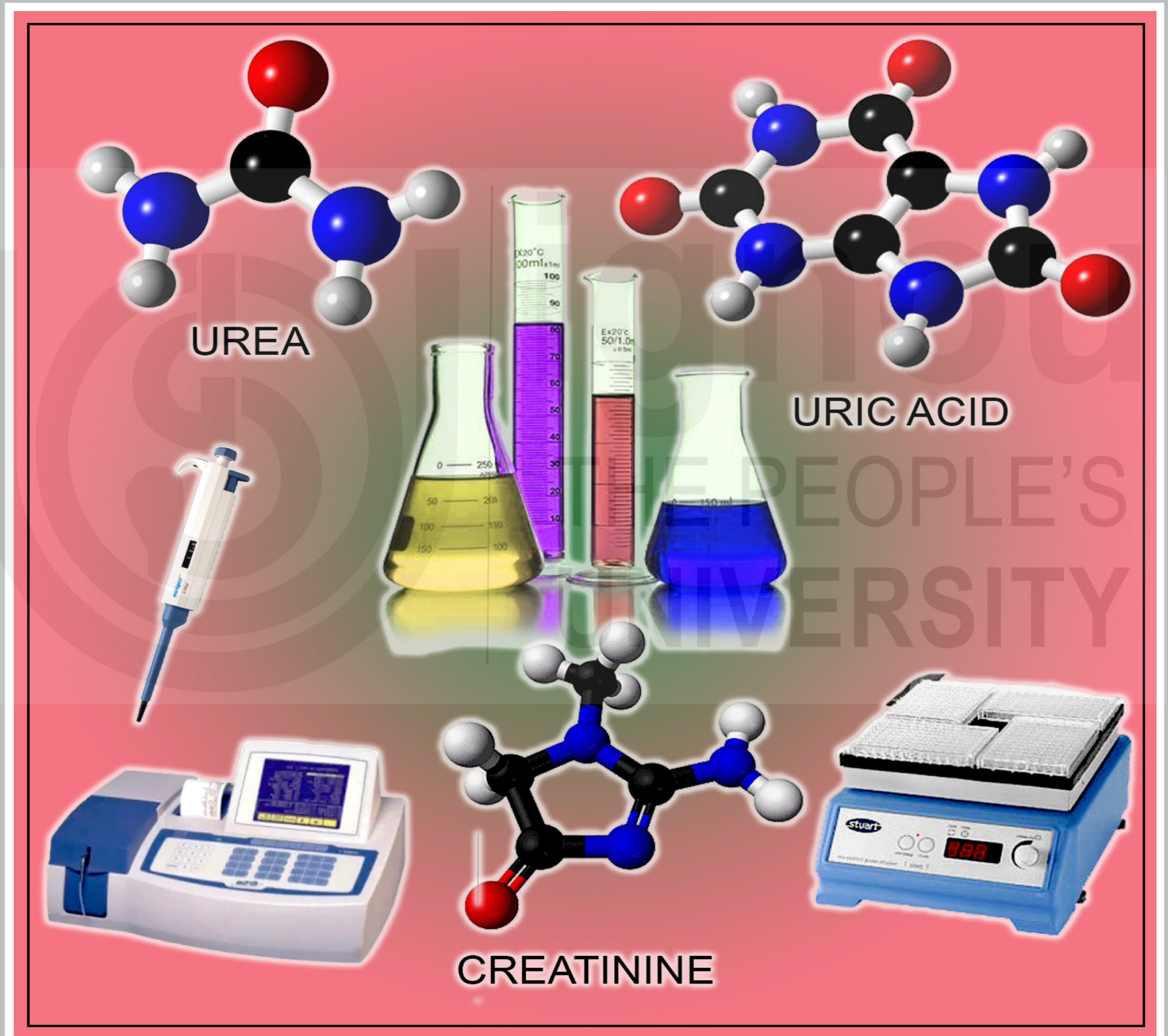
BBCCL-114
METABOLISM OF AMINO ACIDS AND NUCLEOTIDES: LABORATORY



LABORATORY EXERCISES

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METABOLISM OF AMINO ACIDS AND NUCLEOTIDES

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PROGRAMME AND COURSE DESIGN COMMITTEE

Prof. Bechan Sharma
Dept. of Biochemistry
University of Allahabad

Prof. Reena Gupta
Dept. of Biotechnology
H.P. University, Shimla

Prof. D. V. Devaraju
Dept. of Biochemistry
Bangalore University

Dr. Suneeta Joshi
Associate Professor
Dept. of Biochemistry
Daulat Ram College
University of Delhi

Prof. Ranjit K. Mishra
Dept. of Biochemistry
University of Lucknow

Prof. Sanjeev Puri
UIET, Panjab University

Prof. Seemi Farhat Basir
Dept. of Bio Sciences
Jamia Milia Islamia
New Delhi

Prof. Vijayshri
Director, SOS
IGNOU

Dr. Parvesh Bubber
Associate Professor
SOS, IGNOU

Dr. M. Abdul Kareem
Assistant Professor
SOS, IGNOU

Dr. Arvind K. Shakya
Assistant Professor,
SOS, IGNOU

Dr. Maneesha Pandey
Assistant Professor
SOS, IGNOU

Dr. Seema Kalra
Assistant Professor
SOS, IGNOU

COURSE PREPARATION TEAM

Dr. Sunita Joshi
(All Experiments)
Retd. Associate Professor
Department of Biochemistry
Daulat Ram College
Delhi University, New Delhi

Dr. Niraj Srivastva (Author)
Consultant
Discipline of Biochemistry
SOS, IGNOU
New Delhi-110017
and
Dr. Parvesh Bubber (Author)
Associate Professor
Discipline of Biochemistry
SOS, IGNOU
New Delhi-110017

Course Coordinator: Dr. Parvesh Bubber, Associate Professor, SOS, IGNOU

Print Production Team

Mr. Sumit Verma for CRC Preparation and Word Processing

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BBCCL-114: METABOLISM OF AMINO ACIDS AND NUCLEOTIDES

This biochemistry laboratory course is for the learners of undergraduate programme of biochemistry with emphasis on metabolism of amino acids and nucleotides. The lab course is worth two credits. It is a supplement to the theory aspects of amino acid and nucleotides, learners have read in the course BBCCT-113. These lab exercises have been designed to characterize the metabolic reactions of amino acids.

Nitrogen is an indispensable element present in all amino acids; it is derived from dietary protein intake and is essential for the synthesis of proteins as well as for their maintenance. Nitrogen is excreted by the kidneys mainly in the form of urea and ammonia. Under steady-state environment, excretion of renal nitrogen equals intake of nitrogen.

Urea, uric acid, creatine, and creatinine are four major NPN components and their estimation is used to monitor renal function in clinical settings. In hospitals, these are routinely used tests to diagnose several types of kidney diseases and their progression. Urea constitutes nearly half of the NPN in the blood. Its excretion is regulated by a wide variety of conditions as it play vital roles in normal health and disease, including roles in acid-base homeostasis.

The “how” and “why” of the amino acid biochemical reactions that are required for maintenance of cellular functions are being uncovered by the lab exercises on assay of serum transaminases. The alpha amino group of amino acids is removed by amino transferases. The two most important serum amino transferases are aspartate transaminase (AST) and alanine transaminase (ALT). They make significant contribution to overall transamination and are important prognostic and diagnostic biomarkers.

Each practical exercise on these tests begins with the ‘Introduction’ about the experiment in which theory and relevance have been discussed so that one can understand fully the importance of experiment beforehand. Protocols are covered in detail explaining how to take observations followed by calculations. Precautions to be taken while performing experiment have invariably been included under each practical exercise. Attention has also been drawn to careful handling of chemicals, and the erroneous measurements creeping in methods described for different experiments.

Description of experiments:

Experiment I: The first lab experiment begins with the basic exercise on measuring the enzyme activity of transaminases.

Experiment 2: The second exercise elaborates estimation of urea.

Experiment 3: Third exercise has been designed to determine the level of uric acid.

Experiment 4: The lab experiment provides detailed protocol for the estimation of serum creatinine.

It is very important that you (learner) have an observation notebook wherein you record all the observations, results, doubts if any and difficulties experiences in carrying out the experiments. A good worker in the laboratory always makes extensive and detailed notes on the observations and results of his/her experiments.

Besides the observations notebook, you are expected to prepare a record notebook which you will submit to the counselor at the time of examination. You record your experimental data and write a brief discussion of each experiment. You should try to make scientific illustrations wherever necessary. Assessment of the experiments will be graded and you will have to appear for the viva-voce at the end of the practical session. At the end of the laboratory session you should perform the assigned experiment, which will be graded and final assessment will be made based on the continuous performance during the laboratory sessions, maintenance of log books and records followed by viva-voce.

So interact closely with your counselor to get the best of your counselor and conduct experimental exercises with dedication to make your efforts useful and meaningful.

We wish you best in this endeavour!!

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EXPERIMENT 1

ASSAY OF SERUM TRANSAMINASES

Structure

1.1 Introduction

Expected Learning Outcomes

1.2 Principle

1.3 Materials required

1.4 Protocol

1.5 Observations and Results

1.6 Precautions

1.1 INTRODUCTION

The α-amino group of most amino acids is removed by **amino transferases** or transaminases. These enzymes catalyse the reversible transfer of amino group from an amino acid to a keto acid. In this reaction there is no net deamination. All transaminases are pyridoxal phosphate dependent enzymes, covalently bound to the lysine residue of the enzyme through an aldimine linkage (Schiff's base). The keto acceptor of the amino group in majority of them is α-ketoglutarate thereby generating glutamate as one of the product.

The two most important amino transferases are aspartate transaminase (AST) and alanine transaminase (ALT). They make significant contribution to overall transamination and are important prognostic and diagnostic biomarkers. The levels of both enzymes in blood are low in normal healthy individuals.

ALT is found predominantly in the liver and to some extent in kidney, heart, muscles and red blood cells. High serum ALT indicates liver damage from hepatitis, cirrhosis, infections, etc; hemolytic conditions; skeletal diseases and renal infarct. AST on the other hand is present in almost all cells, especially heart and liver. The levels of AST are elevated after myocardial infarction, liver disorders, hemolytic conditions, etc. In alcoholic hepatitis AST is higher than ALT (generally the ratio is>than 2) whereas both are 10-100 fold elevated in viral hepatitis.

In this lab exercise you shall assay the activity of AST and ALT in serum by 2, 4-dinitrophenyl hydrazine (Reitman and Frankel, 1957) method. It is an end point colorimetric method. You must know that colorimetric methods have

been superseded by coupled enzyme assays in which the ketoacid produced by transamination is reduced by NADH and fall in NADH is monitored at 340nm.

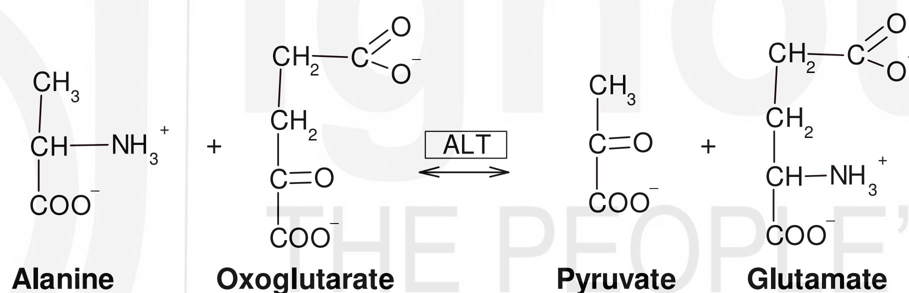
Expected Learning Outcomes

After studying this unit, you should be able to:

- Assay the activity of serum transaminases -SGOT and SGPT;
- Explain the chemistry of colorimetric method; and
- Indicate the clinical significance of assaying them in serum samples.

1.2 PRINCIPLE

Transaminases catalyze pyridoxal phosphate dependent reversible interconversion of amino acids and a keto acid by the intermolecular transfer of amino groups. The reaction catalysed by two important transaminases- aspartate transaminases (AST) or Glutamate oxaloacetate transaminases (GOT; EC 2.6.1.1) and alanine transaminases (ALT) or Glutamate pyruvate transaminases (GPT; EC 2.6.1.2) is given in Fig. 1.1.



Pyruvate formed in ALT catalyzed reaction is coupled to 2, 4-dinitrophenyl hydrazine (2, 4-DNPH) to form brown colour hydrazone derivative under alkaline conditions.

Pyruvate + 2, 4-DNPH ⟶ hydrazone derivative (brown colour)

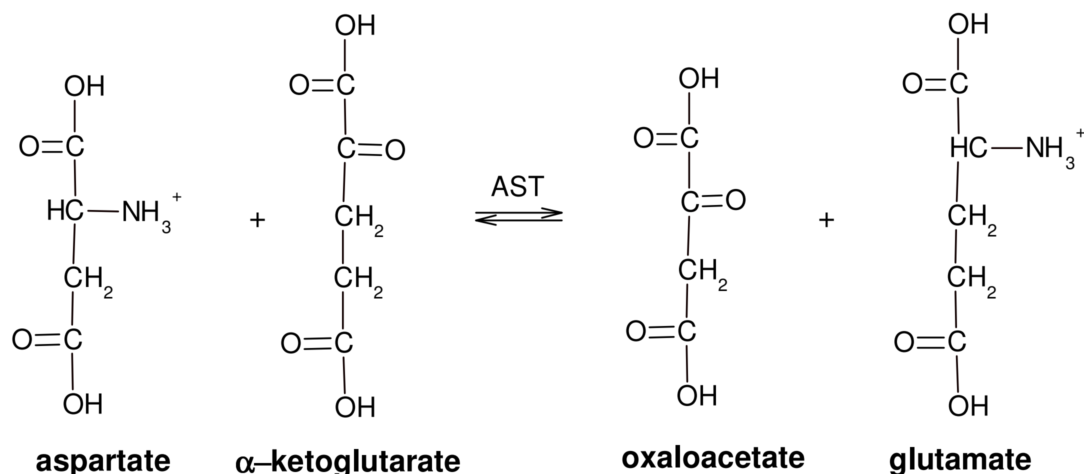


Fig. 1.1: Transaminations reactions catalysed by AST and ALT.

Oxaloacetate formed in AST catalyzed reaction is spontaneously decarboxylated to pyruvate which then gives identical coloured derivative with DNPH as ALT.

1.3 MATERIALS REQUIRED

1. Phosphate buffer (0.1M; pH 7.4)

Given: MW of Na_2HPO4 is 142 and NaH_2PO_4 is 120; pKa_2 is 6.8

Dissolve 11.22 g of anhydrous disodium hydrogen phosphate and 2.52 g of anhydrous sodium dihydrogen phosphate and make volume to 700-800 ml. Adjust pH to 7.4 and make up the volume to 1 litre.

2. SGOT substrate [L-aspartic acid (0.2M), MW 133; α-KG (0.02M), MW 146]

Dissolve 13.3g of L-aspartic acid in 90ml of 1M sodium hydroxide. Add 1.46g of α-keto glutaric acid and dissolve by adding another 10 ml of 1M NaOH. Adjust pH to 7.4 and make up the volume to 500 ml with phosphate buffer. Store it in deep freezer.

3. SGPT Substrate [alanine (0.2M), MW 89; α-ketoglutaric acid (0.02M)]

Dissolve 8.9 g of L-alanine in 90ml water with addition of 6ml of 1M NaOH. Add 1.46g of α-Keto glutaric acid and 4ml of1M NaOH. Adjust pH to 7.4 and make up the volume to 500 ml with phosphate buffer. Store the substrate in deep freezer.

4. Stock pyruvate standard (20mM; MW 110 g/ mol)

Dissolve 220mg of sodium pyruvate in 100ml of phosphate buffer. Store the solution in a refrigerator.

Working standard (4mM)**:** Dilute 4ml of stock solution with 16ml of phosphate buffer.

5. 2, 4-Dinnitrophenylhydrazine (DNPH) [0.2 mg percent (w/v)].

Dissolve 200mg of DNPH in 10ml of conc. HCl. Make up the volume to 100ml with double distilled water.

6. Sodium Hydroxide [0.4 M; MW 40]

Dissolve 16g NaOH pellets in water and make up the volume to one litre with double distilled water. Standardize molarity of NaOH with standard oxalic acid.

1.4 PROTOCOL

i) Took four clean and dry test tubes and labeled them test ('T'), control (C), standard (S) and blank (B).

ii) Added 0.5 ml substrate to each tube

iii) Next pipetted 0.1 ml serum is added to test ('T') and control (C) tubes; 0.1 ml working standard to standard (S) tube and 0.1 ml double distilled water to blank (B) tube.

iv) Incubated the tubes for 30 minutes (SGPT) / for 60 minutes (SGOT) in a water bath at 37°C.

v) At the end of incubation period 0.5 ml DNPH reagent is added to all four tubes.

vi) Left the tubes at room temperature for 20 minutes.

vii) Terminated the reaction by addition of 5 ml of 0.4N NaOH in all tubes.

viii) Record absorbance at 520 nm within 15minutes.

1.5 OBSERVATION AND RESULTS

Additions	**T (test)**	**C (control)**	**S (standard)**	**B (blank)**	**Absorbance (520 nm)**
Substrate	0.5ml	0.5ml	0.5ml	0.5ml	
Serum	0.1ml	0.1ml	-	-	
Standard (Working)	-	-	0.1ml	-	
Double distilled water	-	-	-	0.1ml	
DNPH	0.5ml	0.5ml	0.5ml	0.5ml	

Calculations

$$\text{Activity of ALT (SGPT)} = \frac{E_{(T)} - E_{(C)}}{E_{(s)} - E_{(B)}} \times 4 \times 1000 \times \frac{1}{30}$$

$$= \frac{E_{(T)} - E_{(C)}}{E_{(C)} - E_{(B)}} \times 133 \text{ units}$$

E=Extinction Value

Normal range of SGPT =10-40 IU/L

$$\text{Activity of AST (SGOT)} = \frac{E_{(T)} - E_{(C)}}{E_{(s)} - E_{(B)}} \times 4 \times 1000 \times \frac{1}{60}$$

$$= \frac{E_{(T)} - E_{(C)}}{E_{(C)} - E_{(B)}} \times 67$$

Normal range of SGOT = 8-20 IU/L

1.6 PRECAUTIONS

i) During the collection of blood, minimise haemolysis.

ii) If the enzyme activity is > than 150IU/L, dilute the serum with normal saline.

iii) Avoid variation in incubation time and maintain temperature during assay.

iv) Bring reagents to assay temperature before use.

v) Check buffer pH before preparing reagents

SAQ 1

Q.1. What are the transaminases?

A.1. They are a group of enzymes which catalyse freely reversible interconversion of amino acids and ketoacids by transfer of amino groups.

Q.2. Why GPT and GOT are termed SGPT and SGOT, respectively?

A.2. The alphabet 'S' preceding 'GPT and GOT' in SGPT and SGOT stands for serum.

Q.3. What is the clinical significance of ALT (SGPT) and AST (SGOT) assay?

A.3. High serum ALT indicates liver damage, hemolytic conditions, skeletal diseases and renal infarct. The levels of AST are elevated after myocardial infarction, liver disorders, hemolytic conditions, etc. Both are sensitive indicators of liver damage.

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EXPERIMENT 2

ESTIMATION OF SERUM UREA

Structure

2.1 Introduction

Expected Learning Outcomes

2.2 Principle

2.3 Materials required

2.4 Protocol

2.5 Observations and Results

2.6 Precautions

2.1 INTRODUCTION

Urea is primarily the product of protein catabolism. The synthesis of urea is a mechanism of detoxification of ammonia (released from amino acids), prior to excretion. In humans it is synthesized in the liver, released into blood and excreted almost entirely by the kidneys. On a balanced diet, urea accounts for 80-90% of the total urine nitrogen which may fall to 60% on a low protein diet.

The clinical significance of serum urea estimation is to screen for renal function. Serum urea concentration is a function of balance between hepatic urea production and renal urea excretion in urine. The normal range of serum urea is wide due to variations in protein intake and / endogenous protein catabolism coupled with rates of synthesis and excretion of urea.

An imbalance in the physiological function of liver and kidneys leads to abnormal blood urea levels. In this regard, enhanced level of urea in serum can be caused by enhancing the production of urea, decreased level of urea elimination or a combination of the two. Some of the conditions that result in altered urea levels are due to renal dysfunction (gastroenteritis, burns, diabetic ketoacidosis, hemolytic anemia, etc) and renal diseases (glomerulonephritis, bilateral renal tuberculosis) and hepatic diseases (liver cirrhosis).

In case of renal dysfunction and renal diseases, there is retention of blood urea due to reduction in renal blood flow and glomerular filtration rate (GFR) whereas in hepatic diseases the blood urea levels go down due to decreased synthesis. A good correlation exists between GFR and severity of kidney disease.

Blood urea levels are also elevated in high protein diet, starvation, muscle wasting, exercise and drugs.

The methods available for measurement of urea nitrogen are either non enzymatic or enzymatic. In the former category, the condensation of urea with compounds possessing adjacent ketonic groups to coloured quantifiable products is an example while the latter methods are based on specific hydrolysis of urea to ammonia and carbonic acid by urease. The products are assayed by a variety of ways. In this lab exercise, you will be learning to perform the estimation of serum urea by diacetylmonoxime method.

Expected Learning Outcomes

After studying this unit, you should be able to:

- separate serum from blood;
- prepare protein free serum and perform urea estimation;
- explain the principle of the colorimetric method used;
- indicate the limitations of diacetylmonoxime method; and
- explain the clinical significance of serum urea estimation.

2.2 PRINCIPLE

The diacetylmonoxime method is based on the property of urea to undergo condensation reaction when heated with compounds containing adjacent ketonic groups as in diacetylmonoxime (Fig. 2.1). The pinkish / red diazine derivative is produced in an acidic medium whose intensity is directly proportional to the concentration of urea in protein free serum sample. The colour is further enhanced in presence of thiosemicarbazide and ferric ions. The chromogen is quantified by measuring absorbance at 550nm. Nowadays most of the methods are automated and modified for use in autoanalysers.

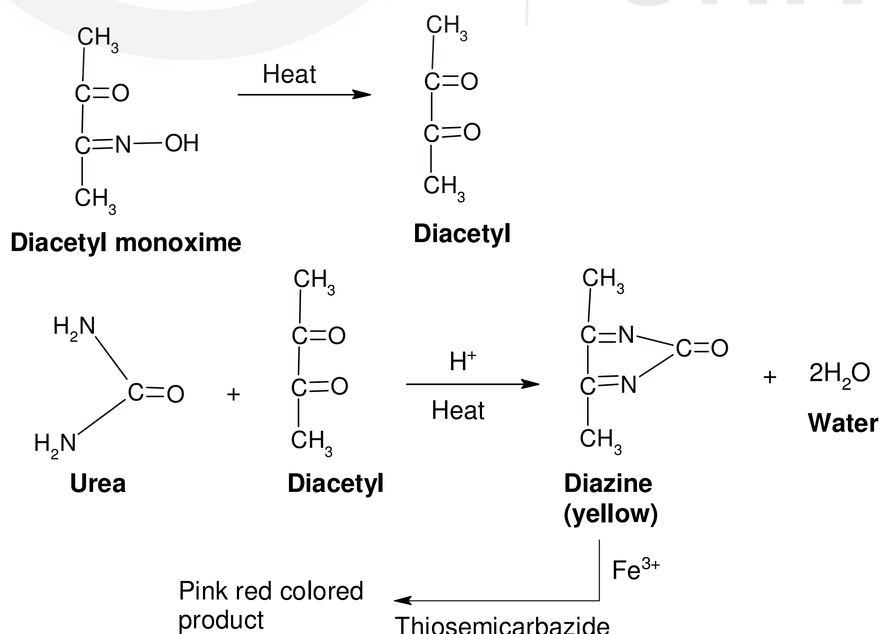

Fig. 2.1: Diacetyl or Fearon reaction for urea estimation.

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2.3 MATERIALS REQUIRED

1. Trichloroacetic acid [10 percent (w/v)]

Weigh 10 g of trichloroacetic acid (TCA) and dissolve it in 100 ml double distilled water. Store the solution at 4℃.

2. Sulfuric acid [20 percent (v/v]

Transfer 80 ml of double distilled water in a beaker and carefully added 20 ml conc. sulphuric acid to it.

3. Ferric chloride-phosphoric acid reagent.

Dissolve 3.24g of ferric chloride in ortho-phosphoric acid (56%). Make up the final volume to 100 ml. Store the reagent in a brown bottle to protect from direct light.

4. Acid reagent.

It is prepared by mixing ferric chloride -phosphoric acid reagent and sulfuric acid (20%) in the ratio 1:1000 (1 ml of ferric chloride phosphoric acid reagent in one litre of 20 percent sulfuric acid). The acid reagent must be prepared freshly.

5. Diacetylmonoxime reagent.

Dissolve 0.7g of diacetylmonoxime in 100 ml of water.

6. Thiosemicarbazide solution

Dissolve 18mg of thiosemicarbazide in 100ml of water. Store the solution in amber coloured reagent bottle.

7. Urea (1mg/ml stock solution)

Dissolve 100mg of pure urea crystals in double distilled water and make up the volume to 100ml in a volumetric flask. The working urea standard (1mg/dL or 0.01mg /ml) is made by diluting 1ml of stock to 100 ml with double distilled water.

8. Refrigerated centrifuge or microfuge

9. Colorimeter or spectrophotometer

10. Vortex mixer

11.Test tubes

12. Eppendorf tubes and pipettes

2.4 PROTOCOL

(a) Separation of serum from blood

i) Blood is collected into fluoride vials and gently shacked for two to three times.

ii) The collected blood is left undisturbed at room temperature (37℃) for 30 minutes to allow clot formation.

iii) Transferred clotted blood to Eppendorf tubes (2ml) and centrifuged at 1000 g (4° C) for 10 minutes.

iv) After centrifugation, the upper light yellow colour fluid (serum) is separated using clean pipette into 2.0 ml Eppendorf tube.

(b) Preparation of protein free filtrates

i) 0.2 ml of serum is pipetted into a clean, dry, test tube.

ii) Added 6.8 ml of double distilled water and mixed thoroughly.

iii) Next 3 ml of TCA (10%) is added slowly with mixing. The tube is left undisturbed for 10 minutes.

iv) The precipitated protein is separated by filtration through Whatman no. 1 filter paper. The protein free filtrate is now ready for estimation of urea.

(c) Estimation of serum urea by diacetylmonoxime method.

i) Three clean and dry test tubes are taken.

ii) Marked the tubes as test ('T') standard (S) and blank (B).

iii) Added 1.0 ml protein free filtrate to tube marked ('T'); 1.0 ml urea working standard to tube (S) and 1.0 ml double distilled water to blank (B) tube.

iv) Pipetted1.0 ml of diacetylmonoxime to all three tubes [('T'), (S) and (B)].

v) Next added 1.0 ml of thiosemicarbazide solution to all tubes.

vi) Finally pipetted 3.0 ml of acid reagent to test, standard and blank tubes.

vii) Mixed thoroughly the contents of each tube.. Put aluminium foil pieces on top of each tube and roll the foil down to form end caps

viii) Placed the tubes in a boiling water bath (100℃) for 15 minutes.

ix) Cooled the tubes to room temperature by placing them in a stream of running water or a beaker containing cold water.

x) Record absorbance within 15 minutes at 540 nm as the colour is not very stable.

2.5 OBSERVATION AND RESULTS

S.No.	Additions to each tube (ml)	Test (T)	Standard (S)	Blank (B)	Absorbance at 540 nm
1.	Protein free filtrate	1.0	-	-	
2.	Standard urea solution	-	1.0	-	
3.	Double distilled water	-	-	1.0	
4.	Diacetylmonoxime	1.0	1.0	1.0	
5.	Thiosemicarbazide	1.0	1.0	1.0	
6.	Acid reagent	3.0	3.0	3.0	

Calculations

Concentration of urea in blood (mg/dL)

$$= \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \frac{\text{Concentration of standard}}{\text{Volume of test sample}} \times 100$$

$$= \frac{(E(T) - E(B))}{(E(S) - E(B))} \times \frac{0.01}{0.02} \times 100$$

$$\text{OR } \frac{(E(T) - E(B))}{(E(S) - E(B))} \times 50$$

E= Extinction Value

Note: The volume of serum used is 0.2 /10 =0.02ml and standard uric acid concentration is 0.01mg /ml

Biological reference range

- ❖ Normal blood urea levels: 10-40mg/dL. It is generally higher in men than in women.
- ❖ Total daily excretion of urea is approx. 30-40g

Based on the given reference range interpret your results.

2.6 PRECAUTIONS

i) During collection of blood take care to minimise haemolysis.

ii) Do not leave the tubes in boiling water bath for more than 15 minutes.

iii) Reconstitute the acid reagent freshly.

iv) Record absorbance of the samples within 15 minutes after cooling.

v) Handle acids carefully and keep first aid ready to manage any mishap.

SAQ 1

Q.1. What does normal serum urea concentration indicate?

A.1. Serum urea concentration reflects the balance between hepatic urea production and urea elimination by the kidneys.

Q.2. Explain the clinical significance of serum urea estimation?

A.2. It is used to screen for renal function; for instance in advanced renal disease there is marked reduction in glomerular filtration rate (GFR)-a parameter that defines kidney function.

EXPERIMENT 3

ESTIMATION OF SERUM URIC ACID

Structure

3.1 Introduction
Expected Learning Outcomes
3.2 Principle
3.3 Materials required
3.4 Protocol
3.5 Observations and Results
3.6 Precautions

3.1 INTRODUCTION

You may recall from Unit 11 (Block 4) of core course (BBCCT-113) on 'Amino acid and Nucleotide metabolism' that uric acid (2, 6, 8 trihydroxypurine) is the end product of purine catabolism in humans and other primates. Uric acid levels in serum are dependent on exogenous (dietary) and endogenous purines (derived from de novo synthesis and turnover of tissue nucleic acids), urinary excretion and intestinal uricolysis. In humans almost all dietary purines are converted to uric acid. It is a weak acid that exists as sodium urate at physiological pH and changes to unionized uric acid at acidic pH (pH5.75) encountered in urine; the salt is more soluble than the acid. Interestingly the solubility of urate in plasma at 37°C is close to the upper limit of urate in adults. The kidney is the major site (⅔ -¾ of total) for excretion of uric acid and the rest (⅓ – ¼) is disposed of by uricolysis catalysed by gut flora. The excretion by kidneys is dependent on the optimal functioning of glomerular filtration, proximal tubular reabsorption, secretion and post secretory reabsorption.

Elevated levels of uric acid (hyperuricemia; >8mg/dL) in serum are associated with gout, Lesch Nyhan syndrome (LNS), diabetes, high cholesterol, obesity, kidney & heart diseases, etc. You may also refer to unit 12 of core course (BBCCT-113) for a detailed account of gout and LNS. Most people have a defect in the renal handling of uric acid which may be age related or due to renal diseases, drugs (like low doses of salicylate and diuretics) and organic acids produced in diabetics and lactic acidosis. Enhanced cell death during

chemotherapy in cancer patients or other conditions also results in high uric acid.

When serum urate concentration is <2mg/dL then the condition is called hypouricemia. It is generally asymptomatic and can result from malignant diseases, liver disorders (cirrhosis), total parenteral nutrition and certain drugs (such as high doses of salicylates and allopurinol) which decrease renal reabsorption.

The methods generally used to estimate uric acid levels are either non enzymatic or enzymatic; the former gives an overestimate as they also detect other substances while the latter are specific for uric acid. The enzymatic methods are oxidation based in which the first step is specific oxidation of urate to allantoin by uricase. The formation of allantoin may be spectrophotometrically analysed by exploiting differential absorption of urate at 293nm or by coupled assays using peroxidase and a chromogen. In this exercise we shall use phospho tungstate method.

Expected Learning Outcomes

After studying this unit, you should be able to:

- explain the principle of colorimetric method used;
- prepare reagents for the assay;
- perform estimation of uric acid;
- express the concentration of uric acid in mg/dL; and
- interpret the possible condition of the patient.

3.2 PRINCIPLE

The phospho tungstate (Wendell T. Caraway, 1955) method is a popular end point assay of uric acid estimation. Uric acid present in protein free serum reduces phosphotungstic acid in alkaline medium to a coloured chromogen-tungsten blue. The intensity of blue color can be measured spectrophotometrically at 660 nm and is directly proportional to the concentration of uric acid in the sample. The absorbance is linear up to 15mg/dL. This method is nonspecific as endogenous serum constituents like vitamin C, glutathione and cysteine are interfering substances.

3.3 MATERIALS REQUIRED

1. Stock Folin's reagent is prepared by transferring 1 gm of sodium tungstate, 20gm of phosphomolybdic acid and 750 ml of water in a one litre flask fitted with a reflux condenser. Reflux for 10 hours and then cool to room temperature. Transfer the solution with washing to a 1 liter volumetric flask and make up the volume to litre. Store the reagent in a brown bottle.

   Working Folin's reagent: Transfer 10 ml of stock reagent into a 100 ml volumetric flask and make the volume 100 ml. Mix the contents well.

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2. Trichloroacetic acid (20 % w/v): Dissolve 20gm of trichloroacetic acid in distilled water and make up the volume to 100ml.

3. Sodium Carbonate Solution: Prepare saturated solution of sodium carbonate in distilled water.

4. Standard Uric Acid (20 mg/dL): Weigh 20mg uric acid, transfer it to a volumetric flask; add 50ml water and few drops of lithium carbonate (0.3%) or 0.5N NaOH. Mix and allow uric acid to dissolve; heat if necessary at 60°C. Make up the volume to 100ml. Use freshly prepared solution.

   Working solution (20mg/L ,2mg/dL): Dilute 1ml of stock solution to 10ml with0.3% lithium carbonate solution (preservative diluent).

5. Refrigerated centrifuge or microfuge

6. Colorimeter or spectrophotometer

7. Vortex mixer

8. Test tubes

9. Eppendorf tubes and pipettes

3.4 PROTOCOL

(A) Processing of serum* sample

Transfer 1.5 ml of serum sample of to a centrifuge tube. Then add 1.5 ml of distilled water and 1.5 ml of 20%TCA solution. Mix the contents well by vortexing. Leave the tube undisturbed for 5 minutes. Centrifuge at 3000 rpm for 10 minutes. Transfer 3 ml of the supernatant to tube labeled T for further analysis.

* Refer to experiment 2 for separation of serum from blood.

(B) Uric acid estimation

Pipette out the following reagents (Table 1) into clean, dry test tubes labeled blank (B), test (T) and standard (S).

Table 3.1: Additions for uric acid estimation

Reagent	Blank (mL)	Test (mL)	Standard (mL)
Test supernatant		3.0	
Standard Uric acid			1.0
TCA solution	1.0		1.0
Distilled water	2.0		1.0
Saturated Sodium Carbonate soln.	1.5	1.5	1.5
Diluted Folin's reagent	1.0	1.0	1.0
Mix properly and incubate at 37°C for 10 minutes.			

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3.5 OBSERVATIONS AND RESULTS

(a) Record absorbance of standard followed by test sample at 540 nm.

(b) Calculate the results.

Serum / plasma uric acid concentration (mg/dL) can be calculated by using the formula given below**:**

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \text{concentration of standard (mg/dL)}$$

$$= \frac{(E(T) - E(B))}{(E(S) - E(B))} \times 2$$

E= Extinction value

Reference Values:

- Pre-puberty: 3.6mg/dL
- Male: 3.5-7 mg/ dL
- Female: 2.4-6 mg/dL
- Urine: 250-750 mg/day (the upper limit may be still higher on a purine unrestricted diet.

Note that uric acid levels are influenced by age and sex. These values may vary from lab to lab primarily depending on the method used.

3.6 PRECAUTIONS

i) If the absorbance of uric acid reagent exceeds 0.2 against water, discard the reagent.

ii) In case uric acid concentration exceeds 15mg/dL, repeat the assay after diluting the sample appropriately. Multiply with the dilution factor to get the final results.

iii) Inspect and discard turbid solutions. The blank should be almost colorless.

iv) Uric acid levels are elevated by diet such as increased ingestion of red meat and treatment with some drugs (thiazide and salicylate).

v) Lipemic and increased bilirubin samples should be avoided.

SAQ 1

Q1. The excretion of urate in humans is increased on purine unrestricted diet, why?

A1. Almost all dietary purines are degraded to uric acid. They are not utilised for the synthesis of tissue nucleic acids.

Q.2. The solubility of uric acid is pH dependent. Explain.

A.2. The solubility of urate is higher than uric acid. At pH 7.5, most of it exists as monosodium urate and is the predominant form in plasma. The pH of urine is acidic (pH 5. 7) at which it is protonated.

Q.3. Indicate two conditions each that result in hyper- and hypo-uricemia, respectively.

A.3. (i) Hyperuricemia- Gout; kidney diseases; diabetes

(ii) Hypouricemia- Liver cirrhosis; high dose of salicylate

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EXPERIMENT 4

ESTIMATION OF SERUM CREATININE

Structure

4.1 Introduction
Expected Learning Outcomes
4.2 Principle
4.3 Materials required
4.4 Protocol
4.5 Observations and Results
4.6 Precautions

4.1 INTRODUCTION

You may recall from Unit 6, Block 3 of core course (BBCCT-113) entitled 'Amino acid and Nucleotide metabolism' the synthesis of creatine (methyl guanidoacetic acid) from three amino acids namely, arginine, glycine and methionine. It is taken up almost entirely by the skeletal muscles for conversion to creatine phosphate, a high energy reservoir. During muscle contraction, creatine phosphate is the phosphoryl donor for resynthesis of ATP in a reaction catalysed by creatine kinase. Each day 1% to 2% of muscle creatine / creatine phosphate is converted non- enzymatically and irreversibly to its internal anhydride, creatinine (Fig. 4.1) and excreted. The amount of creatinine produced is related to total muscle mass, which changes very little from day to day.

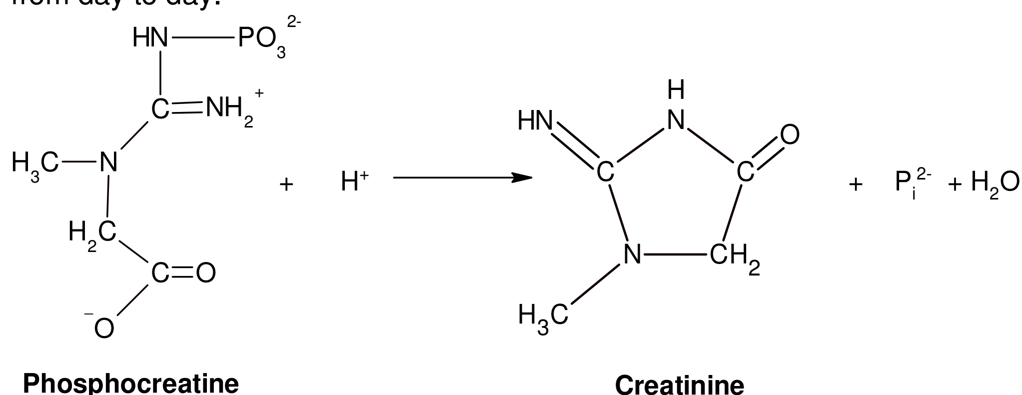

Fig. 4.1: Non enzymatic conversion of creatine/ creatine phosphate to creatinine.

As you know, kidneys filter blood and remove creatinine for excretion in urine. Blood generally has low creatinine as it is rapidly cleared by the kidneys. It is not reabsorbed or affected by urine flow rate. Creatinine is one of the non-protein nitrogen (NPN) substances excreted in urine. Measurement of creatinine clearance is used as an indicator of glomerular filtration rate (GFR) although insulin clearance is more precise. The levels of serum creatinine is found to be reliable indicator of kidney function. Elevated serum creatinine signals (>2mg/dL) is suggestive of impaired kidney function or kidney disease. In chronic renal failure creatinine value in blood can go up to 30mg/dL.

There are two broad types of methods available for creatinine estimation. These methods are either based on Jaffe reaction and its improvements or specific enzyme reactions. An example of non Jaffe assay is automated dry slide method in which ammonia produced is measured as it is hydrolysed by creatinine imino hydrolase. It is a simple, precise and high speed method. In this exercise we shall estimate serum creatinine concentration by Jaffe reaction.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the principle of the method used;
- indicate its limitations and advantages;
- independently prepare the reagents;
- perform the experiment in serum sample and repeat it with urine sample;
- calculate serum creatinine value (mg/dL) of the sample; and
- interpret the condition of the patient.

4.2 PRINCIPLE

Max Jaffe in 1886 discovered a reaction of creatinine with picric acid in an alkaline environment. At that time he did not publish an analytical method but used it to discover the nature of urinary compounds that react with picric acid. It was in 1900, Otto Folin shifted from the earlier Neubauer reaction to alkaline picrate solution and called it Jaffe's reaction. Even today after 134 years it is still a very popular method despite its limitations and development of specific enzymatic analysis essentially because it is a simple low cost procedure.

In Jaffe reaction, a 1:1 yellow-orange coloured Janovsky complex is formed between the methylene group or enolised carbonyl group of creatinine with the metaposition on the picrate anion (Fig.4.2). The colour intensity has been found to be proportional to the concentration of creatinine which is measured at 540nm.

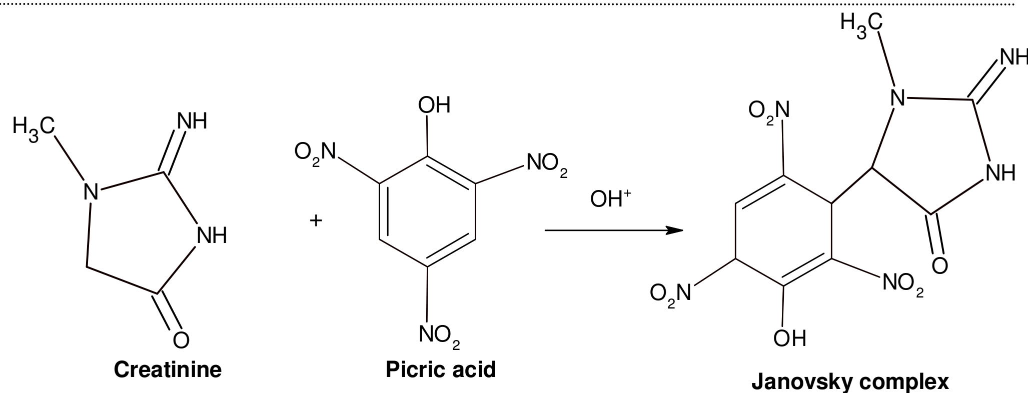

Fig. 4.2: Jaffe reaction.

The alkaline picrate method is non-specific and subject to interference from many non creatinine chromogens (pseudochromogens) such as acetoacetate, vitamin C, pyruvate, glucose, barbiturates, cephalosporin antibiotics, uric acid, urea and proteins. These substances may either cause fading or enhancement of colour depending on the conditions. Jaffe reaction is also sensitive to pH and temperature changes and affected by incubation time. There is no standard protocol for Jaffe reaction although a number of adaptations have been developed to reduce non specificity.

4.3 MATERIALS REQUIRED

1. Sodium hydroxide [NaOH] (0.75 N)

   Dissolve 30 g of sodium hydroxide (MW 40) in water and then make up the volume to 1 litre.

2. Sodium tungstate [10 % (w/v)]

   Dissolve 10 g of sodium tungstate in water and make up the volume to 100 ml.

3. Sulphuric acid [H_2SO_4; 0.66 N]

   Add slowly 18 ml conc. sulphuric acid (36N) to 200 ml double distilled water. Stir the solution and make up the volume to 1litre.

4. Standard creatinine (Stock solution)-1mg/ml

   Dissolve 50 mg of creatinine in 1-3ml of 0.1N HCl and make up the volume to 50ml with double distilled water in a volumetric flask.

   Working standard (0.01mg /5ml or 1mg/500ml): Dilute 1ml of stock solution to make up the volume to 500ml with distilled water.

5. Picric acid solution (0.04 M; MW 229)

   Dissolve 4.6 g picric acid in 50 ml double distilled water and make up the volume to 500ml. Picric acid is hygroscopic (1:2 w/v) so weigh double the amount carefully.

6. Test tubes.

7. Eppendorf pipettes.

8. Colorimeter or spectrophotometer

4.4 PROTOCOL

(A) Preparation of protein free serum

i) In a dry centrifuge test tube pipetted 1.0 ml serum*, 7.0 ml distilled water, 1.0 ml sodium tungstate and 1.0 ml of 0.66 N H_2SO_4.

ii) Mixed the contents well and centrifuge at 3000 rpm for 5 minutes.

iii) 5 ml of the supernatant is collected in another tube labeled T (Test) for creatinine estimation.

* Refer to experiment 2 for separation of serum from blood.

(B) Estimation of creatinine in serum sample by Jaffe reaction

i) The test tubes are marked test (T), standard (S) and blank (B) in duplicates.

ii) Pipetted 5.0 ml of protein free supernatant in tube (T); 5ml standard in tube (S) and 5ml water in tube (B).

iii) Added 2.0 ml of picric acid solution and 2ml ml of sodium hydroxide (0.75 N) to each tube.

iv) Left the tubes at room temperature for 20 minutes.

v) Record absorbance at 540 nm against water blank within 30minutes.

4.5 OBSERVATIONS AND RESULTS

Observation table

Test tubes	Distilled water (ml)	Standard creatinine (ml)	Protein free filtrate (ml)	Picric acid (0.04 M)	NaOH (0.75 N)		Absorbance at 540 nm
B	5	-	-	2 ml	2 ml	Mix and leave the tubes stand for 20 minutes at RT	
B	5	-	-	2 ml	2 ml		
S	-	5	-	2 ml	2 ml		
S	-	5	-	2 ml	2 ml		
T	-	-	5	2 ml	2 ml		
T	-	-	5	2 ml	2 ml		

Note: B = Blank, S = Standard & T = Test.

Calculations

Creatinine concentration (mg/dL) is:

$$= \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \frac{\text{Concentration of standard}}{\text{Volume of test sample}}$$

$$= \frac{(E(T) - E(B))}{(E(S) - E(B))} \times \frac{0.01}{0.5} \times 100 \quad \text{or} \quad \frac{(E(T) - E(B))}{(E(S) - E(B))} \times 2$$

E= Extinction value

Note: 5ml of protein free filtrate contains 0.5ml serum

Biological reference range

- Normal range of serum creatinine is 0.4 to 1.5 mg / dL

  Males: 0.6-1.2; Females: 0.5-1 mg / dL

- The normal daily urinary excretion of creatinine ranges from 1-2 g

  Normal males – 14-26 mg/ kg/ day

  Normal females - 11-20 mg/ kg / day

4.6 PRECAUTIONS

i) Jaffe reaction must be performed under controlled temperature and pH as they profoundly affect colour development.

ii) Record absorbance within 30 minutes.

iii) Handle sulphuric acid carefully. It must be mixed slowly in double distilled water (exothermic reaction).

iv) Take care while weighing hygroscopic substances (NaOH and picric acid).

SAQ 1

Q.1. What is difference between creatine and creatinine?

A.1. Creatinine is an anhydride of creatine.

Q.2. How is creatinine produced in muscle?

A.3 It is produced by irreversible non-enzymatic dehydration of creatine / creatine phosphate.

Q.3. What is the clinical significance of creatinine estimation in serum?

A.3. The levels of serum creatinine are a reliable indicator of kidney function. Elevated serum creatinine signals impaired kidney function or kidney disease. Creatinine levels in blood are elevated due to poor clearance by the kidneys.

Q.4. What is the normal daily range of urinary creatinine excretion?

A.4. It ranges from 1-2 g / day.

Q.5. Enlist two advantages and disadvantages of Jaffe's method.

A.5. Advantages: Simple and coat effective.

Disadvantages: Needs rigidly controlled condition and gives positive reaction with many non creatinine chromogens.