KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2017 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : Practical 8: Metabolism of amino acids and nucleic acids
SEMESTER : III
SUBJECT CODE : 16BCU312 CLASS : II B.Sc.(BC)

Programme Objective: To learn and understand liver and kidney markers and it's clinical significance

Programme learning outcome:

The students after completion of this course will have

- Clear understanding of normal values of liver and kidney markers.
- The students can get clear understanding of disease conditions in which the markers are altered and their clinical significance.
- The students will have a clear knowledge on handling of colorimeter and centrifuge.
- The students will develop the skill of preparing molar and normal solutions.

Experiments

1. Assay of serum transaminases – SGOT and SGPT.
2. Estimation of serum urea.
3. Estimation of serum uric acid.
4. Estimation of serum creatinine.

REFERENCES

Nelson, D.L. and Cox, M.M., (2013). Lehninger: Principles of Biochemistry 6th ed., W.H. Freeman and Company (New York), ISBN:13:978-1-4641-0962-1 / ISBN:10:1-4641-0962-1.

Devlin, T.M., (2011). Textbook of Biochemistry with Clinical Correlations 7th ed., John Wiley & Sons, Inc. (New York), ISBN: 978-0-470-28173-4 / BRV ISBN: 978-0-470-60152-5.

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SEMESTER : **III**
SUBJECT CODE : **16BCU312** **CLASS** : **II B.Sc., BC**

Practical 8 – Metabolism of amino acids and nucleic acids

LAB MANUAL

1. ASSAY THE ACTIVITY OF SGOT (OR) ASPARTATE TRANSAMINASE

AIM

To determine the activity of aspartate transaminase in the given serum sample.

Principle

Aspartate transaminase catalyses the reverse interconversion between aspartate and glutamate and their 2 oxo analogues.

L-Aspartate +α-oxoglutarate $\xleftrightarrow{SGOT}$ Oxaloacetate + L-glutamate

The oxalo acetate is measured by colorimeter by a reaction with 2, 4 – dinitro phenyl hyrdrazine giving a brown coloured hydrazone derivative after the addition of 0.4N sodium hydroxide. The colour developed is read at 520 nm.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)

   Solution A: Disodium hydrogen phosphate – 14.2g/L

   Solution B: Potassium dihydrogen phosphate – 13.6g/L

   The buffer is prepared by mixing 420ml of Solution.A and 80 ml of solution.B.

2. **SGOT Buffered substrate:**

   Dissolve 2.66 g of aspartic acid and 29.2 mg of α keto glutaric acid in 1N NaOH and made up to 100 ml with phosphate buffer. The pH is adjusted to 7.4.

3. **Dinitro phenyl hydrazine**

20 mg of dinitro phenyl hydrazine in 100 ml of 1N hydro chloric acid.

4. **Stock pyruvate (20 mmol)**

   220 mg of sodium pyruvate dissolved in 100 ml of phosphate buffer and stored in refrigerator. The concentration is 2.2mg/ml.

5. **Working standard**

   10 ml of stock standard is diluted to 100 ml with phosphate buffer. The concentration is 220µg/ml which is 20µm/ml.

PROCEDURE

Into a series of test tubes pipetted out 0.1 to 0.5ml of working standard pyruvate solution corresponding to the concentration range 0.2 to 1.0 µg/ml and made up the volume in all the test tubes to 1.0 ml by adding buffered substrate. 1ml of buffered substrate is taken in different test tubes labelled as control and test. 0.2 ml of serum is added to the test alone.

0.2ml of phosphate buffer is added to all the test tube and the standard tubes are incubated at 37^0 C for 30 minutes and the control and test are incubated for 1hour at 37^0 C.

At the end of the incubation 1.0 ml of DNPH is added to all the test tubes and 0.2 ml of serum is added to control tubes, were incubated at 37^0 C for 15mins.

Then 10.0 ml of sodium hydroxide is added to all the tubes and the brown colour development was read at 540nm.

From the graph obtained by plotting concentration range on x axis and the corresponding optical density values on y axis the enzyme activity is calculated.

Normal range – 3-16IU/Litre.

REAGENTS	B	S1	S2	S3	S4	S5	C	T1	T2
Volume of working standard (ml)	**-**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**-**	**-**	**-**
Concentration of working standard (µg)	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**-**	**-**	**-**
Volume of buffered substrate (ml)	**1.0**	**0.9**	**0.8**	**0.7**	**0.6**	**0.5**	**1.9**	**1.0**	**1.0**
Volume of phosphate buffer (ml)	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**0.2**
Incubate at 37ºC for one hour									
Volume of DNPH (ml)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**-**	**-**
Incubate at 37ºC for 15 minutes									
Volume of sodium hydroxide (ml)	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**
Optical density at 540 nm									

CALCULATION

Colorimeter reading ___ corresponds to ____ µm of pyruvate

That is 0.2 ml of serum contains ___ µm of pyruvate

Therefore 1000 ml of serum liberate $\frac{0.1 \times 1000}{0.2}$

= ____ µm of pyruvate

Activity of SGOT is given by $\frac{=}{60}$

= _____ IU/litre

RESULT

The serum aspartate transaminase activity was found to be ----------

2. ASSAY THE ACTIVITY OF SGPT (OR) ALANINE TRANSAMINASE

Aim

To assay the activity of alanine amino trasferase in serum.

Principle

When serum is incubated with substance containing α keto glutarate and L-alanine L-glutamate and pyruvate are formed.

The enzyme catalyses the following reaction:

L-Alanine + α-oxoglutarate $\longrightarrow$ Pyruvate + L-glutamate

Pyruvate so formed is coupled with 2, 4- dinitro phenylhydrazine to give the corresponding hydrazone which gives a brown colour which is measured at 540 nm.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)

   Add 11.3 g dry anhydrous disodium hydrogen phosphate and 2.7 g dry anhydrous potassium dihydrogen phosphate in one litre volumetric flask and make up to the mark with water. Check the pH and store at 4ºC.

2. Buffered substrate:

   Dissolve 1.78 g of alanine and 29.2 mg of α keto glutaric acid in 1N NaOH and made up to 100 ml with phosphate buffer. The pH is adjusted to 7.4.

3. Dinitro phenyl hydrazine

   20 mg of dinitro phenyl hydrazine in 100 ml of 1N hydro chloric acid.

4. Stock pyruvate (20 mmol)

   220 mg of sodium pyruvate dissolved in 100 ml of phosphate buffer and stored in refrigerator. The concentration is 2.2mg/ml.

5. Working standard

   10 ml of stock standard is diluted to 100 ml with phosphate buffer. The concentration is 220µg/ml which is 20µm/ml.

6. 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water

Procedure

0.1 – 0.5 ml of pyruvate standard of concentration range 0.2 – 1µm is pipette out in to a series of test tubes. The volume is made up to 1.0 ml with buffered substrate. 1.0 ml of buffered substrate serves as blank.

Added 1.0 ml of buffered substrate and 0.2 ml of phosphate buffer to the test and control tubes. Added 0.2 ml of serum to the test alone. The standards, test and control were incubated along with the blank at 37^{o}C for 30 minutes. At the end of incubation period added 1.0 ml of 2, 4 dinitro phenyl hydrazine to all the tubes. To the control tube added 0.2 ml of serum after arresting the reaction with 2, 4 dinitro phenyl hydrazine. All the tubes are incubated at 37^{o}C for 15 minutes. At the end of incubation period added 10.0 ml of 0.4N NaOH to all the tubes. The brown colour developed was read at 540 nm after 10 minutes.

From the standard graph obtained by plotting the concentration of pyruvate on x axis and optical density value on y axis the enzyme activity is measured.

Normal value: 4 – 17IU/litre.

REAGENTS	**B**	**S1**	**S2**	**S3**	**S4**	**S5**	**C**	**T1**	**T2**
Volume of working standard (ml)	**-**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**-**	**-**	**-**
Concentration of working standard (µg)	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**-**	**-**	**-**
Volume of buffered substrate (ml)	**1.0**	**0.9**	**0.8**	**0.7**	**0.6**	**0.5**	**1.9**	**1.0**	**1.0**
Volume of phosphate buffer (ml)	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**	**0.2**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**0.2**
Incubate at 37ºC for about 30 minutes									
Volume of DNPH (ml)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**-**	**-**
Incubate at 37ºC for 15 minutes									
Volume of sodium hydroxide (ml)	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**
Optical density at 540 nm									

CALCULATION

Colorimeter reading ___ corresponds to ____ µm of pyruvate

That is 0.2 ml of serum contains ___ µm of pyruvate

Therefore 1000 ml of serum liberate $\frac{0.1 \times 1000}{0.2}$

= ____ µm of pyruvate

Activity of SGOT is given by $\frac{=}{30}$

= _____ IU/litre

Result

The activity of SGPT in serum is found to be _____ IU/litre.

3. ESTIMATION OF UREA
DAM TSC Method

Aim

To estimate the amount of urea present in the given unknown and serum sample.

Principle

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

Reagents

1. Diacetyl monoxime:

   1.56g of diacetyl monoxime in 250ml of distilled water.

2. Thiosemi carbazide:

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

3. Acid reagent:

   (a) Ferric chloride reagent:

   324mg of ferric chloride is dissolved in 10ml of 56% ortho phosphoric acid (Stored in brown bottle).

   (b) 20% sulphuric acid:

   20ml of sulphuric acid in 80ml of water.

   In 1 litre of 20% sulphuric acid added 1ml of ferric chloride reagent.

4. Stock standard urea solution:

   Dissolved 100mg of urea in 100ml of distilled water. 1.0ml of this solution contains 1mg of urea.

5. Working standard urea solution:

   2.0ml of stock standard solution was made upto 100ml with distilled water. 1.0ml of this solution contains 20 µg of urea.

Procedure

Into a series of test tubes pipetted out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of working standard urea solution corresponding to 10, 20, 30, 40 and 50 µg of urea respectively. The given unknown solution was made up to 100 ml with distilled water and from this 1.0 ml was taken for the experiment. In a centrifuge tube added 0.2 ml of serum and 1.8 ml of 10% TCA and centrifuged for 10 minutes at 3000 rpm.

S.No	SOLUTION		Volume of distilled water(ml)	Volume of DAM (ml)	Volume of TSC	Volume of acid reagent (ml)	Allowed to stand for 15 minutes	Optical density at 540nm
	Volume in ml	Concentration in µg						
1	Blank	-	3.0	1.0	1.0	3.0		
	Standard				1.0	3.0		
2	0.5	10	2.5	1.0	1.0	3.0		
3	1.0	20	2.0	1.0	1.0	3.0		
4	1.5	30	1.5	1.0	1.0	3.0		
5	2.0	40	1.0	1.0	1.0	3.0		
6	2.5	50	0.5	1.0	1.0	3.0		
	Unknown				1.0	3.0		
7	1.0	-	2.0	1.0	1.0	3.0		
8	1.0	-	2.0	1.0	1.0	3.0		

From this 1.0 ml of supernatant is taken for the experiment. The volume in all the test tubes were made-up to 3.0 ml with distilled water. Then added 1.0ml of diacetyl monoxime, 1.0 ml of thiosemi carbazide and 3.0ml of acid reagent to all the test tubes. Mixed well and heated vigorously in a boiling water bath for 20 minutes. Along with this a blank was also treated in a similar way. Remove the tubes, cooled and read against the reagent blank at 540 nm in a colorimeter.

A standard graph was drawn by plotting the concentration of urea on x axis and colorimetric reading on y axis. From the standard graph the concentration of urea in the given unknown solution and serum sample was calculated.

Result

The amount of urea present in the given unknown solution is ---------- mg/dl

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

4. ESTIMATION OF URIC ACID
Caraway Method

Aim

To estimate the amount of uric acid present in the given unknown and serum sample.

Principle

Uric acid reduces sodium phosphotungstic acid in the given alkaline medium to give a blue colour which is measured colorimetrically using a red filter at 640nm.

Reagents

1. 14% Sodium carbonate solution
2. Uric acid Reagent:

   Dissolved 5g of sodium tungstate in 400 ml of distilled water. Added 40 ml of 84% phosphoric acid and refluxed gently for 2 hours. Cooled, transferred to 500 ml standard flask and made up to the mark with distilled water. Stored the reagent in a brown bottle. Diluted 1ml to 10ml before use.

3. Stock standard:

   Weighed about 100 mg of uric acid in a small beaker. Also weighed 60 mg of lithium carbonate into the beaker. Added 15-20 ml of water. Heated the solution to about $60^{o}C$ and poured into 100 ml standard flask and made up to the mark with distilled water.

4. Working standard solution:

   Diluted 2 ml of stock standard to 100 ml with distilled water. 1ml of the working standard solution contains 20 µg of uric acid.

Procedure

Into a series of test tubes pipetted out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of working standard solution corresponding to the µg values 10, 20, 30, 40 and 50 respectively. The given unknown solution was made upto 100ml with distilled water. From that 1.0 ml was taken for the experiment. The volume of all the test tubes were made upto 3.0 ml with distilled water. To all the test tubes added 1ml of uric acid reagent follower by 1.0 ml of 14% sodium carbonate

solution and allowed it to stand for 15minutes and the blue colour developed was read in the colorimeter at 640 nm against a reagent blank in a colorimeter.

A standard graph was drawn by plotting the concentration of uric acid on x-axis and the colorimeter reading on y-axis. From this the concentration of uric acid present in the unknown solution was calculated.

Estimation of Uric Acid

S.No	SOLUTION		Volume of distilled water(ml)	Volume of sodium carbonate (ml)	Volume of Uric acid reagent (ml)	Allowed to stand for 15 minutes	Optical density at 640nm
	Volume in ml	Concentration in µg					
1	Blank	-	3.0	1.0	1.0		
	Standard						
2	0.5	10	2.5	1.0	1.0		
3	1.0	20	2.0	1.0	1.0		
4	1.5	30	1.5	1.0	1.0		
5	2.0	40	1.0	1.0	1.0		
6	2.5	50	0.5	1.0	1.0		
	Unknown						
7	1.0	-	2.0	1.0	1.0		
8	1.0	-	2.0	1.0	1.0		

Calculation:

Optical density of __x_ corresponds to __y_ μg of uric acid

1.0 ml of the given unknown solution contains _y_ μg of uric acid.

∴100 ml of the given unknown solution contains $\frac{y \times 100}{1.0 \times 1000}$

= __z__ mg

Result

The amount of uric acid present in 100ml of the given unknown solution is found to be ---------- mg.

5. ESTIMATION OF CREATININE

Caraway method

Aim:

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

Principle:

Creatinine is estimated by making use of jaffe s reagent in which the creatinine gives an orange yellow color with alkaline picrate solution whose value is measured calorimetrically at 540 nm**.**

Reagents:

1. **Stock standard solution**

   100 mg of creatinine in 100 ml of 0.1N HCL. The concentration is 100 µg/ml.

2. Working standard solution

   4.0 ml of the stock is made upto 100 ml with distilled water. Concentration. is 0.04mg/ml.

3. 10% NaOH
4. 1% Picric acid solution
5. 5% sodium tungstate
6. 2/3N sulphuric acid

Preparation of serum sample:

Dilute 2ml of serum/plasma with 2ml of distilled water and precipitate the protein by adding 2ml of sodium tungstate and 2ml of 2/3N sulphuric acid. Added the reagent dropwise with constant shaking, allow to stand for 10 mins and centrifuged. Collect 3ml of supernatant and 1ml of picric acid and 1ml of sodium hydroxide.

Procedure:

0.4, 0.8, 1.2, 1.6 and 2.0ml of working standard creatinine solution corresponding to µg respectively 16,32,48,64 & 80 µg values are pipetted out into a series of test tubes.

3ml of supernatant collected from serum sample pipetted out into another test tube.

The volume of the tubes are made upto 8ml with distilled water. A blank is also made upto 8ml with distilled water. 1ml of 10% sodium hydroxide and 1ml of 1% picric acid to all the test tubes and mixed well.

The orange yellow color was developed after 15min was read at 540nm against the reagent blank. A standard graph was drawn by plotting creatinine concentration on x axis and optical density an y axis through which the amount of creatinine was calculated.

Estimation of creatinine

S.No	SOLUTION		Volume of distilled water(ml)	Volume of Na_2CO_3 (ml)	Volume of Picric acid	Allowed to stand for 15 minutes	Optical density at 540nm
	Volume in ml	Concentration in µg					
1	Blank	-	8.0	1.0	1.0		
	Standard				1.0		
2	0.4	16	7.6	1.0	1.0		
3	0.8	22	7.2	1.0	1.0		
4	1.2	48	6.8	1.0	1.0		
5	1.6	64	6.4	1.0	1.0		
6	2.0	80	6.0	1.0	1.0		
	Unknown				1.0		
7	3.0	-	5.0	1.0	1.0		
8	3.0	-	5.0	1.0	1.0		

Calculation:

Optical density of __x_ corresponds to __y_ µg of creatinine

1.0 ml of the given unknown solution contains _y_ µg of creatinine.

∴100 ml of the given unknown solution contains $\frac{y \times 100}{1.0 \times 1000}$

= __z__ mg

Result:

The amount of creatinine present in the given blood sample is found to be-------

The amount of creatinine present in the 100ml of unknown solution is found to be -------

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Practical 8 – Metabolism of amino acids and nucleic acids

Viva questions

1. Name some liver markers.
2. Name some kidney markers.
3. Give the principle of assay of aspartate transaminase activity.
4. Give the principle of assay of alanine transaminase.
5. What are transaminases?
6. What is the reaction taking place in transaminases?
7. What is the normal value of aspartate transaminase.
8. What is the normal value of alanine transaminases?
9. What is the indication of liver damage?
10. Give the clinical significance of transaminases.
11. How will you identify kidney damage?
12. What is the normal value of urea in serum?
13. What is the normal value of uric acid in serum?
14. What is the normal value of creatinine in serum?
15. Give the principle of assay of urea in serum.
16. Give the principle of assay of uric acid in serum.
17. Give the principle of assay of creatinine in serum.
18. How will you confirm kidney damage?
19. What are the reagents used in urea estimation?
20. What are the reagents used in uric acid estimation?
21. What are the reagents used in creatinine estimation?

22. What is the colour developed in urea estimation?
23. What is the colour developed in uric acid estimation?
24. What is the colour developed in creatinine estimation?
25. How will you prepare normal solution?
26. How will you prepare molar solution?
27. Write the preparation of 1N NaOH.
28. What is the nanometer range of urea.
29. What is the nanometer range of uric acid.
30. What is the nanometer range of creatinine.
31. What is the colouring reagent used in urea estimation?
32. What is the colouring reagent used in uric acid estimation?
33. What is the colouring reagent used in creatinine estimation?
34. Write the clinical significance of urea.
35. Give the clinical significance of uric acid.
36. Give the clinical significance of creatinine.
37. Name some end products of kidney clearance.
38. What is the colour developed in SGOT assay?
39. What is the colour developed in SGPT assay?
40. Write the reaction cataysed by transaminases.