

Appendices

Appendix I

Determination of Kidney-Body Weight Ratio

The percentage body weight ratio was calculated by dividing the fresh weight of each organ by the weight of the life animal and then multiplied by 100.

$$\text{Percentage Organ to Body Weight} = \frac{\text{Fresh Weight of Organ} \times 100}{\text{Weight of Animal}}$$

Determination of Total Protein Concentration

The protein concentrations of homogenate and serum were determined using the Biuret method described by Gornall *et al.*, (1949).

Principle

In alkaline solution, Cu^{2+} forms coloured complexes; purple/pink, with peptides or proteins in which the Cu^{2+} is linked to more than one group of the peptide.

Procedure

Calibration Curve Procedure

The calibration curve used for protein determination was obtained using varying concentrations of bovine serum albumin (BSA). The concentration of the BSA stock solution used was 10 mg/ml, and from this, varying volumes were pipetted into test tubes and made up to 1.0 ml with distilled water, such that concentrations of 1 – 10 mg/ml were achieved. Thereafter, 4.0ml of Biuret reagent was added to each of the test tubes. The resulting mixture was mixed thoroughly and allowed to stand for 30 minutes at room temperature for maximum colour development. The blank was made up of 1.0 ml of distilled water and 4.0 ml of Biuret reagent. The absorbance was read at 540nm against the blank. The absorbance values obtained were used for plotting of the calibration curve for protein determination.

After the addition of Biuret reagent, the content was mixed thoroughly and allowed to stand for 30 minutes at room temperature for maximum colour development, after which the absorbance was read at 540 nm against a reagent blank (Appendix VI). The absorbance values obtained were used for plotting of the calibration curve for protein concentration determination (Appendix I).

Determination of Total Protein concentration in serum and tissue homogenate

For the test, 1.0 ml of the appropriately diluted tissue homogenate was pipetted into test tubes and 4.0 ml of Biuret reagent was added. The mixture was thoroughly mixed and allowed to stand for 30 minutes. The blank was made up of 1.0 ml of distilled water and 4.0 ml of Biuret reagent. The absorbance was read against reagent blank at 540 nm.

Calculation

The corresponding protein concentrations for the absorbance were extrapolated from the calibration curve and used for the calculation of protein concentration.

$$\text{Protein Concentration (mg/ml)} = C_s \times D.F$$

Where;

C_s =Corresponding concentration obtained from the calibration curve.

D.F = Dilution factor.

DETERMINATION OF SPECIFIC ACTIVITY OF ENZYMES

Determination of Alkaline Phosphatase activity in homogenate and serum

The method described by Wright *et al.* (1972) was employed.

Principle

The amount of phosphate ester hydrolyzed within a given period of time is a measure of the phosphatase enzyme. Para-nitrophenyl phosphate (pNPP) is hydrolyzed to para-nitrophenol and phosphoric acid at pH of 10.1 by the enzyme. The para-nitrophenol confers a yellowish colour on reaction mixture and its intensity is measured at 400 nm.

Procedure

Unto test tube, 2.2 ml of carbonate buffer (0.1 M), 0.1 ml of MgSO₄·7H₂O (0.1 M) and 0.2 ml of appropriately diluted tissue supernatant or serum were mixed and incubated at 37°C for 10 minutes. Afterwards, 0.5 ml of p-Nitrophenyl phosphate (10mM) was added and the resulting mixture was mixed and incubated at 37°C for 10 minutes. Thereafter, 2.0 ml of NaOH (1 N) was added to the mixture. The resulting mixture was allowed to stand and the absorbance was read at 400 nm against a blank. Meanwhile, the blank was likewise prepared but 0.2 ml of distilled water was used instead of the 0.2 ml tissue supernatant/serum.

Enzyme activity was calculated using the following expression:

$$\text{Enzyme Activity (nmol/min/ml)} = \frac{\Delta\text{OD}/\text{min} \times 1000 \times \text{TV} \times \text{F}}{18.8 \times \text{SV} \times \text{L}}$$

Where:

$\Delta\text{OD}/\text{min}$ = Change in optical density of reaction mixture per minute.

TV = Total volume of the reaction mixture.

F = Dilution factor. SV = Volume of enzyme source

L = Light pathlength (cuvette width: 1cm)

18.8 = Extinction co-efficient of 1 μm of p-nitrophenol in an alkaline solution of one ml volume and 1 cm path length.

1000 = The factor introduced to enable the enzyme activity to be expressed in nmol/min/ml.

$$\text{Specific enzyme activity (nmol/min/mg protein)} = \frac{\text{Enzyme activity}}{\text{Protein concentration}}$$

Determination of Aspartate Aminotransferase activity in homogenate and serum

The method described by Reitman and Frankel (1957) was used to determine the activity of aspartate aminotransferase.

Principle

The enzyme, aspartate aminotransferase, catalyzes the reversible reaction involving α -ketoglutarate and L-aspartate to form L-glutamate and oxaloacetate. The oxaloacetate formed reacts with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. After a given time, the 2,4-dinitrophenylhydrazone of oxaloacetate is then determined spectrophotometrically in alkaline solution. Some of the oxaloacetate formed spontaneously decarboxylate to pyruvate. The assay mixture therefore contains oxaloacetate, pyruvate and α -ketoglutarate, all of which form 2,4-dinitrophenylhydrazones with absorption maxima at different wavelengths. During the reaction, the concentration of α -ketoglutarate decreases while those of oxaloacetate and pyruvate increases. To keep low the contribution of the α -ketoglutarate hydrazone to the colour, the measurements are made at wavelengths between 500-550nm, higher than the absorption maximum which brings about the greatest differences between the extinctions of the three possible hydrazones.

Calibration Curve Procedure

A calibration curve was prepared using varying concentrations of sodium pyruvate solution as standards. The concentration of the sodium pyruvate stock solution used was 2 mM. From this, different concentrations of the standard solution were made by pipetting different volumes (0.1 to 0.5 ml) into five different test tubes and making them up to 1.0 ml with varying volumes (0.9 to 0.5 ml) of AST-buffered substrate. Thereafter, 0.2 ml of distilled water and 1.0 ml of 2,4-DNPH (1 mM) were added. This was properly mixed and allowed to stand for 20 minutes. Then, 10.0 ml of NaOH (0.4 N) was added and the mixture was mixed and allowed to stand for 5 minutes. The reagent blank was prepared by pipetting 1.0 ml of AST buffered substrate, 0.2 ml of distilled water and 1.0 ml of DNPH which was also allowed to stand for 20 minutes, then the addition of 10.0 ml of NaOH (0.4 N). The absorbance was read at 546 nm against the reagent blank (Appendix VIII). The obtained values were used in plotting the calibration curve (Appendix II).

Determination of AST activity in tissue homogenate and serum

For the test, 0.1 ml of appropriately diluted supernatant/serum was added to a test tube to which 0.5 ml of AST buffered substrate was also added and the resulting mixture was mixed and incubated at 37°C in water bath for 60 minutes. Thereafter, 0.5 ml of 2,4-DNPH (1 mM) was added and the mixture was mixed and allowed to stand at room temperature for 20 minutes. 5.0 ml of NaOH (0.4 N) was then added. The mixture was mixed and allowed to stand for 5 minutes. The blank was likewise prepared but 0.1 ml of distilled water was used instead of the 0.1 ml tissue supernatant/serum. The absorbance of test sample was read against blank at 546nm.

Calculation

The corresponding pyruvate concentration of the absorbance was obtained from the calibration curve and used for calculating the specific activity of the enzyme using the formula:

$$\text{Specific activity (nmol/ml/mg protein)} = \frac{\text{P.C} \times 1 \times \text{D.F}}{\text{I.P} \times \text{P}}$$

Where: P.C= pyruvate concentration

D.F= Dilution factor

I.P= incubation period

P= Protein concentration

Determination of Alanine Aminotransferase activity in homogenate and serum

The method described by Reitman and Frankel (1957) was used to determine the activity of alanine aminotransferase.

Principle

Alanine aminotransferase acts on the L-alanine in the presence of α -ketoglutarate to yield pyruvate and glutamate, in a reversible reaction. After a given time, the pyruvate formed is determined spectrophotometrically by treating the 2,4-dinitrophenylhydrazone of pyruvate with alkali. The residual α -ketoglutarate also forms a hydrazone but its maximum absorption wavelength in alkali solution is different from that of pyruvate hydrazone.

Calibration curve Procedure

A calibration curve was prepared using varying concentrations of sodium pyruvate solution as standard. The concentration of the sodium pyruvate stock solution used was 2 mM. From this, different concentrations of the standard solution were made by pipetting different volumes (0.1 to 0.5 ml) into five different test tubes and making them up to 1.0 ml with varying volumes (0.9 to 0.5 ml) of ALT-buffered substrate. Thereafter, 0.2 ml of distilled water and 1.0 ml of 2,4-DNPH (1 mM) were added. This was properly mixed and allowed to stand for 20 minutes. Then, 10.0 ml of NaOH (0.4 N) was added and the mixture was mixed and allowed to stand for 5 minutes. The reagent blank was prepared by pipetting 1.0 ml of ALT-buffered substrate, 0.2 ml of distilled water and 1.0 ml of DNPH which was also allowed to stand for 20 minutes, then the addition of 10.0 ml of NaOH (0.4 N). The absorbance was read at 546 nm against the reagent blank (Appendix IX). The obtained values were used in plotting the calibration curve (Appendix III).

Determination of ALT activity in tissue supernatant and serum

The ALT activity in sample was determined using appropriately diluted tissue supernatant. 0.1 ml of this appropriately diluted supernatant was added to a test tube to which 0.5 ml of ALT buffered substrate was added and the resulting mixture was mixed and incubated at 37°C in water bath for 30 minutes. Then, 0.5 ml of 2,4-DNPH (1 mM) was added and the mixture was mixed and allowed to stand at room temperature for 20 minutes. 5.0 ml of NaOH (0.4 N) was then added. The mixture was mixed and allowed to stand for 5 minutes. The blank was likewise prepared but 0.1 ml of distilled water was used instead of the 0.1 ml tissue supernatant. The absorbance of test sample was read against blank at 546nm.

Calculation

The corresponding pyruvate concentration of the absorbance was obtained from the calibration curve and used for calculating the specific activity of the enzyme using the formula:

$$\text{Specific activity (nmol pyruvate/ml/mg protein)} = \frac{\text{P.C} \times 1 \times \text{D.F}}{\text{I.P} \times \text{P}}$$

Where: P.C= pyruvate concentration D.F= Dilution factor I.P= incubation period P= Protein concentration.

Determination of Glutamate Dehydrogenase Activity in homogenate

The method described by Bradley *et al.* (1979), with little modification, was used for the determination of glutamate dehydrogenase activity.

Principle

L-Glutamic acid is oxidized by nicotinamide-adenine dinucleotide (NAD^+) in the presence of glutamate dehydrogenase (GLDH), leading to the formation of α -ketoglutarate, reduced nicotinamide-adenine dinucleotide (NADH) and ammonium ions (NH_4^+).

Procedure

A known volume 800 μl of Tris-HCl (62.5 mM, pH 8.0) was added to a test tube to which 100 μl of NAD^+ (15 mM) was also added. 200 μl of appropriately diluted serum was added and the mixture was well mixed and allowed to stand for 3 minutes at 25°C. The absorbance A1 was measured and after 5 minutes the absorbance A2 was measured. 100 μl of L-Glutamate (150 mM) was then added to the reaction mixture. This was well mixed and the absorbance A3 was taken. The mixture was allowed to stand for 5 minutes at 25°C and then absorbance A4 was taken. The absorbance was taken at 340 nm.

Calculation

$$\text{Enzyme Activity } (\mu\text{mol/ml/min}) = \frac{\Delta A/\text{min} \times V_t \times D.f}{6.22 \times 1.0 \times V_s}$$

Where;

$$\Delta A/\text{min} = (A_4 - A_3) - (A_2 - A_1)$$

5 min _____

V_t = Total reaction mixture volume, V_s = Volume of enzyme source used, D.f = Dilution factor, 6.22 = Extinction coefficient factor of NADH, 1.0 = Cuvette light path

$$\text{Specific Activity } (\mu\text{mol/min/mg protein}) = \frac{\text{Enzyme activity}}{\text{Protein Concentration}}$$

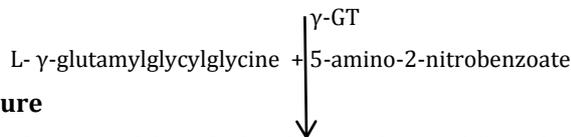
Determination of Gamma-glutamyl Transferase Activity in homogenate and serum

The activity of gamma-glutamyl transferase activity in the serum was determined by the method described by Teitz (1987).

Principle

The substrate L- γ -glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylglycine is converted by γ -GT in the sample to 5-amino-2-nitrobenzoate which can be measured at 405 nm.

L- γ -glutamyl-3-carboxy-4-nitroanilide + glycylglycine



Procedure

The protocol described in the assay kit was followed.

Protocol for the Determination of γ -GT Activity in the homogenate and serum

A known volume of 0.10 ml of the test sample was pipetted into a test tube; 1 ml of Reagent was added to it. It was mixed and initial absorbance at 405 nm was read and timer was started simultaneously. Absorbance was also read again after 1, 2 and 3 minutes.

Calculation

$$U/L = 1158 \times \Delta A \text{ 405 nm/min}$$

Where:

$$\Delta A = \text{Change in absorbance } (A_4 - A_3 - A_2 + A_1)$$

Histological study

Portions of the kidney were fixed in 10% (v/v) formaldehyde, dehydrated through ascending grades of ethanol (70%, 90%, and 95%, v/v), cleaned in xylene, and embedded in paraffin wax (melting point 56 °C). Organ sections prepared according to the procedure described by Drury and Wallington and stained with Hematoxylin and Eosin (H & E). The photomicrographs were captured at x 100 with software, Presto Image Folio package.

APPENDIX II

PREPARATION OF REAGENTS

Sucrose Solution (0.25M)

85.575g sucrose was weighed and dissolved in distilled water and made up to 1,000ml in a standard volumetric flask with more distilled water. The solution was refrigerated before usage.

Biuret Reagent

1.5g of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 6.0g of sodium potassium tartarate ($\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) were dissolved in distilled water to make 500ml solution A.

30g of sodium hydroxide (NaOH) was dissolved in distilled water to make 300ml solution (10% w/v). This is solution B.

Solution B was then added slowly to solution A with continuous stirring and the resulting solution transferred into a 1,000ml standard flask and the volume made up with distilled water. This was then stored.

Standard Bovine Serum Albumin (BSA, 10mg/ml)

0.5g of bovine serum albumin was dissolved in a little amount of 0.5N NaOH and then made up to 50ml with the same solution.

ALKALINE PHOSPHATASE

Carbonate Buffer (0.1M, pH 10.1)

0.84 g of sodium bicarbonate (NaHCO_3) was dissolved in 100 ml of distilled water (0.1M NaHCO_3) to give SOLUTION A. 1.06 g of sodium carbonate (Na_2CO_3) was dissolved in distilled water and made up to 100ml (0.1M Na_2CO_3) to give SOLUTION B. Solution A was added to Solution B with continuous stirring until pH of the mixture comes to 10.1.

MgSO₄ (0.1M)

1.2 g of MgSO_4 was dissolved in distilled water and consequently made up to 100 ml in a standard volumetric flask.

Para-Nitro Phenyl Phosphate (pNPP, 10mM)

0.37114 g of para-nitro phenyl orthophosphate was dissolved in distilled water and made up to 100 ml in a 100 ml standard volumetric flask

1N NaOH

20 g of sodium hydroxide pellet was dissolved in distilled water and made up to 500 ml in a standard volumetric flask.

AMINOTRANSFERASES

AST-Buffered substrate solution (0.1M, pH 7.4)

1.5g K_2HPO_4 and 0.2g KH_2PO_4 were dissolved in 10ml distilled water after which 1.32g L-aspartic acid was added and then 0.03g α -ketoglutarate. 60ml of distilled water was then added and the pH adjusted to 7.4 with 0.4N NaOH solution and the resulting solution was made up to 100ml using distilled water.

ALT-Buffered substrate solution (0.1M, pH 7.4)

1.5g K_2HPO_4 and 0.2g KH_2PO_4 were dissolved in 10ml distilled water after which 1.78g DL-alanine was added and then 0.03g α -ketoglutarate. 60ml of distilled water was then added and the pH adjusted to 7.4 with 0.4N NaOH solution and the resulting solution was made up to 100ml using distilled water.

0.4N NaOH

16g of NaOH pellet was dissolved in a little amount of distilled water and then made up to 1,000ml in a standard volumetric flask.

Pyruvate standard solution for AST and ALT (2mM)

0.022g of sodium pyruvate was dissolved in distilled water and made up to 100ml with more distilled water.

2,4-Dinitrophenylhydrazine (1mM)

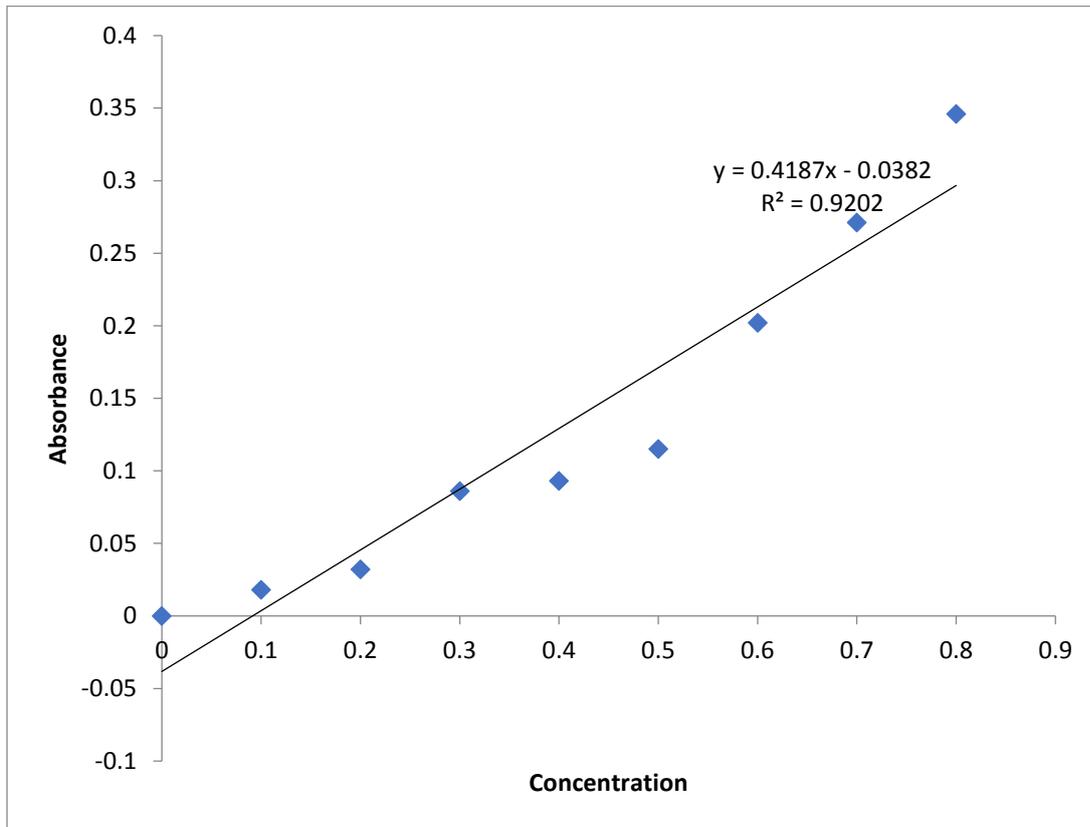
200mg of 2,4-dinitrophenylhydrazine was dissolved in hot 1N HCl and allowed to cool. The solution was then made up to 1,000ml, using more 1N HCl, in a standard flask.

GLUTAMATE DEHYDROGENASE

150mM L-Glutamate 0.55174 g of L-glutamate was dissolved in about 10 ml of distilled water and made up to 25 ml with more distilled water in a standard 25 ml volumetric flask. 135

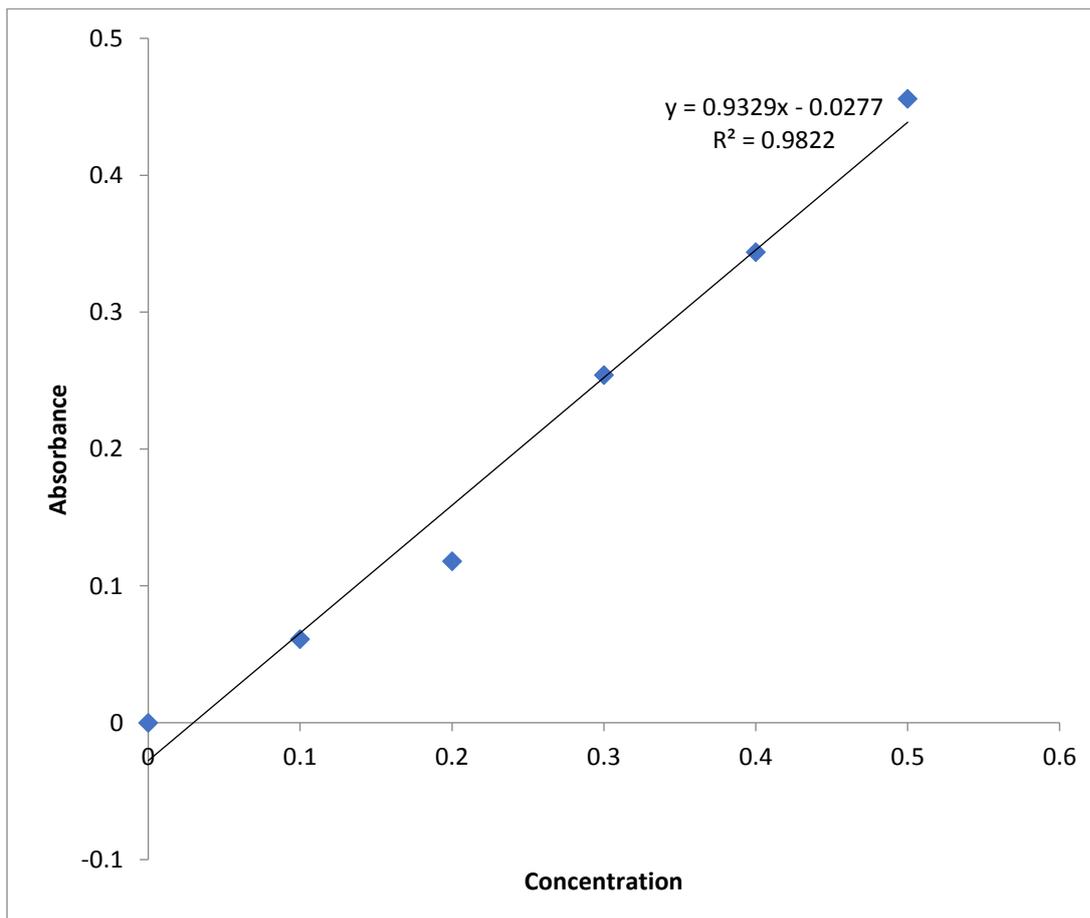
15mM NAD⁺ 0.14927 g of β -Nicotinamide adenine dinucleotide salt was dissolved in 15 ml of distilled water. 62.5mM Tris-HCl 2.4625 g of Tris-HCl was dissolved in about 200 ml of distilled water. The pH was adjusted to 8.0. The resulting volume was transferred and made up to 250 ml, with more distilled water, in a standard 250 ml volumetric flask.

APPENDIX III



Protein Calibration Curve

APPENDIX III



Aspartate Aminotransferase Calibration Curve

APPENDIX VI

Table 2: Protocol for calibration curve of Total Protein concentration

Assay	Dilution factor
ALP	6
GGT	6
AST	6
ALT	6
GLDH	6

APPENDIX VII

Table 3: Protocol for calibration curve of Alkaline phosphatase

Reagents	Blank (ml)	Test (ml)
Carbonate buffer (0.1M)	2.2	2.2
MgSO ₄ .7H ₂ O (0.1 M)	0.1	0.1
Distilled water	0.2	---
Enzyme source	---	0.2
(appropriately diluted) Incubate at 37°C for 10 min		
P-nitrophenyl phosphate (10mM)	0.5	0.5
Incubate at 37°C for 10 min NaOH (1N)	2	2

APPENDIX VIII

Table 4: Protocol for calibration curve of Aspartate amino transferase

Reagent	Blank	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Pyruvate standard (ml)	---	0.1	0.2	0.3	0.4	0.5
AST buffered substrate (ml)	1	0.9	0.8	0.7	0.6	0.5
Distilled water (ml)	0.2	0.2	0.2	0.2	0.2	0.2
1 mM 2,4-DNPH (ml)	1	1	1	1	1	1
The mixture was mixed properly and allowed to stand for 20 min 0.4N NaOH (ml)	10	10	10	10	10	10

APPENDIX IX

Table 5: Protocol for calibration curve of ALT

Reagent	Blank	1	2	3	4	5
Pyruvate standard (ml)	----	0.1	0.2	0.3	0.4	0.5
ALT buffered substrate (ml)	1	0.9	0.8	0.7	0.6	0.5
Distilled water (ml)	0.2	0.2	0.2	0.2	0.2	0.2
1 mM 2,4-DNPH (ml)	1	1	1	1	1	1
The mixture was mixed properly and allowed to stand for 20 min 0.4N NaOH (ml)	10	10	10	10	10	10