NUTRITIONAL BIOCHEMISTRY

PRACTICAL MANUAL - I

M.Sc., FOODS AND NUTRITIONAL SCIENCES **(First Year)**

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M.Sc. (Foods & Nutritional Science), First Year

PRACTICAL - I : NUTRITIONAL BIOCHEMISTRY
SYLLABUS

Practical Exercises:

1. Qualitative analysis of carbohydrates and proteins.
2. Qualitative tests for normal and abnormal constituents in urine.
3. Estimation of urinary creatinine.
4. Blood glucose.
5. Blood urea and uric Acid.
6. Serum protein
7. Serum cholesterol
8. Serum iron

1. QUALITATIVE TESTS FOR CARBOHYDRATES

OBJECTIVE:

- To characterize carbohydrates present in the unknown solution on the basis of various chemical assays

MATERIALS REQUIRED:

- Glass ware
- Test tubes
- Test tube holder
- Water bath
- Spatula
- Dropper

REAGENTS:

- Iodine Solution: Add a few crystals of iodine to 2% potassium iodide solution till the colour becomes deep yellow.
- Fehling's Reagent A: Dissolve 34.65g copper sulphate in distilled water and make upto 500mL.
- Fehling's Reagent B: Dissolve 125g potassium hydroxide and 173g Rochelle salt (potassium sodium tartrate) in distilled water and make upto 500mL.
- Benedict's Qualitative Reagent: Dissolve 173g sodium citrate and 100g sodium carbonate in about 800mL water. Heat to dissolve the salts and filter, if necessary.

  Dissolve 17.3g copper sulphate in about 100ml water and add it to the above solution with stirring and make up the volume to 1L with water.
- Barfoed's Reagent: Dissolve 24g copper acetate in 450ml boiling water. Immediately add 25mL of 8.5% lactic acid to the hot solution. Mix well. Cool and dilute to 500mL.
- Seliwanoffs Reagent: Dissolve 0.05g resorcinol in 100ml dilute (1:2) hydrochloric acid.
- Bial's Reagent: Dissolve 1.5g orcinol in 500mL of concentrated HCL and add 20 to 30 drops of 10% ferric chloride.

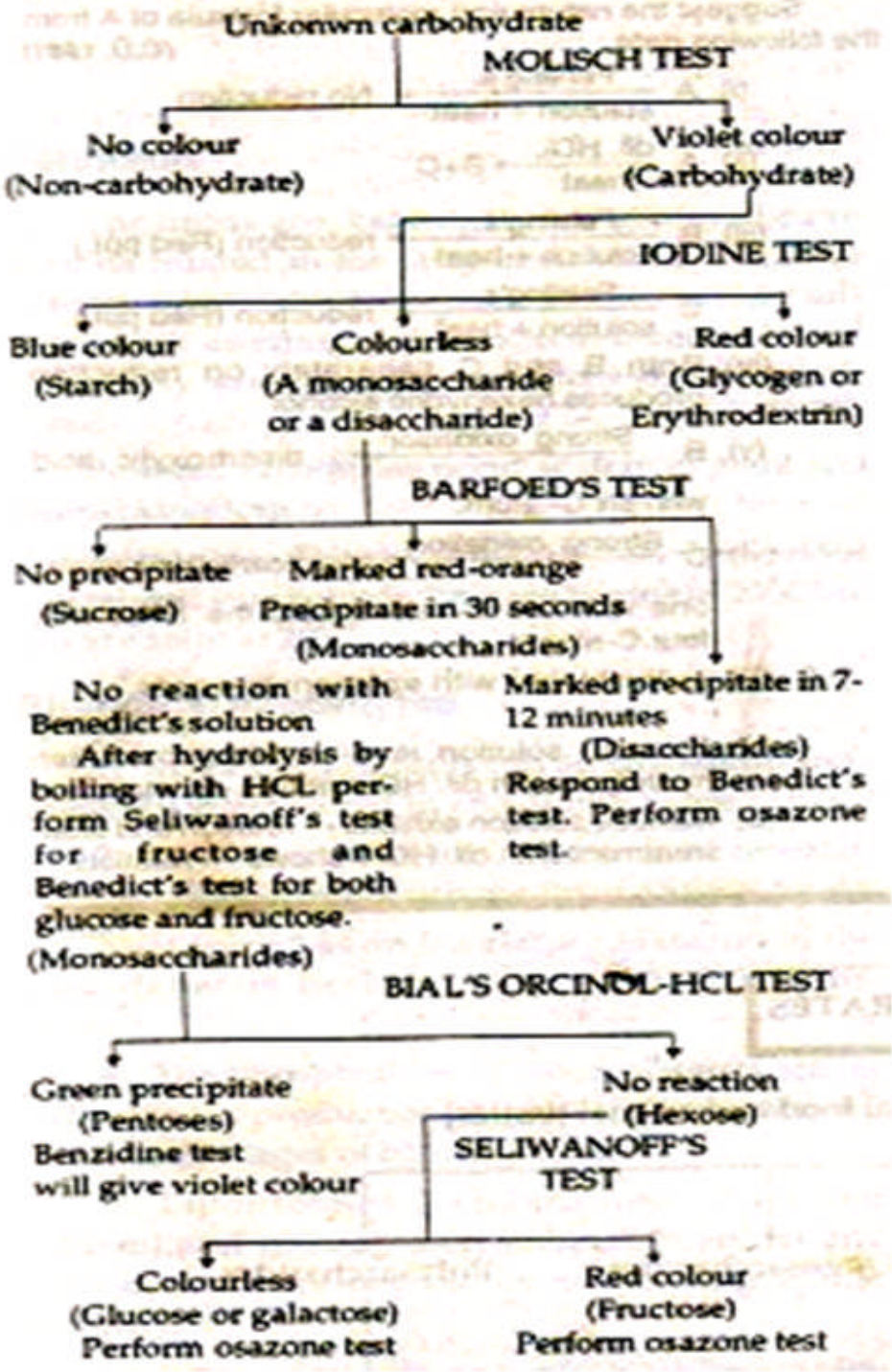

FIG: 1 QUALITATIVE TEST FOR IDENTIFICATION OF CARBOHYDRATES

Table 1: Reactions of carbohydrates

No.	Experiment	Observation	Inference	Reaction
1.	Molisch's test: Add two drops of Molisch's reagent (5% 1-naphthol in alcohol) to about 2mL of test solution and mix well. Incline the tube and add about lml of concentrated sulphuric acid along the sides of the tube. Observe the colour at the junction of the two liquids.	A red-cum-violet ring appears at the junction of the two liquids	Presence of carbohydrates.	The colour formed is due to the reaction of alpha naphthol with furfural and/or its formed by the dehydration of sugars by concentrated sulphuric acid All carbohydrates react positively With this reagent
2.	Iodine Test: Add a few drops of iodine solution to about lmL of the test solution,	Appearance of deep blue colour,	Presence of polysaccharide.	This indicates the presence of starch in the solution. The blue colour is due to the formation of starch-iodine complex
3.	Fehling's Test: To lmL of Fehling's solution 'A', add lmL of Fehling's solution 'B' and a few drops of the test solution. Boil for a few minutes.	Formation of yellow or brownish-red precipitate.	Presence of reducing sugar	The blue alkaline cupric hydroxide present Fehling's solution, when heated in the presence of reducing sugars, gets reduced to yellow or red cuprous oxide and it gets precipitated. Hence, formation of the coloured precipitate indicates the presence of reducing Sugars in the test solution.

4.	Benedict's Test: To 2mL of Benedict's reagent add five drops of the test solution. Boil for five minutes in a water bath. Cool the solution.	Formation of red, yellow or green colour! Precipitate.	Presence of reducing sugars	As in Fehling's test, the reducing sugars because of having potentially free aldehyde or keto group reduce cupric hydroxide in alkaline solution to red coloured cuprous oxide. Depending on the sugar concentration yellow to green colour is developed.
5.	Barfoed's Test: To lmL of the test solution add about 2mL of Barfoed's reagent. Boil it for one minute and allow to stand for a few minutes.	Formation of brick-red precipitate.	Presence of reducing sugars. Appearance of a red precipitate as a thin film at the bottom of the test tube within 3-5 min. is indicative of reducing mono-saccharide. If the precipitate formation takes more time, then it is a reducing disaccharide.	Only monosaccharides answer this test. Since Barfoed's reagent is weakly acidic, it is reduced only by monosaccharides.

6.	Seliwanoff's Test: To 2mL of Seliwanoff's reagent add two drops of test solution and heat the mixture to just boiling.	Appearance of deep red colour.	Presence of ketoses [Sucrose gives a positive ketohexose test] Presence of aldoses	In concentrated HCl, ketoses undergo dehydration to yield furfural derivatives more rapidly than do aldoses. These derivatives form complexes with resorcinol to yield deep red colour. It is a timed colour reaction specific for ketoses.
7.	Bial's Test To 5mL of Bial's reagent add 2-3mL of solution and warm gently. When bubbles rise to the surface cool under the tap.	Appearance of green colour or precipitate.	Presence of pentoses.	It is specific for pentoses They get converted to furfural. In the presence of ferric ion orcinol and furfural condense yield a coloured product.
8.	Test for non-reducing sugars such as sucrose: a) Do Benedict's test with the test solution. b) Add 5 drops of concentrated HCl to 5mL test solution in another test tube. Heat for five minutes on a boiling water bath. Add 10% sodium hydroxide solution give a slightly alkaline solution (test with red litmus paper). Now perform Benedict's test with this hydrolysed solution.	No characteristic colour formation. Appearance of red or yellow colour.		Indicates the absence of reducing sugars in the given solution Indicates the formation of reducing sugars from non-reducing sugars after hydrolysis with acid

9.	Mucic Acid Test: Add a few drops of cone. HNO3 to the concentrated test solution or substance directly and evaporate it over a boiling water bath till the acid fumes are expelled. Adda fewdrops of water and leave it overnight.	Formation of crystals.		The both end carbon groups are oxidized to carboxylic groups. The resultant saccharic acid of galactose is called mucic acid which is insoluble in water.
10.	Osazone Test: To OSg of phenylhydrazine hydivchlonde add 0.lg of sodium acetate and 10 drops of glacial acetic acid. To this mixture add 5mL of test solution and heat on a boiling waler bath for about half an hour. Allow the tube to cool siowl' and examine the crystals under a microscope	Glucose, fructose and mannose produce needle-shaped yellow osazone crystals, whereas lactosazone is mushroom-shaped. Different osazones show crystals different shapes Maltose produces flower-shaped crystals.	Glucose/fructose Presence of lactose Presence of maltose	The ketoses and aldoses react with phenylhydrazine to produce a phenylhydrazone which in turn reacts with another two molecules of phenylhydrazine to form the osazone.

Notes

1. For osazone test, the reaction mixture should be between pH 5 and 6. Fructose takes 2mm to form the osazone whereas for glucose it is 5mm. The disaccharides take a longer time to form osazones. Dissacharides form crystals only on cooling.

2. When a mixture of carbohydrates is present in the test sample, chromatographic methods should be employed to identify the individual sugars.

Questions:

1. Identify given sugar solution by qualitatively analysis

References:

1. Sadasivam S and Theymoli Balasubramanian (1985). Practical Manual (Under Graduate) Tamil Nadu Agricultural University, Coimbatore.2.

2. Sadasivam S and Manickam A (1997). Biochemical methods. New age International (p) limited. Ansari road, Daryaganj, New Delhi. 1-5.

2. QUALITATIVE TESTS FOR PROTEINS

OBJECTIVE:

- To know about the identification of unknown proteins

MATERIALS REQUIRED:

- Glass ware
- Test tubes
- Test tube holder
- Water bath
- Spatula
- Dropper

Table 2: Reactions of Proteins

Test	Observation	Remarks
BIURET REACTION: To 2mL of the test solution add 2mL of 10% NaOH Mix. Add two drops of 0.1% CuSO4 solution.	Violet or pink colour	Compounds with two or more peptide bonds give a violet colour with alkaline copper sulphate solution.
NINHYDRIN TEST: To 4m1, of the solution which should be at neutral pHadd lmL of 0.1% freshly prepared ninhydrin solution. Mix the contents and boil for a couple of minutes. Allow to cool.	Violet or purple colour	The ninhydrin test is answered by amino acids and proteins. The formation of a complex called Rheumann's purple due to the condensation of two molecules of ninhydrin with one molecule of ammonia from amino acid is responsible for the violet colour. The α-amino group is the reactive group.

XANTHOPROTEIC REACTION: To 5mL of the solution add lmL of conc. HNO3. Boil the contents. After cooling add excess 40% NaOH.	On adding acid, yellow colour will be noticed. When NaOH is added deep orange colour will develop.	The yellow colour is due to the nitro derivatives of the aromatic amino acids present in the protein. The sodium salts of nitro derivatives are orange in colour
GLYOXYLIC REACTION FOR TRYPTOPHAN: (Hopkins-Cole test) Add 2mL o glacial acetic acid to 2mLof the test solution. Then add about 2mL of conc. H2SO4 carefully down the sides of the test tube. Observe the colour change at the junction of the two liquids.	Violet ring is formed at the junction.	The indole group of tryptophan reacts with the glyoxylic acid released by action of conc. H2SO4 acetic acid to give a purple colour.
SAKAGUCHI REACTION To 5mL of the solution cooled on ice add lmL of 10% NaOH solution and lmL of 0.02% α-Naphthol solution. After few minutes add 10 drops of alkaline hypobromide solution.	Intense red colour	The guanidine group of arginine reacts with α-napthol to form a bright red coloured complex.
SULPHUR TEST To 2mL of the solution add 2ML of 40% NaOH and 10 Drops of 2% lead acetate solution. Boil for a minute and cool.	Black precipitate	The sulphur in sulphur containing amino acids of the proteins in presence NaOH, is changed intoNa2S which forms black lead sulphide when reacted with lead acetate.
MODIFIED MILLON'S TEST (a) To lmL of solution add 1mL of 10% mercuric sulphate in 10% sulphuric acid. Boil gently for half a minute. (b) Cool under a tap and add a drop of'1% NaNO2 solution and warm gently.	Yellow precipitate A red colour develops.	The yellow precipitate is due to the precipitation of protein. Mercury combines with tyrosine of the protein. The red colour is due to reaction of the precipitate with nitrous acid.

QUESTIONS:

1. How to identify unknown amino acid in given protein solution

REFERENCES:

1. Sadasivam S and Theymoli Balasubramanian (1985). Practical Manual (Under Graduate) Tamil Nadu Agricultural University, Coimbatore p2.
2. Sadasivam S and Manickam A (1997). Biochemical methods. New age International (p) limited. Ansari road, Daryaganj, New Delhi. 33-34.

3.QUALITATIVE TESTS FOR NORMAL AND ABNORMAL CONSTITUENTS IN URINE

OBJECTIVE:

- To identify the normal chemical constituents in urine
- To identify the abnormal constituents in urine

INTRODUCTION:

Many kidney and other medical problems can be detected by routine urinalysis, which includes chemical pH measurement, tests to detect protein, sugar, and ketones and microscopic examination to detect red and white blood cells (not done in this experiment). In a doctor office, dipsticks or strips (strips of plastic impregnated with chemicals) are routinely used in urine testing. The chemicals in the stick react with substances in the urine and change color. Protein presence in urine (proteinuria) is usually a sign of kidney disease, but it may occur normally after strenuous exercise. Glucose in the urine (glucosuria) is usually caused by diabetes. Ketones in the urine (ketonuria) may appear as a result of starvation, uncontrolled diabetes, and alcohol intoxication. Ketones are formed when the body breaks down fat. In some instances, doctors prescribe specialized tests for the levels of inorganic ions, such as chloride, sulfate, calcium, ammonium, and phosphate. Although normal urine contains these ions, some medical disorders (e.g. resulting from biochemical dysfunction) may cause either lower or increased concentration of some of these ions.

Materials required:

- Test tubes
- Test tube holder
- Water bath
- Spatula
- Dropper

A. Normal constituents in urine
Tests for Inorganic compounds

Chloride Test: Usually excretion of may be decreased in some fevers, chronic nephritis, fasting and diarrhea.

Silver nitrate test: Take 5 ml of urine in a test tube and add a few drops of nitric acid to acidify it. Then add a few drops of silver nitrate solution. A white precipitate of silver chloride is produced which darkens on standing. The precipitate is soluble I ammonium hydroxide.

CALCIUM AND PHOSPHORUS:

To 20ml of urine add few drops of liquid ammonia and boil. A white flaky precipitate indicates phosphates of calcium and magnesium.

Filter and dissolve the precipitate in 5ml of dilute acetic acid to one half of this solution add potassium oxalate solution. A white precipitate of calcium oxalate is formed. To the other half of the solution add and equal volume of concentrated nitric acid and 5ml of ammonium molybdate. Boil a yellow crystalline precipitate indicates phosphate.

SULPHUR TEST:

This exist in three forms

1. Inorganic sulphate
2. Ether and sulphates. These are ethers of sulphuric acid with phenolic substance like phenol, cresol, indoxyl.
3. Organic sulphur traces of sulphur containing amino acids cystine are also present, but not usual methods.

To 10ml of urine and a few drops of concentrated hydrochloric acid and 8ml of solution of barium chloride. A bulky precipitate indicates the presence of inorganic sulphate.

TESTS FOR ORGANIC COMPOUNDS

TEST FOR UREA

1. Biuret test: Take a small amount of urea in a dry test tube and heat gently in a low flame. Urea melts and liberates ammonia. Continue heating until the fused mass begins to solidify. Cool the tube and dissolve the residue in dilute NaOH solution. Then add copper sulphate solution. A purple colour is formed due to the presence of biuret.
2. Decomposition test: Urea can be decomposed by the enzyme urease liberating ammonia. Take 5ml of fresh urine and add 1ml of urease (fresh) solution in a test tube. Keep it for 10 minutes for the reaction to proceed. Then boil the contents. Odour of ammonia comes through the mouth of the tube.

TEST FOR CREATININE

Creatinine is the anhydride of creatine and under normal conditions about 0.1g of creatinine per 100ml of urine is present.

1. Niroprusside test: Take 5ml of urine in a test tube and add few drops of sodium nitroprusside solution. Make the contents of the tube alkaline by adding a few drops of sodium hydroxide solution. A ruby red colour will develop which soon turns yellow.

2. Picric acid test: Take 5ml of urine in a test tube. Add a few drops of aqueous solution of picric acid and make the mixture alkaline with NaOH solution. A red colour is produced which turns yellow if the solution is acidified.

TEST FOR GLUCOSE:

About 0.15% of reducible sugars are present in urine which may include pentose, lactose and other type of carbohydrates. Normal urine does not contain glucose. However, in glucosuria and diabetes 3% to 10% or more glucose may be present in urine. Hence glucose has to be detected in urine by conventional methods.

Benedict's test: take 5ml of Benedict's reagent in a test tube and add 8 drops of urine to be tested. Boil the contents for 1-2 minutes and then cool slowly. If glucose is present, the solution will be filled with greenish/yellow/red precipitate depending upon the quantity of glucose present. Greenish precipitate would indicate very small amount of glucose. If no glucose is present the solution will remain clear.

ACETONE BODIES:

Acetone bodies or ketone bodies are compounds that appear in body during fatty acid oxidation as intermediate products. In normal conditions these compounds do not appear in the blood or urine, but in conditions such as ketonemia and ketonuria these compounds accumulate in the body. In ketonuria these appear in the urine. These compounds are acetoacetic acid, acetone and β hydroxylbutyric acid. During starvation more of these bodies accumulate in the body.

Nitroprusside test: Take 2ml of urine in test tube and add a few drops of 5% aqueous sodium nitropusside solution. Make alkaline by adding a few drops of NaOH solution. A persistent red colour indicates the presence of acetone bodies.

Another test can be performed. Take 5ml of urine and a few drops of sodium nitroprusside solution. Mix well and add concentrated ammonium hydroxide solution down the sides of the tube to form a layer. A purple ring at the zone of contact indicates the presence of acetone bodies.

B. ABNORMAL CONSTITUENTS IN URINE

PROTEIN IN URINE:

Heat coagulation test:

Take about 10ml of urine in a clean and dry test tube. Heat the upper portion of the test tube. Lower portion is used as a control. Add 2 to 3 drops of 1% acetic acid. If a coagulum is formed, indicates the presence of proteins in the urine.

BLOOD PIGMENTS IN URINE:

Benzidine test:

Take a clean and dry test tube. Mix two drops each of benzidine and hydrogen peroxide. Add a drop of this mixture to the urine. If blue-green color is formed (which is stable for only a few minutes), it indicates the presence of blood in the urine sample. This test can be used to detect the presence of blood in the feces (occult blood).

heme of hemoglobin decomposes hydrogen peroxide and liberates oxygen. Oxygen combines with the benzidine to form a colored complex.

Presence of blood in the urine is called hematuria. The commonest cause of hematuria is injury caused to urethra during the introduction of catheter. Other causes are urinary tract infection, stones in the urinary tract, glomerular nephritis, tuberculosis of the urinary tract etc.

Benedict's test:

Principle: Copper sulphate of Benedict's qualitative solution is reduced by reducing substances on boiling to form the coloured precipitate of cuprous oxide.

Test for Glucose:

Procedure	Observation	Inference
To about 5 ml of Benedict's reagent add 0.5 ml of urine and boil for 2 min.	Blue color appears	Sugar Absent
	Light green precipitate appears	0.1-0.5 % 0f reducing sugar present
	Green precipitate appears	0.5 to 1.0 % of reducing sugar present
	Yellow precipitate appears	1-2 % reducing sugar present
	Brick red precipitate appears	Above 2 % reducing sugar present

Normal urine also contains a trace of glucose and glucuronates, but their amount is too small to cause reduction in Benedict's test. In *Diabetes mellitus* and in renal glycosuria, glucose is found in urine. This gives a Benedict's test positive.

Bile salt (Hay's test) and bile pigment (Fouchet's test):

Hay's test:

Take 3 ml of urine and sprinkle a little of sulphur powder. If sulphur sinks to the bottom of the test tube, it indicates the presence of bile salt in the given urine sample. Bile salts reduce the surface tension, so sulfur sinks to the bottom of the tube.

Fouchet's test:

To five ml of urine, add 2 ml of barium chloride and a little of magnesium sulphate and mix well. Barium sulphate is precipitated. The bile pigments get adsorbed to barium sulphate and are present in the precipitate. Filter the precipitate by using a filter paper. Add a few drops of Fouchet's reagent to the precipitate.

Green or blue color formation indicates the presence of bile pigment. Fouchet's reagent oxidizes the bile pigment to form blue colored bilicyanine or green colored biliverdin.

Bile salts and bile pigments are present in the urine in case of obstructive jaundice and hepatic jaundice.

QUESTIONS:

1. How to assess the normal constituents in urine
2. How to assess the abnormal constituents in urine

REFERENCES:

1. Ashok kumar, J (2007). Text book of biochemistry for nurses, International publishing house Pvt. Ltd. New Delhi.205-208.

4. ESTIMATION OF CREATININE IN URINE

OBJECTIVE:

- To estimate the amount of creatinine in urine

PRINCIPLE:

The method makes use of the jaffee's reaction, the production of a mahogany red colour with an alkali picrate solution. The intensity of the colour developed is compared in the colorimeter against a reagent blank at 520nm.

REAGENTS:

1. Picric acid (0.04 molar solutions): 9.16g of crystalline picric acid is dissolved in 100ml of water.
2. 0.75 Sodium hydroxide: 30g of sodium hydroxide in liter of water.
3. Stock solution of creatinine: Dissolve 100mg of creatinine in 0.1N hydrochloric acid and made to 100ml with the same.
4. Working standard: Diluted 2ml of the stock solution to 100ml with water. This contains 20 micro grams of creatinine per ml.

PROCEDURE:

Dilute 5 ml. of urine to 500 nil, in a volumetric flask. Pipette 3 ml. of the diluted urine into a test tube and add 1 ml. of 004 M picric acid followed by 1 ml. of 075 N sodium hydroxide. Treat 3 ml. (= 0.03 mg.) of the standard solution for use in the same way and put up a blank consisting of 3 ml. water, and 1 ml. of each of the reagents. Allow to stand for fifteen minutes and then read in the colorimeter during the next half hour with a blue-green filter, or transmission at 500 millimicrons.

CALCULATIONS:

Since the standard contains 003 rug. creatinine, and 3 ml. of diluted urine corresponds to 003 ml. of the original urine.
Grams creatinine per litre of urine

$$= \frac{\text{OD of the Test}}{\text{OD of the Standard}} \times 0.03 \times \frac{1000}{0.03} \times \frac{1}{1000}$$

$$= \frac{\text{OD of the Test}}{\text{OD of the Standard}}$$

STANDARD CURVE:

Since Beer's law may not he followed sufficiently closely, prepare a standard curve to check this. For this curve dilute 2 ml. of the stock standard to 100 ml. to obtain a solution which contains 002 mg. per ml. Set up tubes as follows: For the preparation of standard curve, follow the procedure listed in table below

Reagents	**B**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**
Grams per litre of urine	0.0	0.4	0.8	1.2	1.6	2.0
Mg. per 100 ml. urine	0.0	40	80	120	160	200
Ml. of standard solution(0.02 mg. per ml)	0.0	0.6	1.2	1.8	2.4	3.0
Add distilled water (ml)	3.0	2.4	1.8	1.2	0.6	0
Add picric acid and sodium hydroxide as above						

CLINICAL ASPECTS:

Urine creatine is necessary in the process of muscle contraction, which is related to the amount of phosphocreatine broken down, resynthesis occurring after contraction. Creatinine is derived from creatine and is a waste product. The normal daily excretion of creatinine ranges from 1 to 2 grams, being as a rule nearer to the higher limit in men and to the lower in women. Creatinine is largely endogenous in origin, so that the amount excreted in the urine is little influenced by the diet, provided this is a normal one and does not contain considerable amounts of creatine- or creatinine-rich foods such as meat. Such endogenous creatinine is related rather to the amount of muscle tissue and so of phosphocreatine in the body. This remains relatively constant, for which reason it can be used to check the reliability of 24 hour urine collections. It is particularly useful in this respect when a series of daily specimens is being collected.

QUESTIONS:

1. How to determine the urine creatinine levels?

REFERENCES:

1. Harold Varley (2005). Practical clinical biochemistry, chapter 11 Determination of creatinine in urine, CBS publishers and distributors 11, Daryaganj, New Delhi, India. 197-198

5.ESTIMATION OF GLUCOSE IN BLOOD

OBJECTIVE:

- To estimate the amount of glucose present in blood

PRINCIPLE:

Glucose reacts with o-toluidine in glacial acetic acid in the presence of heat, to yield a bluish green, N-glucosylamine. Proteins in blood are precipitated with trichloroacetic acid. Equal volume of protein-free filtrate containing glucose and a standard solution and blank containing trichloroacetic acid are separately treated with the reagent and optical density values are read in a photoelectric colorimeter using a red filter (625).

REAGENTS:

1. 0-Toluidine reagent: To 5.0 g thiourea (reagent grade) adds 90.0 ml of o- toluidine and dilute to 1 litre with glacial acetic acid. Store in a brown or amber colored bottle and keep the reagent in a refrigerator.
2. 10%Trichioroacetic acid.
3. Glucose standard: Dissolve 10mg glucose in about 50 ml of distilled water in a 100 ml of

   volumetric flask. Add 30 ml of 10% trichloroacetic acid. Make up to 100 ml mark with distilled water.
4. Blank solution: dilute 30 ml of I 0% trichloroacetic acid to 100ml

PROCEDURE:

Preparation of protein-free filtrate:

Into a dry test-tube, pipette 3.0 ml f distilled water and 0.5 ml of blood. Mix well. Add 1.5 ml of 10% trichloroacetic acid and mix thoroughly. Let stand for 10 minutes and filter into a dry test tube.

Development of colour:

Label three test tubes as T, S, and B. Pipette 1.0 ml of protein-free filtrate into T. 1.0 ml of standard into S and 1.0 ml of blank solution into B. To all the tubes add 5.0 ml of o-toluidine reagent and mix thoroughly. Keep the tubes in a boiling water bath for 10 minutes. Cool and read the optical densities at 630 nm.

Additions	B(ml)	S (ml)	T (ml)
Distilled water	1	-	-
Glucose standard	-	1	-
Protein-free filtrate	-	-	1
o-toluidine reagent	5	5	5

Calculations:

$$\text{Glucose (mg) in 100 ml blood} = \frac{\text{OD of the Test} - \text{OD of the blank}}{\text{OD of the Standard} - \text{OD of the blank}} \times \frac{0.1}{0.1} \times 100$$

$$= \frac{T - B}{S - B} \times 100$$

STANDARD CURVE:

For the preparation of standard curve, follow the procedure listed in table below

Reagents	B	S_1	S_2	S_3	S_4	S_5
Take standard (ml) (1 ml =0.1mg)	0.0	0.5	1	1.5	2.0	2.5
Amount of glucose present (mg)	0.0	0.05	0.1	0.15	0.2	0.25
Blood equivalent (mg %)	0.0	50	100	150	200	250
Add distilled water (ml)	2	1.5	1	0.5	-	-
Add o-toluidine (ml)	5	5	5	5	5	4.5

CALCULATION OF BLOOD EQUIVALENT:

Standard concentration/volume of sample × 100 = glucose in mg%
For S1
0.05/ 0.1×100 = 50mg%
The advantage of this method is its simplicity and it gives the true glucose value. The colour developed is stable due to the presence of thiourea as an antioxidant.

CLINICAL ASPECTS:

The normal level of blood glucose in the fasting state varies between 70 and 110 mg / dl. The level rises post-prandially, i.e., following the ingestion of a regular meal to not more than 140 mg% (this value is not to be confused with post – glucose load).

QUESTIONS:

1. How to determine the blood glucose level

REFERENCES:

2. Biswajit Mohanty and Sharbari Basu (2007). Text book of fundamentals of practical clinical biochemistry, BI Publications Pvt. Ltd. 54, Janpath, New Delhi. New Delhi.54-57.

3. Harold Varley (2005). Practical clinical biochemistry, chapter 3 blood sugar determination ,CBS publishers and distributors 11, Daryaganj, New Delhi, India. 82-83

6. DETERMINATION OF UREA IN BLOOD

OBJECTIVE:

- To estimate the amount of urea present in blood

PRINCIPLE:

Diacetyl monoxime decomposes on heating to give hydroxylamine and diacetyl ($CH_3COCOCH_3$), a compound that has adjacent carbonyl groups. On heating under acidic conditions, urea forms a yellow coloured product with diacetyl by the condensation mechanism. The OD of the colour developed can be read at 480 nm.

REAGENTS:

1. Sodium tungstate, 10%.
2. Diacetyl monoxime reagent, 2% solution in 2% acetic acid.

   Add 2 g of compound to 60 ml of water followed by the addition of 2ml of glacial acetic acid. Shake to dissolve with slight warming, if necessary, and then make up to 100ml.
3. Sulphuric acid – phosphoric acid reagent: Add 150 ml of 85% phosphoric acid to 140 ml water, mix well and add 50 ml of conc. Sulphuric acid slowly while mixing.
4. Sulphuric acid,2/3 N
5. Stock standard urea solution: Dissolve 250 mg of urea in 100ml water. Working standard: Dilute 1 ml of stock to 200ml with water (0.0125 mg/ml).

PROCEDURE:

For deproteinisation, to 0.1 ml blood, add 3.3 ml of distilled water and mix well. Add 0.3 ml of sodium tungstate followed by 0.3 ml of 2/3N sulphuric acid and mix thoroughly (blood diluted 40 times). Keep aside for 10 min. centrifuge the mixture to get a clear protein – free supernatant. Prepare test, standard and blank samples as indicated in table.

Table: preparation of samples

Reagents	**Blank(ml)**	**Standard (ml)**	**Test (ml)**
Protein – free supernatant	-	1	1
Distilled water	2	1	1
DAM reagent	0.4	0.4	0.4

Phosphoric acid-sulphuric acid reagent	1.6	1.6	1.6
Total volume	4	4	4

Mix well and place the three tubes in a boiling water bath for 15 minutes. Cool and take readings at 480 nm.

CALCULATION:

$$\text{Blood urea in mg\%} = \frac{OD_T}{OD_S} \times \frac{\text{Conc. of }_S\ (0.0125)}{\text{Vol. of blood } (0.025)} \times 100$$

$$= OD_T/OD_S \times 50$$

Dilute the stock standard 1: 100 to obtain 0.025 mg per ml.

STANDARD CURVE:

Follow the procedure listed in Table for preparing the standard curve

REAGENTS

	B	S_1	S_2	S_3	S_4	S_5
Take standard (1 ml=0.025 mg)	0	0.4	0.8	1.2	1.6	2.0
Amount of urea present (in mg)	0	0.01	0.02	0.03	.04	0.05
Blood equivalent of urea/100 ml (in mg)	0.0	40	80	120	160	200
Add DAM reagent (in ml)	0.4	0.4	0.4	0.4	0.4	0.4
Add distilled water (in ml)	2.0	1.6	1.2	0.8	0.4	0
Add sulphuric – phos. acid reagent (in ml)	1.6	1.6	1.6	1.6	1.6	1.6

Wait for 15 min for final colour to develop. Measure the OD at 480nm. For calculating blood equivalent, e.g., for S_1, mg of urea present in 4 ml assay volume. The concentration of urea, 0.01/ 0.025× 100= 40 mg%

Plot the curve taking OD on Y-axis against different concentrations on X-axis. The curve may not obey Beer's law for lower urea concentrations.

CLINICAL ASPECTS:

Normal blood urea level is 15-40 mg% and it can be expressed in terms of BUN by dividing the urea value by 2.14. The urea content is influenced by the amount of protein in diet. Blood urea is lower in pregnant and lactating women and growing children because of negative nitrogen balance. Pre-renal causes of increase in blood urea are mainly related to volume contraction, for example in dehydration. In renal parenchymal damage, urea cannot be filtered and gets accumulated and hence rises. Post- renal rise of urea is due to obstruction to the flow of urine. This may reduce effective filtration pressure at glomeruli; if prolonged, irreversible kidney damage results.

QUESTIONS:

1. How to determine the blood urea level

REFERENCES:

1. Biswajit Mohanty and Sharbari Basu (2007). Text book of fundamentals of practical clinical biochemistry, BI Publications Pvt. Ltd. 54, Janpath, New Delhi. New Delhi.62-64.

2. Harold Varley (2005). Practical clinical biochemistry, blood urea and uric acid determination, CBS publishers and distributors 11, Daryaganj, New Delhi, India. 170,203

7. DETERMINATION OF URIC ACID IN SERUM

OBJECTIVE:

- To estimate the amount of uric acid present in serum

PRINCIPLE:

Serum is deproteinised by treating it with tungstic acid. Uric acid present in the protein-free filtrate when treated with phosphotungstic acid and sodium carbonate gives a blue colour. The intensity of the colour so developed is directly proportional to the concentration of uric acid present and thus uric acid can be estimated colorimetrically at 650 nm (red filter).

REAGENTS:

1. 10% Sodium tungstate.
2. 2/3 N Sulphuric acid.
3. 10% Sodium carbonate (w/v).
4. Phosphotungstic acid: Dissolve 50g of sodium tungstate in about 400 ml of water. Add 40 ml of 85% phosphoric acid and reflux for 2 hr., cool, transfer to a 500ml flask and make to the mark with water. Keep in brown bottle. Dilute 1 in 10 for use.
5. Tungstic acid: Add 50 ml 10% sodium tungstate, 50 ml 2/3 N sulphuric acid and a drop of phosphoric acid with mixing to 800 ml water. Discard when cloudy. Keep in brown bottle.
6. Stock standard Uric acid – 100 mg/dl. Weigh out 100mg of uric acid in a small beaker. Dissolve 60 degree C and pour on to the uric acid. Stir until dissolved, heating further in warm water if necessary. When dissolved, transfer with washing to a 100 ml flask. Add 2 ml of 40% formalin and then, slowly with shaking, add 1 ml of 50% v/v acetic acid. Make to the mark with water and mix. Keep in well – stoppered bottle away from sun light.
7. Working standard: for one point caliberation, dilute 1 ml of stock to 200ml with distilled water (1 ml = 0.005 mg). For standard curve, 1 ml of stock is diluted to 100 ml with distilled water (0.01 mg/ml).

PROCEDURE:

Add, while shaking, 5.4 ml of dilute tungstic acid to 0.6 ml serum and centrifuge. Into three test tubes, measure 3 ml of the diluted standard and 3 ml of water as blank (Table)

Table: sample preparation for uric acid determination

Reagents	**Test (ml)**	**Standard (ml)**	**Blank(ml)**
Uric acid standard (0.01 mg/ml)	-	3	-
Protein free filtrate	3	-	-
Water	-	-	3
Sodium carbonate	0.6	0.6	0.6
Phosphotungstic acid	0.6	0.6	0.6

Mix well and place in a water bath at 25^0Cfor 30 minutes. Take OD reading at 650 nm (red filter).

CALCULATION:

$$\text{Concentration of test} = \frac{OD_T}{OD_S} \times \frac{\text{Conc. of S}}{\text{Vol. of sample}} \times 100$$

$$= \frac{OD_T}{OD_S} \times \frac{0.015}{0.3} \times 100$$

$$= (OD_T/OD_S) \times 0.05 \times 100$$

$$= (OD_T/OD_S) \times 5 \text{ mg/dl}$$

STANDARD CURVE:

For preparing the standard curve, arrange the tubes as shown in Table. Wait for 15 minutes to allow the final colour to develop. Measure the OD at 650 nm.

Table: Preparation of standard curve

Reagents	**B**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**
Take standard in ml (1 ml=0.01mg)	0.0	0.6	1.2	1.8	2.4	3.0
Amount of uric acid in mg present	0.0	0.006	0.012	0.018	0.024	0.03
Blood equivalent of uric acid in mg%	0.0	2	4	6	8	10
Add distilled water in ml	3	2.4	1.8	1.2	0.6	-
Add sodium carbonate in ml	0.6	0.6	0.6	0.6	0.6	0.6
Add phosphotungstic acid in ml	0.6	0.6	0.6	0.6	0.6	0.6

For calculating blood equivalent, e.g., for $S_{1:}$ 0.006 mg uric acid present in 3 ml assay volume, i.e., 0.006/0.3 × 100 = 2 mg% uric acid.

Plot the curve taking OD on Y-axis against different blood concentrations on X-axis.

CLINICAL ASPECTS:

Uric acid concentration in normal serum is between 2.5 and 6.0 mg/dl. It is increased in gout, renal failure and in common conditions in which blood urea is increased. Uric acid estimation in serum is of diagnostic importance in gouty arthritis. Elevated levels are observed in leukemia and psoriasis due to enhanced turnover of cells. Also, in toxemia of pregnancy, the level of serum uric acid is increased. Blood levels of uric acid may be decreased in patients receiving steroid therapy or drugs that decrease re-absorption of urates by renal tubules (aspirin, probenecid, etc.).

QUESTIONS:

1. How to determine the serum uric acid level

REFERENCES:

1. Biswajit Mohanty and Sharbari Basu (2007). Text book of fundamentals of practical clinical biochemistry, BI Publications Pvt. Ltd. 54, Janpath, New Delhi. 68-70.

2. Harold Varley (2005). Practical clinical biochemistry, blood urea and uric acid determination, CBS publishers and distributors 11, Daryaganj, New Delhi, India. 205-206.

8. DETERMINATION OF SERUM PROTEINS

OBJECTIVE:

- To estimate the amount of protein present in serum

PRINCIPLE:

The peptide bonds (-CO-NH) present in the protein react with copper sulphate in an alkali medium (biuret reagent) to give a blue/purple colour. The optical density of this solution at 540 nm is compared with the colour developed by a standard protein solution similarly treated to get the serum total protein level.

Total protein estimation by the biuret method can be further extended to determine the amount of globulins (G) and albumin (A), and determine A/G ratio. The globulins are precipitated using a sulphate - sulphite solution. The albumin present in the globulin – free solution is estimated using the biuret procedure. Globulin = total protein – albumin.

REAGENTS:

1. Sulphate – sulphite solution: Dissolve 208 g of sodium sulphate and 70 g of sodium sulphite in 900 ml of water, add 2 ml of conc.H_2SO_4 and make up the volume to 1L, pH>7.0

2. Biuret reagent:

   Stock solution – Take 45 g of Rochelle salt in 400 ml of 0.2N NaOH, and add 15g of $CuSO_4$ while stirring. Into it, pour 5 g KI and make up volume to 1L with 0.2N NaOH.

   Working solution – Prepare the working solution by 5-fold dilution of the stock solution with 0.2 N NaOH containing 0.5 g% KI.

3. Tartarate iodine solution: Take 9 g of Rochelle salt in 1L of 0.2N NaOH containing 6.5 g % KI.

4. Ether .

5. Standard solution: Stock solution 6 g%.

   Working standard: 625 mg% (6.25 mg/ml) – Dilute 0.66ml of stock in 6.4 ml of sulphate – sulphite solution for preparing standard curve. For one point calibration, dilute the stock 1:16 times, i.e., 1 ml = 3.75 mg.

PROCEDURE:

To 6 ml of sulphate – sulphite solution, add 0.4ml of plasma while stirring. Pipette out 2 ml of mixture and add 5ml biuret reagent. The OD of the colour developed is measured. This reading gives the value of total protein. In another test tube take 2 ml of the above mixture and add 3 ml of ether. The tube is stoppered and shaken by inversion 40 times. The solution is then centrifuged at 3000rpm for 5 minutes. The tube is then tilted, taking care not to disturb the precipitate, 2 ml of the clear solution below the globulin layer is pipette out and 5 ml of biuret reagent is added. For blank, add 2 ml of sulphate – sulphite solution to 5 ml of biuret reagent. Mix the test tubes properly and place at 37^0 C for 10 min in a water bath. Then cool it at room temperature. Read the final colorimeter reading (OD) at 540nm

Calculation:

Amount of total protein present in g% =

$$= \frac{OD_T}{OD_S} \times \frac{\text{Conc. of S}}{\text{Vol. of sample}} \times 100$$

Or,

$$= \frac{OD_T}{OD_S} \times \frac{7.5}{0.125} \times 100$$

$$= (OD_T/OD_S) \times 60 \times 100$$

Follow the same procedure for calculating albumin.

STANDARD CURVE:

Follow the procedure listed in Table for preparing the standard curve.

Table: Standard curve preparation

Reagents	**B**	**S_1**	**S_2**	**S_3**	**S_4**
Take standard (1 ml=6.25mg)	-	0.4	0.8	1.2	1.6
Amount of protein present (in g)	-	2.5	5	7.5	10
Blood equivalent of protein/100ml (in g)	-	2	4	6	8
Add sulphate - sulphite (in ml)	2	1.6	1.2	0.8	0.4
Add biuret reagent (in ml)	5	5	5	5	5

Mix the test tubes properly and place at 37^0C for 10 min in a water bath, then cool it at room temperature. Read the final OD value at 540 nm.

For calculating blood equivalent, e.g., for S_1:2.5/0.125×100 = 2 g%

Plot the curve taking OD on the Y-axis against different concentrations on the X-axis.

CLINICAL ASPECTS:

The total protein in adults ranges from 6.0 to 8.0 g% and albumin varies from 3.5 to 5.2 g%. The normal A/G ratio is 1.7: 1. Liver synthesizes albumin and most of globulins except the immunoglobulins, which are synthesized by the plasma cells. Very minute quantity of albumin is excreted (<100 mg/day in urine), which cannot be detected by the normal heat coagulation test. Thus, diseases of the liver kidney and the state of hydration affect the serum protein level. In acute conditions of liver diseases, the albumin concentration remains apparently normal because of its long half life, whereas in chronic liver diseases, like cirrhosis of liver, albumin concentration starts falling. This in turn leads to reversal of A/G ratio. Therefore, in cases of cirrhosis, reversal of A/G ratio is a bad prognostic sign as it depicts total parenchymal damage. Similarly, nephritic syndrome is associated with massive proteinuria, albumin being a lower molecular weight protein leaks out early leading to reversal of A/G ratio. In dehydration, because of haemoconcentration there is an apparent increase in serum protein. In monoclonal diseases like multiple myeloma (abnormal proliferation of plasma cells), serum protein concentration is found to be increased.

QUESTIONS:

1. How to determine the serum protein level

REFERENCES:

1. Biswajit Mohanty and Sharbari Basu (2007). Text book of fundamentals of practical clinical biochemistry, BI Publications Pvt. Ltd. 54, Janpath, New Delhi. 59-61.
2. Harold Varley (2005). Practical clinical biochemistry, serum protein determination,CBS publishers and distributors 11, Daryaganj, New Delhi, India. 236-238.

9.DETERMINATION OF CHOLESTEROL IN SERUM

(Zak's Method)

OBJECTIVE:

- To estimate the amount of cholesterol present in serum

PRINCIPLE:

Ferric chloride reagent precipitates the proteins from serum and extracts cholesterol. Cholesterol present in the protein – free filtrate is treated with concentrated sulphuric acid (Analar). The intensity of the red colour developed with $FeCl_3$-H_2SO_4 is compared colorimetrically at 540 nm with that of a standard cholesterol solution similarly treated.

REAGENTS:

1. $FeCl_3$ reagent: 0.05% $FeCl_3.6H_2O$ in glacial acetic acid (aldehyde – free).
2. Concentrated H_2SO_4
3. Cholesterol stock standard: 100 mg% in glacial acetic acid.
4. Working solution: 4 ml of stock is made up to 100ml with ferric chloride-acetic reagent (0.04 mg/ml)

PROCEDURE:

For preparation of protein – free filtrate, add 0.1ml of serum to 9.9ml of FeCl3 reagent in a glass-Stoppered centrifuge tube. Mix the contents well and let stand for 20-30min in order to allow proteins to precipitate. Centrifuge and take 5ml of the supernatant liquid for the test. Treat the working standard similarly and take 5ml for the test.

Table: Preparation of samples for the colorimetric determination of cholesterol in serum

Reagents	**Blank**	**Test**	**Standard**
Standard solution (ml)	-	-	3
Supernatant fluid (ml)	-	5	-
Ferric chloride reagent (ml)	5	-	2
Conc. H_2SO_4 (drops)	3	3	3

Mix the contents well and allow standing for 20 minutes for colour development to take place. Read OD using a colorimeter at 560 nm against the blank solution.

CALCULATION:

$$\text{Serum cholesterol (mg/dl)} = \frac{OD_T}{OD_S} \times \frac{\text{Conc. of Std.}}{\text{Vol. of sample}} \times 100$$

$$= \frac{OD_T}{OD_S} \times \frac{0.02}{0.05} \times 100$$

STANDARD CURVE:

For preparing standard curve, proceed as shown Table below

Table: Standard curve preparation

Reagents	**B**	**S_1**	**S_2**	**S_3**	**S_4**
Take standard in ml(1 ml=0.08g)	-	1	2	3	4
Amount of cholesterol (in mg)	-	0.04	0.08	0.12	0.16
Blood equivalent of cholesterol (in mg)	-	80	160	240	320
Add $FeCl_3$ acetic acid reagent (in ml)	5	4	3	2	1

Mix thoroughly and proceed as above by adding 3 ml of conc. sulphuric acid.
For calculating blood equivalent, e.g., for S_1: 0.04/0.05×100 = 800 mg%

CLINICAL ASPECTS:

Normal level of cholesterol is 130-200 mg/dl. However, it is desirable that level should not exceed 200 mg/dl.

The serum cholesterol concentration is low at birth but it increases with the growth of the child. At the time of growth, it reaches slightly above the adult value and then comes back within normal limits by the end of second decade. The cholesterol value is slightly lower in women till they attain menopause.

Hypercholesterolemia is seen in the cases of severe diabetes mellitus, biliary obstruction, myxedema (hypothyroidism), nephritic syndrome, chronic renal failure and familial hypercholesterolemia. A lowered level of cholesterol, i.e., hypercholesterolemia is observed in cases of malabsorption, cirrhosis of liver, hyperthyroidism, anemia and starvation.

QUESTIONS:

1. How to determine the serum cholesterol level
2. Why is HDLc called good cholesterol?

REFERENCES:

1. Biswajit Mohanty and Sharbari Basu (2007). Text book of fundamentals of practical clinical biochemistry, BI Publications Pvt. Ltd. 54, Janpath, New Delhi. 74-75.
2. Harold Varley (2005). Practical clinical biochemistry, serum cholesterol determination,CBS publishers and distributors 11, Daryaganj, New Delhi, India. 313-315.

10. DETERMINATION OF IRON IN SERUM (DIPYRIDYL METHOD)

OBJECTIVE:

- To determine the amount of iron present in serum

PRINCIPLE:

Ferrous iron gives a pink colour with 2'2'-dipyridyl. A solution of dipyridvl in acetic acid is added to serum followed by a reducing agent. Proteins are removed by heating in boiling water and then centrifuging or filtering.

REAGENTS:

1. 2.2'-dipyridyl: 0.1 per cent, in acetic acid, 3 per cent.v/v.

2. Sodium sulphite: 0.l M. Dissolve 1'26 grams of anhydrous sulphite or 2.52 grams of Na2SO3, 7H2O in water and make up to 100 ml. Prepare freshly every few days.

3. Chloroform.

4. Standard solution: containing 100 micrograms iron per ml. Dissolve 0.498 gram of ferrous sulphate FeSO4, 7H2O in water, add 1 mL concentrated sulphuric acid and make to a litre. Alternatively use a solution of ferrous ammonium sulphate (N114)2SO4, FeSO4, 6H2O, containing 0.702 gram per litre.

5. Working standard: Dilute 3 ml. of the stock solution to 100 ml. with water to obtain a solution containing 3 µg. per ml.

PROCEDURE:

Mix equal volumes of serum, 0.1 M sodium sulphite and dipyridyl reagent in a glass stoppered tube which can be centrifuged. Heat in boiling water bath for five minutes. Cool, add 1 ml. of chloroform, stopper and shake vigorously for thirty seconds. Remove the stopper and centrifuge for five minutes at 300 r.p.m. If the supernatant fluid is not completely clear repeat the shaking and centrifuging. Read at 520 millimicrons or using a green filter. As blank use water instead of serum. For the standard put through the working standard in the same way.

Clean the tubes used by placing them in boiling 5 N hydrochloric acid. Then wash with glass distilled water and keep for this determination only.

CALCULATION:

Micrograms iron per 100ml. of serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 300$$

The readings are linear with concentration to at least 500 µg. per 100 ml. To obtain a calibration curve dilute 5 ml. of the stock standard to 100 ml. with water and set up tubes containing 0.4,0.8, 1.2, 1.6 and 2.0 ml. of this, make each to 2 ml. with water, and develop the colour as described above and read against the blank. These correspond to 100, 200, 300, 400 and 500 µg. per 100 ml.

CLINICAL ASPECTS:

Nearly two-thirds of the body's iron (4 to 5 grams) is present in hemoglobin (338 mg. iron per 100 grams) of which about 90 per cent. is red cell hemoglobin, the rest muscle hemoglobin. The greater part of the remaining iron is stored, apparently combined with protein as ferritin, the liver being the tissue richest in iron. This iron is readily available when required. In addition, iron is present in most tissues in proteins such as cytochrome and catalase, which also contains hm. Iron, is thus concerned with the transport of oxygen by the blood and in cellular oxidations.

The blood iron is almost entirely present in the red cell hemoglobin, but there is a small amount in the plasma, probably as ferric iron, forming a pink complex with a protein which migrates electrophoretically as a α-globulin, and is termed siderophilin or transferrin. This is regarded as iron which is being transported in connection with the metabolism of hemoglobin. Accordingly determinations of serum iron are used in investigating some cases of anemia.

QUESTIONS:

1. How to determine the serum iron level in given sample

REFERENCES:

1. Harold Varley (2005). Practical clinical biochemistry, serum iron determination,CBS publishers and distributors 11, Daryaganj, New Delhi, India. 471-473.