UNIVERSITY OF TIKRIT

COLLEGE OF PHARMACY

THIRD STAGE

PRACTICAL BIOCHEMISTRY

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Experiment No-1-

General urine examination

Urine: is a solution of inorganic substances and waste products of organic metabolism contain 96% water.

For GUE , 24h urine sample sometimes required , for best results fresh sample required , also the examination should be fast for prevention of bacterial contamination that will lead to consuming of sample glucose , conversion of urea into ammonia then change the urine PH (make it alkaline) then precipitate the alkaline sediments , and also produce protein (result from bacterial contamination) also long standing may cause the oxidation of urobilinogen to urobilin .

Composition of Normal Urine

- Water 96%
- Urea 2%
- Uric acid
- Creatinine
- Ammonia
- Sodium
- Potassium 2%
- Chloride
- Phosphate
- Sulphate
- oxalate

Physical properties of the urine

Color , Volume, Specific gravity, Appearance, odor, pH.

1- Color :

• Normally, Urine is clear and amber (yellow) in color due to the presence of urobilin

• the higher the concentration of urine, the deeper is the color.

• Pale urine has a low specific gravity, a dark uine has a high specific gravity.

• The concentration of urine is highest in the a morning specimen (overnight urine)

and is lowest in a specimen passed an hour after much fluid has been taken.

Very pale urine is excreted in diabetes mellitus , diabetes insipidus , sever ferrous deficiency .

Abnormally colored urine may be result from :

Dark red	Bleeding from kidney
Bright red	Bleeding from lower urinary tract
Dark yellow	Presence of urobilinogen or bilirubin as in liver disease
Green	Pseudomonas organism
Dark brown	Presence of mathoemoglobin as in cancer

Milky urine as in :

-Lipid urea presence of fat in urine as in nephritic syndrome.

- Chyluria presence of lymph.

2-Volume : The average of daily output of urine on an average diet and normal fluid intake is 1500 ml/day.

• There are several Factors will affected on urinary output :

1)Physiological factors

Physiological: depends on the fluid intake (which is usually a matter of habit) and on the loss of fluid by other routes (primarily sweating which, in absence of fever, depends on physical activity and on the external temperature). When the water intake is voluntary restricted , the urine volume decreased to 500 ml\day this volume called obligatory volume which the least volume of urine needed to remove the daily waste products .

2)Pathological factors.

Polyuria	Oliguria	Anuria
More than 2500 ml/day	Below 500 ml /day	100 ml /day
Diabetes Mellitus and diabetes insipidus	In case of deficient intake of water	Stones or tumors in the urinary tract creating an obstruction to urinary flow
Chronic renal Insufficiency	excessive loss of fluids by other routs hemorrhage or as diarrhea and vomiting	

The urine volume increase in the following diseases DM , DI , renal failure diuretics .

Decrease in urine volume occur in the following conditions : dehydration , gastroenteritis , pyrexia , poor fluid intake .

3-Specific gravity

Specific gravity: is SG is a measure of the density of the dissolved chemicals in the specimen and refers to the weight of urine to that of D.W (which has sp.gravity of

Specific gravity = weight of volume of urine / wt of the same volume of water

The normal specific gravity of a pooled 24 hour urine sample is between 1.008 – 1.02 . There are direct relation-ship between concentration of substance in urine (Concentration of urine) and SG.

High specific gravity occur in nephritis , decrease fluid intake , presence of glucose (glucoseuria as in DM) , or protein (proteinuria as in nephrotic syndrome) in urine , sweating .

Low specific gravity occurs in chronic nephritis , increase fluid intake .

4-Apperance

This is classified as clear and turbid.

• Normal urine is clear.

• cloudy urine causes of turbidity include the presence of amorphous phosphate and carbonate that sediment in alkaline urine , uric acid that sediment in alkaline urine , high oxalate, pus, epithelial cell, blood cells, yeast , bacteria.

5-Odour

Normally Urine smells aromatic due to the presence of volatile organic acids .

• The urine of patients with diabetes mellitus may have a fruity (acetone) odor because of ketosis.

• Urine which is infected with Gram-negative organisms often has a distinctive unpleasant smell.

6-PH

The PH of urine normally slightly acidic (4.8-8) with an average of 5.5 . Acidic urine as in case of diarrhea , acidifying salts (NH_4Cl) intake , DM (due to formation of H_3PO_4). Alkaline urine as in excessive vomiting , intake of bicarbonate , some cases of bacterial infections where bacteria convert urea to ammonia , in anuria (renal damage) , vegetarian diet (formation of alkaline carbonate) .

Experiment No -2-

Chemical tests of urine

1- **Protein in urine**

Normally protein is not present in urine because the space of the glomerular filtrating membrane is too small to allow the passage of large particles like: protein , up to 20-50mg/day of protein in urine regards normal.

Proteinuria refers as albuminuria because albumin is the major protein .

False albuminuria

A-Boiling of acetic acid test :

Fill the test tube 5ml with urine and boil for 5minutes coagulation occurs indicates protein or phosphate . Add one drop of glacial acetic acid if the coagulation disappeared this means phosphate otherwise its protein.

B- Add 2drops of 20% solution of sulphosalicylic acid to 2ml of filtered urine . The turbidity indicates protein is present.

2-Bence Jones protein : normally urine does not contain this protein the presence of this protein indicates multiple myeloma , which is a light chain protein of immunoglobulin found in 75% of patients with multiple myeloma and metastasized tumor to the bone and chronic lymphocytic leukemia .

Bence Jones protein precipitated at (40-60) and resolved at the temperature reaches to the boiling point .

3-Reducing sugars in urine

Sugars types that may present include : glucose , galactose , lactose , fructose , and pentose (xylose and arabinose) .

Glucoseuria : glucose appears in urine when the blood glucose level exceeds the renal threshold (180mg/dl) mainly occur in uncontrolled DM .

Add 0.5 ml of urine to 5ml of Benedicts reagent . Place in boiling water bath for 5 minutes if turbidity or precipitate occurs indicates the presence of reducing sugar .

4- Ketone bodies in urine

Ketone bodies are (acetone , aceto-acetic acid , B-hydroxybutyric acid) they may be present in urine in cases of DM , persistent vomiting and prolong starvation .

Ketonaemia elevated level of ketone bodies in blood

Ketonuria elevated level of ketone in urine

Both ketonaemia and ketonuria are ketosis

A/ Rothera's test

Saturated 5ml of urine by shaking with solid ammonium sulphate then add 2drops of 2% sodium nitroprusside and 1ml of ammonium hydroxide , violet color indicates the presence of acetone bodies .

B/Gerhard test

Only aceto-acetic acid gives positive results and also salicylate gives positive results , B-hydroxybutyric acid gives negative results unless the urine boiled with H_2O_2 for oxidation of this compound to aceto-acetic acid and then apply Rothera's

test . Add few drops of 10% ferric chloride solution to 2ml of urine in a test tube purplish color appears if ketone bodies present .

5-Blood in urine

Blood may be present in urine as intact red cells which may be seen under the microscope or as hemoglobin resulting from hemolysis of red blood cell and either examined by spectroscopic or by chemical tests .

Add few drops of glacial acetic acid to 3 ml of urine saturated with benzidine then add 1ml of 3%H_2O_2 to give blue color which means urine contains blood .

Experiment No -3-

Bile pigments and bile salts in urine

1-Bile pigments

Urobilinogen : small amount normally present in urine this pigments on standing becomes oxidized to urobilin .

Indican : urine contains this pigment becomes deep brown when urine becomes ammonical on standing on standing some indigo may form .

Most tests used depend on the oxidation of bilirubin to differently color compounds .

Fouchet's test

Add few ml of 10% barium chloride solution to about 10ml of urine in the test tube , filter it and add drop of fouchet's reagent a greenish blue color if bilirubin is present this is very sensitive test .

2-Bile salts

Bile salts when present in the urine lower the surface tension .

Add sulphur powder on five ml of urine . The sulphur particles sink in the presence of bile salts but remain floating if they are absent .

Experiment No -5-

Unknown of urine

TEST	OBSERVATION	RESULTS
1-Detection of protein		
a)heat coagulation test	a)white coagulum by adding a drop of dilute acid b)No white coagulum	a)The urine may contain protein or albumin b)The urine may not contain protein or albumin
b)Sulpho salicylic acid	a) white cloud b)no white clouds	a-The urine contains protein b-The urine does not contain protein
2-Detection of glucose		
a)Benedicts test	a) colored ppt b)No colored ppt	a) the urine contain glucose b)the urine does not contain glucose
3-Detection of ketone bodies		
a)Rothera's test	a)purple color b)no purple color	a)the urine contains acetone and or aceto acetic acid b)the urine not contain acetone
b)Gerhard's test	the yellow color of ferric chloride increase by boiling	Absence of acetoacetic acid

4-Detection of bile

a)Hay's test	a)the sulphur flower sinks in thread link manner b)the sulphur flower float on the surface	a)the urine contain bile salts b)the urine not contain bile salts
5-Detection of blood		
a)Benzidine test	a)blue color b)no blue color	a)the urine contain blood b)the urine does not contain blood

Experiment No -5-

Sugars in cerebrospinal fluid

CSF: is clear colorless fluid placed in intraventicular and subarachnoidal spaces formed in chorioidal plexi of brain ventricles and subarachnoidally circulates round brain and spinal cord resorbed to venous (80%) and lymphatic (20%) systems.

Function of cerebrospinal fluid

• mechanic protection of brain and spinal

cord, protection against microorganisms

• transport of biomolecules to the brain

• clearance of catabolites (CO_2

, lactate)

• maintenance of constant intracranial

Pressure

The sugar content of normal lumber fluid is usually between 50-80mg/100ml , its value is about 60% of blood sugar value . The most important pathological change is the decrease which occur in meningitis . Meningitis due to various cocci-like meningococci , pneumococci , staphylococci , the glucose often disappears completely from the fluid . In the tuberculosis meningitis the sugar content is reduced but rarely completely absent (being 10-40mg/100ml) , also in hypoglycemia there is decrease in cerebrospinal fluid glucose .

Small increase are found in some cases of encephalitis , poliomyelitis , and cerebral abscess and may reach to 150-180mg/dl , even this is of little diagnostic value while in diabetes mellitus there is high increase in CSF glucose when there is

increase in blood sugar . Examination should not be done slightly after intrathecal streptomycin injection because streptomycin can reduce alkaline copper solution so impair the reliability of glucose estimation .

Determination of CSF glucose :

This done by any of the blood sugar determination methods.

Procedures :

In centrifuge tube put 3.7ml of isotonic solution add 0.1ml of CSF sample , 0.2ml of sodium tungstate . Shake then centrifuge , take 1ml of supernatant fluid into test tube mix with 1ml of benedicts reagent , cover this solution with cotton and place in boiling water bath for 10 minutes , then cool the mixture , add 3ml of arsenomolybdic solution , add 5ml of water , read at 700nm .

For blank use 3.8ml of isotonic solution without sample addition .

For standard 0.1ml of standard glucose solution use instead of sample .

CSF glucose (mg/100ml)=(T-B/S-B)XSX(100/0.025)

Note : sod. Tungestate use to precipitate protein .

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Experiment No -6-

Proteins in cerebrospinal fluid

The protein of normal lumber fluid lies between 15-45mg/100ml is almost entire albumin , normally CSF contain 60% albumin , 8% immunoglobulin and 32% other proteins . An increase in the total protein is the commonest abnormality and this result from the breakdown of the blood CSF and brain CSF barriers as a result of inflammation reaction but occasionally if the flow of CSF is obstructed the protein in such cases are mixture of albumin and globulin , fibrinogen may be present in the fluid when permeability of the blood –brain barrier is markedly increase in which there is considerable increase in protein and may give rise to a clot on standing . The extent which protein rise is important for the diagnosis of the diseases in meningitis , polyneuritis , tumors it may be reach to 400mg/100ml . Cell increase with protein increase is found in inflammatory lesions , meningitis , poliomyelitis . Isolated increase in protein found in tumors .

Determination of total protein :

Turbidimetric methods mainly used

Colorimetric methods depend mainly on biuret method .

Turbidimetric methods are simple and quick but they are affected by several facts which modify the particle size produced , temperature affects the turbidity so the samples and standards should be measured at the same temperature . The presence of red cell and bacteria lead to interference also contamination of CSF with blood affect on results due to the presence of plasma proteins .

Procedure : in the test tube mix 1ml of CSF with 4ml of 3% trichloroacetic acid wait 10 minutes then mix and read the optical density of the turbid solution at 450nm . For standard put 1ml of standard solution (contain 50mg/100ml) instead of sample . For blank put 1ml of water instead of sample .

CSF protein (mg/100ml) = (T-B/S-B) X 50 .

Experiment No -7-

Chloride in cerebrospinal fluid

Normally CSF contain 120-130mEq/L of chloride (700-760mg/100ml) , this value higher than that found in plasma (98-106mmol/L) , most important indications for the measurements of CSF chloride are during the diagnosis of meningitis as there is reduction in the CSF chloride accompanied this disease , the reduction is more marked in tubercles than in the coccal meningitis and may reach to 102mEq/L , in benign lymphocytic choriomeningitis chlorides are normal , in other diseases of the nervous system chloride are within normal limits . When there is marked reduction in plasma chloride in diseases not involving the CNS (vomiting and burns) also accompanied by low concentration of CNS chloride . In hypertension and in renal disease there is increase in CSF chloride .

Measurements:

This done by titration with silver nitrate solution using potassium chromate as indicator.

Technique: in test tube put 2-3 ml of distilled water , add 1ml of CSF , then add 2drops of potassium chromate and titrate with silver nitrate solution , appearance of brick red color indicate the end point which refer to the formation of silver chromate after the reaction of all chloride found in the sample with the added silver nitrate solution .

mEq.chloride /1 CSF = no. of ml of silver nitrate solution X30.

Experiment No -8-

Serum calcium

Calcium is an essential mineral. In the plasma, it is present in three forms:

• Free ionised (Ca^{++})

• Protein-bound (principally to albumin)

• Complexed (primarily with phosphate and citrate).

The protein bound is not diffusible , normally serum contain 4.5-5.4mEq/L ,

venous stasis due to veinpuncture should be avoided as this affected on the result.

Calcium has numerous functions

- The control of ion transport across cell membranes (particularly relevant to excitable tissues)
- Acting as an intracellular second messenger
- Activation of blood coagulation factors, coupling neuromuscular excitatin and providing the strength and rigidity of bones and teeth.

Estimation of calcium

Principles :

Colorimetric determination of calcium without deproteinization using O-cresol phthalein complex. Interference due to Mg^{+2} is eliminated by B-hydroxyquinoline (up to 4mmol/L , 0.01mg/L).

Procedure:

	Reagent blank	Standard	Sample
Sample	-	-	20microliter
Reagent 1 (Std)	-	20microliter	-
Working solution (R2+R3)	1ml	1ml	1ml

Mix, read absorbance after 5minutes at 572nm against blank . The color intensity is stable for 1hr .

Experiment No -9-

Inorganic phosphorus

Blood phosphorus

Present in the blood can be classified as :

1-Inorganic phosphorus , like phosphate

2-Organic phosphorus like glycerophosphates , nucleotide phosphate , hexosephosphate .

3-Lipid phosphorus like lecithin , cephalin.

Phosphorus is an abundant element that is widespread in its distribution. It is a major intracellular anion in mammals. Total body phosphorus in a 70-kg man is about 700 to 800 mg, 85% of which is in the skeleton in hydroxyapatite phase; the remaining 15% is in soft tissues. The majority of the phosphate in the body is in the organic form as a complex with carbohydrates, lipids, and proteins. Phosphorus is an essential element in the cellular structure, cytoplasm, and mitochondria. The normal serum phosphorus concentration is 3.4 to 4.5 mg/dl (1.12 to 1.45 mmol/L). This fluctuates with age (it is higher in children than adults), dietary intake, and acid–base status. There is a diurnal variation, which reaches its nadir between 8 and 11 a.m. Fasting is required before doing the test because phosphorus enter red blood cell with glucose , so lower plasma phosphorus after diet containing carbohydrate , also insulin secretion lower phosphorus level . The timing of blood sample is important , within day the level is higher in the morning than that in the evening , also within seasons the lower value reach during winter season.

Also there is difference between levels in the blood from that in the serum or plasma (whole blood contain higher than plasma or serum) the differences result from that the RBC richer in phosphorus than in plasma mainly because they contain more ester phosphate so it is important to avoid hemolysis of blood samples.

Determination of Inorganic phosphorus

Principles :

Colorimetric determination , without deprotrinization of serum phosphorus using a single reagents which forms a phosphomlybdate complex in the presence of reducing agents (ferrous sulphate).

Procedure

	Reagent blank	Standard	Sample
Sample	-		100microliter
Reagent (std)(5mg/dl,1.61mmol/l)	-	100microliter	-
D.W	100microliter	-	-
Working solution (R2+R3)	2.5	2.5	2.5
Mix and wait 10min , measure the absorbance of sample and standard at 690nm.			

Experiment No -10-

Serum total proteins

Proteins are organic nitrogenous compounds formed of C H O & “N” □ Proteins are the polymers of 20 naturally occurring amino acids. Only tests for the soluble proteins presently are performed on a routine bases in the chemical chemistry laboratory because the emphasis on fluid rather than solid tissue samples . Three common fluids that submitted for these analysis are serum , urine , CSF . Serum is deficient in those coagulation therefore serum protein will be approximately 0.25gm/dl lower than for the plasma protein because of the absence of fibrinogen . Serum protein can be analyzed in total in groups and individually

<table>
<tr><th colspan="6">Serum total proteins</th></tr>
<tr><td rowspan="3">Albumin</td><td colspan="5">Globulin</td></tr>
<tr><td>Alpha-1</td><td>Alpha-2</td><td>Beta</td><td colspan="2">Gamma</td></tr>
<tr><td colspan="5">Gamma (Immunoglobulines)</td></tr>
<tr><td></td><td>IgE</td><td>IgG</td><td>IgM</td><td>IgA</td><td>IgD</td></tr>
</table>

Many proteins including albumin , fibrinogen and most globulins are formed in the liver . The technical methods used for separation serum protein types are :

1-Salt fractionation

2-Electrophoresis

3-Ultracentrifugation

4-Chromatography

5-Gei filtration

6-Immun chemical analysis

The normal value of serum protein (6.2-8.2gm/dl)

Clinical significance

A-Hyperproteinemia

- Dehydration
- Multiple myeloma
- Cirrhosis of liver
- Certain chronic diseases
- Drugs
- Exercise

B-Hypoproteinemia

- Over hydration
- Kidney diseases
- Sever burns
- Sever malabsorption
- Fever
- Extensive bleeding
- Necrosis
- Sever protein deficiency (protein starvation)
- Drugs
- Increase requirement as in growth

Estimation of Serum total protein

Principle

Cupric ions , in an alkaline medium interact with protein peptide bonds resulting in the formation of colored complex .

	Reagent blank	Standard	Sample
D.W	0.02ml	-	-
Standard protein(6g/l)	-	0.02ml	-
Serum	-	-	0.02ml
Biuret reagent	1ml	1ml	1ml

Mix and incubate for 30min at 20-25C^0 Measure absorbance of the sample and standard reagent blank .

Experiment No -11-

Blood urea

Urea metabolism is an important way to excrete ammonia, which is a waste product of proteins metabolism . After breakdown of protein into amino acids which catabolized in the liver to form ammonia . Urea is formed from combination of ammonia with CO_2 to be transported by the blood to kidney for excretion . Urea diffuse freely in and out of the red blood cell and its concentration in red blood cells and plasma is nearly the same , for most purpose it is not essential weathere whole blood , plasma or serum used in analysis. .

Urea kit

Procedure

	Blank	Standard	Sample
Sample	-	-	10microliter
Standard 98.33mmol/l	-	10microliter	-
Working solution	1ml	1ml	1ml

Shake and incubate for 3min at $37C^0$ or 5min at $25C^0$

Reagents 4 200microliter 200 microliter 200microliter

Shake and incubate for 5min at $37C^0$ OR 10min at $25C^0$ -then read at 580nm .

Experiment No -12-

Determination of Uric acid

Uric acid is a waste product of purine metabolism in humans. The purine basis adenine and guanine formed in the course of nucleic acid catabolism and free nucleotide to uric acid as show :

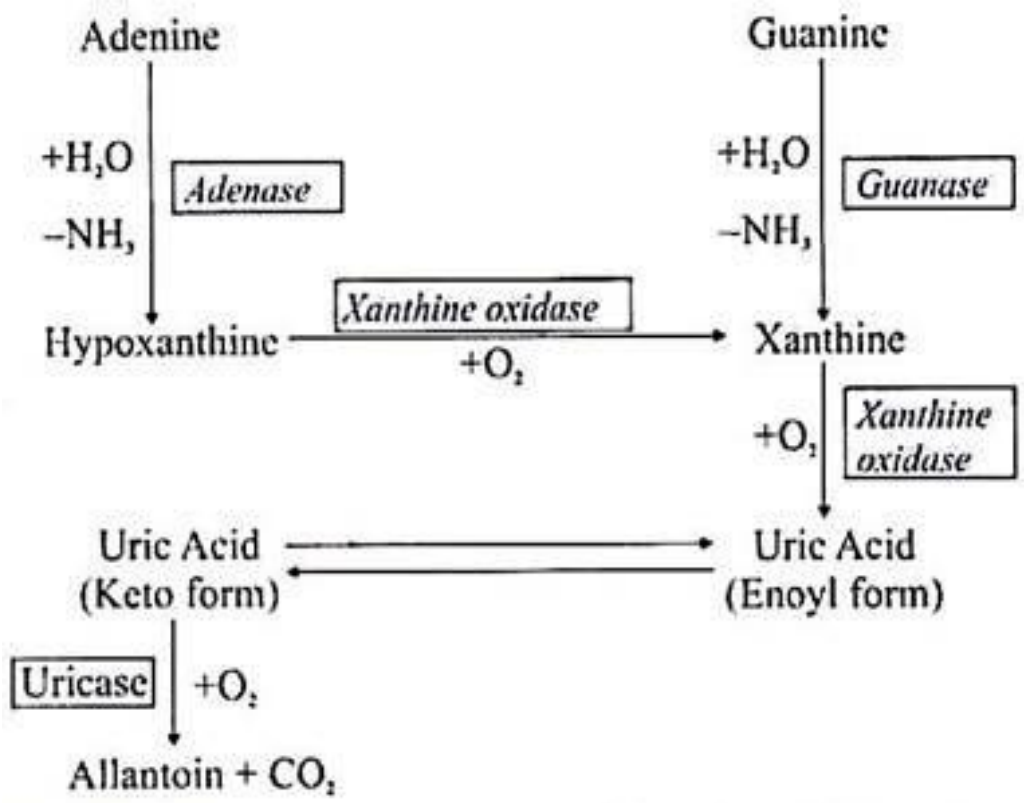

Fig. 10.114 Diagram showing the formation of uric acid.

Other animals degrade uric acid to allantoin by means of uricase enzyme which is missing by humans .

Uric acid sources:

A-Exogenous source : red meat , liver , stimulant in coffee and tea .

B-Endogenous nucleic acid catabolism

Liver is the main site of uric acid formation . Plasma uric acid is filtered by the glomeruli and about 90% reabsorbed by the tubules .

Normal value of uric acid in serum is (3-6.5) mg/dl.

Clinical significance

Determination of uric acid is most helpful in the diagnosis of gout where Sodium Urate are deposited in the solid form in and about the joints .

Hyperurecemia : increase level of uric acid is found in :

- Acute and chronic nephritis
- Urinary obstruction
- High purine diet
- Diabetic ketoacidosis
- Malignant tumors specially with extensive necrosis

Hypourecemia

- Proximal renal damage where urate reabsorption will reduced
- Xanthine oxidase deficiency
- Wilson's disease
- Fanconie's syndrome