KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2015 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : HORMONES: BIOCHEMISTRY AND FUNCTION
SEMESTER : III
SUBJECT CODE : 16BCU313 **CLASS :** II B.Sc.(BC)

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1. Glucose tolerance test.
2. Estimation of serum Ca2+.
3. Estimation of serum T4.
4. HCG based pregnancy test.
5. Estimation of serum electrolytes.
6. Case studies.

REFERENCES

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DEPARTMENT OF BIOCHEMISTRY

SUBJECT : HORMONES: BIOCHEMISTRY AND FUNCTION- Practicals

SEMESTER : III

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Experiment No: 1

Glucose tolearance test

The glucose tolerance test is a medical test in which glucose is given and blood samples taken afterward to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance, impaired beta cell function, and sometimes reactive hypoglycemia and acromegaly, or rarer disorders of carbohydrate metabolism. In the most commonly performed version of the test, an oral glucose tolerance test (OGTT), a standard dose of glucose is ingested by mouth and blood levels are checked two hours later. Many variations of the GTT have been devised over the years for various purposes, with different standard doses of glucose, different routes of administration, different intervals and durations of sampling, and various substances measured in addition to blood glucose.

Procedure

A zero time (baseline) blood sample is drawn.

The patient is then given a measured dose (below) of glucose solution to drink within a 5-minute time frame.

Blood is drawn at intervals for measurement of glucose (blood sugar), and sometimes insulin levels. The intervals and number of samples vary according to the purpose of the test. For simple diabetes screening, the most important sample is the 2 hour sample and the 0 and 2 hour samples may be the only ones collected. A laboratory may continue to collect blood for up to 6 hours depending on the protocol requested by the physician.

Dose of glucose and variations

75g of oral dose is the recommendation of the WHO to be used in all adults, and is the main dosage used. The dose is adjusted for weight only in children The dose should be drunk within 5 minutes.

A variant is often used in pregnancy to screen for gestational diabetes, with a screening test of 50 grams over one hour. If elevated, this is followed with a test of 100 grams over three hours.

Fasting plasma glucose (measured before the OGTT begins) should be below 6.1 mmol/L (110 mg/dL). Fasting levels between 6.1 and 7.0 mmol/L (110 and 125 mg/dL) are borderline ("impaired fasting glycaemia"), and fasting levels repeatedly at or above 7.0 mmol/L (126 mg/dL) are diagnostic of diabetes.

A 1 hour GTT (Glucose Tolerance Test) glucose level below 10 mmol/L (180 mg/dL) is considered normal.

For a 2 hour GTT (Glucose Tolerance Test) with 75g intake, a glucose level below 7.8 mmol/L (140 mg/dL) is normal, whereas higher glucose levels indicate hyperglycemia. Blood plasma glucose between 7.8 mmol/L (140 mg/dL) and 11.1 mmol/L (200 mg/dL) indicate "impaired glucose tolerance", and levels above 11.1 mmol/L (200 mg/dL) at 2 hours confirm a diagnosis of diabetes.

For gestational diabetes, the American College of Obstetricians and Gynecologists (ACOG) recommends a two-step procedure, wherein the first step is a 50g glucose dose. If it results in a blood glucose level of more than 7.8 mmol/L (140 mg/dL), it is followed by a 100 gram glucose dose. The diagnosis of gestational diabetes is then defined by a blood glucose level exceeding the cutoff value on at least two intervals, with cutoffs as follows:

Before glucose intake (fasting): 5.3 mmol/L (95 mg/dL)

1 hour after drinking the glucose solution: 10 mmol/L (180 mg/dL)

2 hours: 8.6 mmol/L (155 mg/dL)

3 hours: 7.8 mmol/L (140 mg/dL)

ESTIMATION OF BLOOD GLUCOSE- Anthrone method

Aim

To estimate the amount of glucose present in a given unknown sample.

Principle

Hot acidic medium sugar is dehydrated to hydroxy methyl furfural. This compounds found a green colour product with anthrone which was read at 630nm.

Reagents

1. **Anthrone reagent**
   Dissolve 200mg anthrone on 100 ml of ice cold 95% H_2SO_4. It should be prepared freshly before use.
2. **Standard Glucose Solution**
   500 mg of glucose was dissolved in 100 ml of distilled water.
3. **Working Standard**
   10 ml of stock was diluted to 100 ml with distilled water.

Procedure

Into a series of test tubes pipette out 0.1 to 0.5 ml of working standard and labeled as S1 to S5. The given unknown solution was made upto 100 ml with distilled water mix well and from that 0.5 ml was taken in two test tubes and marked as U1 and U2 make up the volume of each test tube to 1 ml. 1 ml of distilled water was serves as blank and add 4 ml of anthrone in all test tubes. Test tubes were treated in a water bath for 8 minutes. Cool rapidly and read the dark green colour solution at 630 nm.

Draw the standard graph by plotting concentration of standard on X-axis and absorption at Y-axis. From the graph, the amount of in the unknown was calculated.

Table

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water(ml)	Volume of Anthrone (ml)	Heated in a boiling water bath for 8 minutes	Optical Density at 630 nm
1	Blank	-	-	1.0	4.0		
2	Standard						
	S1	0.2	20	0.8			
	S2	0.4	40	0.6			
	S3	0.6	60	0.4			
	S4	0.8	80	0.2			
	S5	1.0	100	-			
3	Sample	0.1	-	0.9			
		0.1	-	0.9			
4	Unknown	1.0	-	-			
		1.0	-	0.5			

Experiment No: 2

Estimation of serum Calcium – Permanganate Method

Aim :

To estimate the amount of Calcium in the given amount of Serum.

Principle :

Calcium in Serum is precipitated as calcium oxalate with ammonium oxalate (Magnesium is precipitated as the conditions are selected to increase the solubility of Magnesium oxalate).

The precipitate is washed with dilute ammonia to remove the excess ammonium oxalate & then dissolved in normal H_2SO_4 acid. Oxalic acid formed is with std permanganate solution. The end point of titration is indicated by the formation of pink colour which is stable for atleast 30 sec.

Reagent :

1. 4 % Ammonium oxalate solution.
2. 2 % Ammonium – diluted 2 ml of specific gravity 0.88 – 100 ml with H_2O.
3. 0.01 N Potassium Permanganate solution prepared freshly before use by diluting stock Potassium Permanganate (0.1 N) solution.
4. Appr. Normal H_2SO_4 .

Procedure :

Into two centrifuge tubes pipette out 2 ml of dil. H_2O. 2 ml of serum with 1ml of 4 % ammonium oxalate. Mix the tubes and allowed to stand over night at room temperature. Centrifuge for 10 mins at 2000 rpm & carefully collect the supernatant, invert the tubes & allow to drain on a pair of filter paper for 3 mins. Add 3 ml of 2 % Ammonia down the sides of the tube. Mix the precipitate & Centrifuge & decant the solution. This processes was repeated until the precipitate was washed completely and supernatant gave no precipitate with Calcium Chloride.

Pippete out 2 ml of normal H_2SO_4 rotating the tube to wash down. Then place the tubes in water both for 5 mins at 70-80°C to dissolve the precipitate. Remove the titrate the contents while hot with $KMnO_4$ solution taken in the burette until pale pink colour a min. Repeat the titration with duplicate tubes.

Performed a blank titration with 2 ml normal H_2SO_4 kept in H_2O both for 5 mins & titrated with $KMnO_4$ to give pink colour. The difference between two tubes titrate volume given the value of 0.01N $KMnO_4$ required to titrate Calcium oxalate.

Result :

The amount of Calcium present in 100 ml of urine is found to be ___mg/dl.

S.No	Volume of solution (ml)	Biurette reading (ml)		Volume of $KMnO_4$ (ml)	Indicator
		Initial	Final		
1.	Blank				Self
	2.0	0.0	0.2	0.2	
	2.0	0.0	0.2	0.2	
2.	Sample				
	2.0	0.0	2.2	2.2	
	2.0	0. 0	2.2	2.2	

Calculation :

Volume of 0.01 $KMnO_4$ required by 2.0 ml of blank = 0.2ml

Volume of 0.01 $KMnO_4$ required by 2.0 ml of serum= 2.2 ml

2.0 ml serum = 2.2 — 0.2

= ____ ml of $KMnO_4$

V1 N1 = V2 N2

0.01 × 2 =2 × N2

N2 = x N

Amount of Calcium = Equivalent weight of Calcium × Normality (N2)

= 2.0 × x

Amount of calcium present in 100 ml of serum = 0.2/2 × 100

= ____mg/dl.

Experiment No: 3

Estimation of Serum Thyroxine

Principle

This assay is based on the principle of solid phase competitive enzyme immunoassay.

Preparation of Reagents

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (18-25°C).

Working Substrate Solution – Prepare immediately before use

To prepare H2O2/TMB solution, make a 1:1 mixing of Color Reagent A with Color Reagent B up to 1 hour before use. Mix gently to ensure complete mixing. The prepared H2O2/TMB reagent should be made at least 15 minutes before use and is stable at room temperature in the dark for up to 3 hours. Discard excess after use.

Preparation and Collection of Specimen

1. Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques.

2. This kit is for use with serum samples without additives only.

3. Serum samples may be refrigerated at 2-8°C for a maximum period of 48 hours. If the samples cannot be assayed within 48 hours, they may be stored at temperatures of -20°C for up to 30 days.

Assay Procedure:

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (18-25°C).

1. Format the microplates' wells for each serum reference, control, and patient specimen to be assayed in duplicate.

2. Pipette 50 µl of the appropriate serum reference, control and specimen into the assigned well.

3. Add 100 µl of Free Thyroxine (T4) Enzyme Conjugate Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Incubate 60 minutes at room temperature.

6. Remove the incubation mixture by emptying the plate content into a waste container. Rinse and empty the microtiter plate 5 times with distilled water. Strike the microtiter plate sharply onto absorbent paper or paper towels to remove all residual water droplets.

7. Add 200 µl of Working Substrate Solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells. Gently mix for 10 seconds.

8. Incubate at room temperature in the dark for 20 minutes.

9. Stop the reaction by adding 50 µl of 3N HCl (Stop Solution) to each well.

10. Gently mix for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.

11. Read absorbance at 450 nm with a microtiter well reader within 30 minutes.

Data Analysis

1. Calculate the mean absorbance value (OD450) for each set of reference standards, controls and patient samples.

2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/dl on graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.

3. Use the mean absorbance values for each specimen to determine the corresponding concentration of fT4 in ng/dl from the standard curve.

Experiment No: 4

Estimation of hCG

PRINCIPLE

The hCG Card Pregnancy Test is a rapid chromatographic immunoassay for the qualitative detection of human chorionic gonadotropin in urine to aid in the early detection of pregnancy. The test utilizes a combination of antibodies including a monoclonal hCG antibody to selectively detect elevated levels of hCG. The assay is conducted by adding a urine specimen to the specimen well of the test device and observing the formation of colored lines. The specimen migrates via capillary action along the membrane to react with the colored conjugate.

Positive specimens react with the specific antibody-hCG-colored conjugate to form a colored line at the test line region of the membrane. Absence of this colored line suggests a negative result. To serve as a procedural control, a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

REAGENTS

The test card contains anti-hCG particles and anti-hCG coated on the membrane.

Storage and Stability

The kit can be stored at room temperature or refrigerated (2-30°C). The test card is stable through the expiration date printed on the sealed pouch. The test card must remain in the sealed pouch until use. DO NOT FREEZE. Do not use beyond the expiration date.

SPECIMEN COLLECTION AND PREPARATION URINE ASSAY

A urine specimen must be collected in a clean and dry container. A first morning urine specimen is preferred since it generally contains the highest concentration of hCG; however, urine specimens collected at any time of the day may be used. Urine specimens exhibiting visible precipitates should be centrifuged, filtered, or allowed to settle to obtain a clear specimen for testing.

SPECIMEN STORAGE

Urine specimens may be stored at 2-8°C for up to 48 hours prior to testing. For prolonged storage, specimens may be frozen and stored below -20°C. Frozen specimens should be thawed and mixed before testing.

Direction For Use

1. Allow the test device, urine specimen and/or controls to equilibrate to room temperature (15-30°C) prior to testing.

2. Bring the pouch to room temperature before opening it. Remove the test device from the sealed pouch and use it as soon as possible.

3. Place the test device on a clean and level surface. Hold the dropper vertically and transfer 3 full drops of urine (approx. 180 µl) to the specimen well (S) of the test device, and then start the timer. Avoid trapping air bubbles in the specimen well (S). See the illustration below.

4. The result should be interpretated between 3-5 minutes. Please confirm negative results at 10 minutes.

Do not interpretate result exceeding 10 minutes.

Interpretation of Results

POSITIVE:* Two distinct red lines appear. One line should be in the control region (C) and another line should be in the test region (T).

*NOTE: The intensity of the red color in the test line region (T) will vary depending on the concentration of hCG present in the specimen. Therefore, any shade of red in the test region (T) should be considered positive.

NEGATIVE: One red line appears in the control region (C). No apparent red or pink line appears in the test region (T).

Experiment No: 5

Estimation of Sodium

Principle :

The sodium and the proteins are Precipitated Simultaneously by means of a reagent containing magnesium uranyl acetate containing alcohol. The precipitate is seperated by centrifugation. The content of sodium is calculated from the loss in the concentration of magnesium uranyl acetate in the reagent solution in comparison to a standard sodium solution treated similarly. The residusal amount of magnesium uranylacetate is estimated by forming brown (dark) ferrous uranyl acetate. Which is read in a colorimeter.

Step I - Precipitation of sodium and proteins.

Pipette into two clean dry test tubes labelled standard (S) and test (T)

	S	T
Sodium PPT Reagent(l)	1.0 ml	1.0 ml
Standard Sodium/Potassium(2)	0.02 ml	.....
Serum	.....	0.02 ml

Mix well on vortex for one minute and wait for five minutes at room temperature. Centrifuge for one minute at 3000 rpm.

Step II - Color Development.

Pipette into three clean dry test tubes labelled blank (B), standard (S) and test (T)

	B	S	T
Distilled Water	3ml	3ml	3ml
Supernatant from step I	...	0.05ml	0.05ml
Sodium PPT Reagent (1)	0.05ml	....	....
Sodium Color Reagent (3)	0.2ml	0.2ml	0.2ml

Mix well and allow it to stand at room temperature for five minutes. Then measure absorbance of B,S,T against distilled water on a photocolorimeter at 540 nm within 10 minutes.

Calculation :

Sodium in mmol/L =

Absorbance of B-T X 150 (Standard concentration)

_____

Absorbance of B-S

Normal Range :
Sodium :135 to 155 mmol/L

Experiment No: 6

Zollinger-Ellison Syndrome Associated with von Recklinghausen Disease: Case Report

Patient: Female, 28

Final Diagnosis: Gastrinoma

Symptoms: Vomiting • diarrhea • epigastric soreness • heartburn • nausea • significant weight loss

Medication: —

Clinical Procedure: Esophagogastroduodenoscopy • blood tests • abdomen CT scan • surgery

Specialty: Gastroenterology and Hepatology

Objective: Rare co-existance of disease or pathology

Background:

Pancreatic endocrine tumors (PETs) are rare and can occur as part of neurofibromatosis type 1 (NF1). Gastrinomas are functional PETs that are rarely associated with NF1. Only two cases of their occurrence have been reported in the literature.

Case Report:

A 28-year-old woman was admitted for further evaluation of epigastric soreness, heartburn, nausea, vomiting, diarrhea, and a significant weight loss. Physical examination was remarkable for cutaneous findings (axillary freckling and multiple café-au-lait spots) as well as neurofibromas (dermal, plexiform). A diagnosis of NF1 was confirmed. Esophagogastroduodenoscopy (EGD) revealed multiple ulcers in the duodenum and the upper jejunum. A fasting gastrin level exceeded ten times the normal limit. An abdominal multi-slice 128 computed tomography (CT) scan revealed an oval mass of 26 mm in diameter adjacent to

the second segment of the duodenum. The patient was examined carefully to rule out multiple endocrine neoplasia type 1 (MEN1). Surgical resection was performed and a gastrinoma, causing Zollinger-Ellison syndrome (ZES), was diagnosed by histological examinations of the extirpated mass. The serum gastrin level decreased to normal limits shortly after surgery. Continuous follow-up revealed that the symptoms and the EGD findings completely resolved without recurrences.

Conclusion:

Although NF1 has common skeletal, visual, neurological, and cardiovascular complications, it also has a rare association with duodenal or pancreatic gastrinomas. Vigilance for this possible association is important to promote timely and careful management to help eliminate serious and potentially life-threatening complications

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Practical Completion Report

S.No	Name of the Experiment	Date of Completion
1	Glucose tolerance test.	30.06.2017
2	Estimation of serum Ca^{2+}.	14.07.2017
3	Estimation of serum T4.	28.7.2017
4	HCG based pregnancy test.	18.08.2017
5	Estimation of serum electrolytes.	19.08.2017
6	Case Studies	1.09.2017