Endocrinology Laboratory

This laboratory consists of the following main sections:

- **Background information**
- **Protein assay: determination of purified human chorionic gonadotropin (hCG) concentration and pregnancy status**
- **Quantitative determination of glucose in serum**

Background information

Immunoprecipitation

Typically, while a blood or urine sample contains a variety of proteins (crude sample), only one may be of diagnostic interest. Immunoprecipitation is often used to purify a target protein from solution (purified sample). This way, the protein of interest can be further examined for quantity. Immunoprecipitation involves the interaction between a protein (antigen) and its specific antibody. An antibody specific to the target protein is bound to a Protein G support coupled to latex beads (column) and immobilized by a cross-linking agent (DSS: disuccinimidyl suberate). Cross-linking ensures that the immobilized antibody remains bound to the support and does not contaminate the final antigen preparation. Protein G is a bacterial cell wall protein isolated from group G streptococci. Its use in this technique relies on its unique ability to bind the Fc portion of the antibody and not the Fab fragment (see Figure 1).

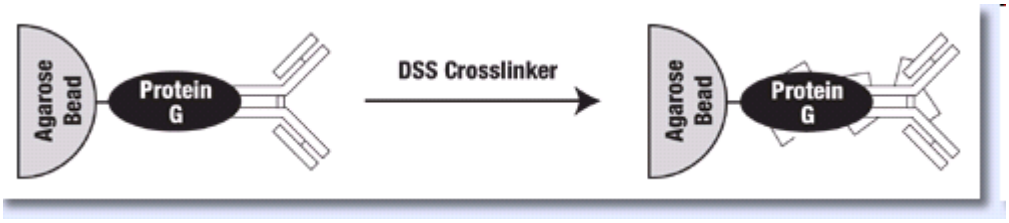

Figure 1. Schematic showing crosslinking of antibody to Protein G immobilized agarose beads

Antigen can thus be isolated from a crude protein mixture by incubation on a Protein G column that has its complementary antibody bound to the immobilized Protein G.

Only the specific antigen should bind to the immobilized antibody to form the immune complex. Unbound proteins are removed by washing the column. The antigen is then dissociated from the antibody using an elution buffer.

Immunoprecipitation steps

(**the following steps have been done for you:** due to time constraints, the immunoprecipitation of hCG from blood serum **will not** be performed during the laboratory).

- A specific anti-hCG antibody was covalently bound to protein G coated beads using the cross-linking agent disuccinimidyl suberate (DSS).
- The serum containing hCG was then incubated with the immobilized antibody to form the immune complex.
- The affinity column (protein G beads – anti hCG antibody) was then washed by centrifugation to eliminate unbound proteins, leaving the antigen bound to the antibody support.
- The immunopurified antigen (hCG) was dissociated from the antibody-protein G beads complex using an elution buffer.

Determination of hCG concentration following its purification

- **Protein assay: the Bradford method of protein quantification generates a protein-dependent color change which is measured by spectrophotometry.**

The Bio-Rad Protein Assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solubilized proteins. It involves the addition of an acidic dye to a protein solution and subsequent measurement at 595nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration. The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465nm to 595nm when binding to protein occurs. The Coomasie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine. Thus, **Beer's law** may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration" (from Biorad protein assay manual)

Beer's law is given by $A = \sum cl$

where **A** is the absorbance of the solution (also termed **OD: optical density**), $\sum$ is the substance specific absorption coefficient (also termed extinction coefficient), **l** is the length traveled by light throughout the sample and **c** is the concentration of the sample. In the cuvettes you utilize, the path length is 1 cm.

To determine the hCG concentration you will measure OD at 595nm of a 1ml sample in spectrophotometer cuvettes so that the length the light travels through the sample is a constant. Under these conditions, OD is directly proportional to the concentration of total proteins (varies linearly). The amount of protein in an unknown sample can be interpolated, using its absorbance and comparing it to a standard curve.
The standard curve is obtained by plotting the OD that is measured for several known concentrations of a protein of reference or standard protein (the purified assayed protein or bovine serum albumin BSA) versus the concentration. You will use bovine serum albumin as the reference standard protein. The data should fit a straight line.

Many detergents and buffers can interfere with the assay owing to interactions with the dye. It is essential to dilute the standards in the same buffer that is used for the protein to be assayed. A potential non-specific effect of the buffer will also be minimized by just adding buffer alone (without protein) in the first spectrophotometer cuvette. The OD measured for this first cuvette will correspond to a zero protein content.

An important consideration in all assays is the determination of "assay sensitivity". This corresponds to the minimal detectable concentration of the entity (molecule or compound) of interest. Assay sensitivity will depend on the uncertainty associated with the "zero" dose as well as the error associated with the mean response of the "unknown".

- **hCG and pregnancy**

During pregnancy, normal trophoblast cells of the placenta secrete a glycoprotein hormone: human chorionic gonadotropin (hCG). Under normal circumstances, hCG is produced only by placental tissue and is detected in the blood and urine of a pregnant woman. Hence, hCG detection becomes a sensitive chemical test for early pregnancy. The trophoblast cells begin to secrete hCG around the time the trophoblast begins to invade the uterine endometrium.

In the absence of pregnancy the corpus luteum degenerates within two weeks after its formation. The

function of hCG is to maintain the corpus luteum in the beginning stages of gestation. This action of hCG is exerted through the LH/CG receptors on luteal cells.

The secretion of hCG reaches a peak 60 to 80 days after the last menstrual period, decreasing rapidly afterwards. By the end of the third month it has reached a low level which will remain constant for the duration of the pregnancy. With this decrease of hCG secretion, the placenta begins to secrete large quantities of estrogen and progesterone and the dependence on the corpus luteum for the maintenance of pregnancy disappears.

You will determine the concentration of the immunoprecipitated hCG sample and report the days from conception according to the following table:

From conception	**LMP (last menstrual period)**	**hCG range (mIU/ml)[1]**
7 days	3 weeks	0 to 5
14 days	28 days	3 to 342
21 days	35 days	18 to 7 340
28 days	42 days	1 080 to 56 500
35-42 days	49-56 days	7650 to 229 000
43-64 days	57-78 days	25 700 to 288 000
57-78 days	79-100 days	13 300 to 253 000
17-24 weeks	2nd trimester	4 060 to 165 000
25 weeks to term	3rd trimester	3 640 to 117 000
Several days postpartum		**Nonpregnant levels (<5)**

Definition of diabetes and diagnostic criteria

Diabetes mellitus is a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both. The chronic hyperglycemia of diabetes is associated with relatively specific long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease. The diagnostic criteria for diabetes are based on thresholds of glycemia that are associated with microvascular disease, especially retinopathy.
A fasting plasma glucose level of ≥ 7.0 mmol/L, a 2-hour plasma glucose value in a 75 g oral glucose tolerance test of ≥ 11.1 mmole/L or a glycated hemoglobin value of ≥ 6.5% can predict the development of retinopathy. Each of these criteria permits the diagnosis of diabetes and is based on non-hemolyzed venous samples and laboratory methods.

Quantitative determination of glucose in serum

Measurement of blood glucose levels was among the first chemical procedures used in clinical laboratory medicine. The reagent which will be used is based on a technique described by Trinder et al. Glucose is oxidized in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under the influence of peroxidase, with phenol and 4-aminoantipyrine to form a red-violet quinone complex (see figure 2). The intensity of the colour, read by a spectrophotometer set at a wavelength of 500 nm, is proportional to glucose concentration. The linearity of the test is from 0 to 650 mg/dL.

[1]Statistically, the ranges are broad as each pregnancy cycle and hormone level is unique to the individual and extreme readings either way are possible in a very small percentage of pregnancies.

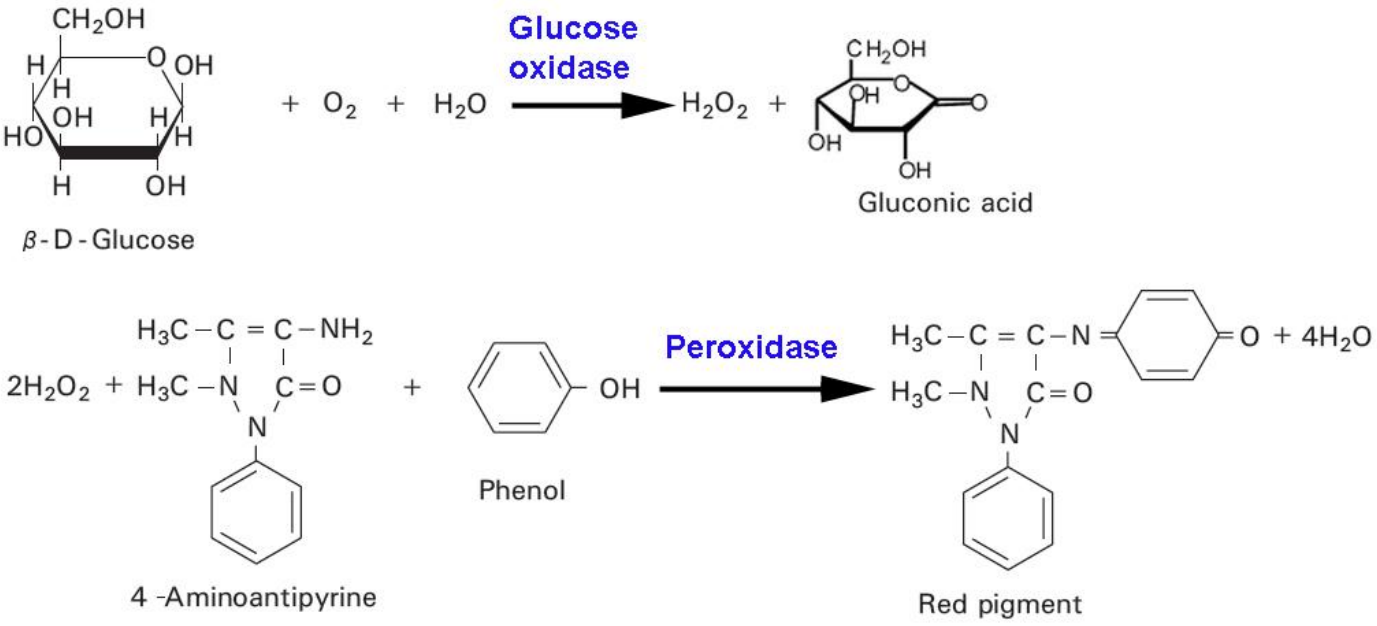

Figure 2. Glucose is oxidized in the presence of glucose oxidase.

Protein assay: determination of hCG concentration and pregnancy status

Aim

- To determine hCG concentration of the purified hormone from serum:
  - using the Bradford method of protein quantitation, measure the absorbance of the Coomassie brilliant blue dye-protein complex by spectrophotometry at a wavelength of 595nm.
  - establish a standard curve with known concentrations of Bovine Serum Albumin (BSA) and use the equation of the curve to calculate the concentration of immunopurified hCG.
  - determine the pregnancy status.

Materials

When handling all samples and the BioRad reagent, wear gloves!
Discard used pipette tips in special containers at your bench.

-Stock immunopure hCG (1 ml)
-Stock BSA standard (concentration: 200 μg/ml; quantity: 1 ml)
-Elution buffer
-BioRad Reagent (contains Coomassie Brilliant Blue G250 dye in a solution of phosphoric acid and methanol)
-adjustable pipettes: 2 μl-20 μl; 10 μl-200 μl; 200 μl-1000 μl
-microcentrifuge tubes (1.5 ml volume)
-Cuvettes for spectrophotometer
-Marker to label tubes

Guidelines

Concentration of immunopurified hCG will be measured based on the Bradford Method.
In this method, the absorbance of a protein-dye complex will be measured with a spectrophotometer set at 595 nm. According to Beer's law and the experimental conditions used in this protocol, the absorbance of the dye-protein complex will be directly proportional to protein concentration.

You will need to determine all appropriate dilutions and volumes needed to process the standards and the samples. **Each student must prepare a protocol showing the volume and concentration handling of the standards (BSA) as well as the hCG samples to be used in the assay. A copy of this protocol will be collected from each student at the start of the laboratory session and will form part of the laboratory report.**

Groups that did not prepare their protocols will not be permitted to start the laboratory session until the protocols are provided. This delay may prevent the group from completing the laboratory session within the allotted time.

Protein standard solutions:

- Prepare 5 different concentrations in triplicate of the protein standard BSA (stock concentration: 200 µg/ml) which is representative of the protein solution to be tested, including tubes containing 0 µg/ml of protein. The linearity of the assay ranges between 2 and 20 µg/ml.

Sample solutions:

- You are provided with a sample to be tested for protein content labeled stock immunopure hCG.
- You will use a fraction of stock hCG as well as prepare dilutions of these stock solutions: 1/2 and 1/3 for hCG. Remember the final volume of these samples should be made up to 800 µl. They should be prepared at the same time as your standard curve so that all tubes (standard curve and samples) receive the Bradford reagent (200 µl) at the same time.

Assay reaction:

- The assay should be done in triplicate. A blank should also be included: the OD measurement from this blank sample corresponds to 0 µg of BSA (or 0 µg of protein). All dilutions should be done in the elution buffer provided.
- Pipet 800 µl of each standard and sample solution into microcentrifuge tubes. The volume of the sample solution represents 1/20 of the 800 µl total volume.
- Add 200 µl of dye reagent concentrate to each tube and immediately invert to mix.
- Incubate at room temperature for 5 minutes.
- Measure OD at 595 nm: handle the spectrophotometer cuvettes by their hatched sides! Detailed instructions on how to manipulate the spectrophotometer will be given during the lab.

Results

Calculate protein concentration:

Using the standard curve, and OD values of your samples, determine the concentration of hCG in the sample, and the time from conception.

Example of a protocol detailing dilutions required to perform the assay:

hCG dilution preparation [2](see footnote below)			
Tube labelled ½ hCG	Total volume required for 3 tubes: ? µl	Volume taken from stock hCG: ? µl + Volume of buffer added: ? µl	Final concentration of hCG: 1/2
Tube labelled 1/3 hCG	Total volume required for 3 tubes: ? µl	Volume taken from stock hCG: ? µl + Volume of buffer added: ? µl	Final concentration of hCG: 1/3

Standard curve: dilutions protocol					
Tube designation		**µl BSA (taken from the 200 µg/ml standard)**	**µl buffer**	**Total volume (µl) before the addition of the BioRad reagent**	**Total assay volume: (after the addition of the BioRad reagent)**
1 (a,b,c)	0 µg	0	800	800	**1000 µl/tube**
2 (a,b,c)	2 µg	?	?	800	
3 (a,b,c)	…			800	
4 (a,b,c)	…			800	
5 (a,b,c)	…			800	

For each tube:			**Volume sample to buffer**	**Total volume (µl) before the addition of the BioRad reagent**	**Total assay volume: (after the addition of the BioRad reagent)**
neat sample	Volume of stock hCG: ? µl	Volume of buffer: ? µl	**1/20**	**800**	**1000 µl/tube**
½ hCG	Volume from prepared ½ hCG tube above: ? µl	Volume of buffer: ? µl	**1/20**	**800**	
1/3 hCG	Volume from prepared 1/3 hCG tube above: ? µl	Volume of buffer: ? µl	**1/20**	**800**	

[2] **Warning:** when making up diluted samples take into consideration a slight volume loss which will occur while pipetting out into each of the triplicate assay tube, i.e.: prepare in a conveniently larger volume.

Quantitative determination of glucose in serum

Aim

- To provide a protocol detailing the dilutions of the glucose standards. The assay is done in triplicate.
- To provide a standard curve of absorbance (OD) versus dilutions from a stock solution of 500 mg/dL glucose.
- To plot for each of the individuals the glucose concentrations of their serum versus time: 0 minute (8 hour fasting baseline), 30 minutes, 60 minutes, and 120 minutes post oral glucose tolerance test.
- To determine which of the two sets of sera originates from a diabetic individual.

Materials/reagents

- Glucose stock solution: 500 mg/dL (in a volume of 1 ml), 0.5 ml capacity microcentrifuge tubes, and distilled water to prepare suggested dilutions of 50 mg/dL, 100mg/dL, 150 mg/dL, 200 mg/dl, 250 mg/dL, and 300 mg/dL glucose standards
  (Suggestion: 0.250 ml could be the total volume of each standard prepared in separate microcentrifuge tubes)

- 4 Serum tubes from individual A and 4 serum tubes from individual B

- Trinder reagent and distilled water

- Spectrophotometer cuvettes

- Adjustable pipettes: 2 µl-20 µl; 10 µl-200 µl; 200 µl-1000 µl

When handling all samples and the Trinder reagent, wear gloves!
Discard used pipette tips in special containers at your bench.

- The spectrophotometer is set at a wavelength of 500 nm and zeroed with distilled water.

Assay

- Add 5µl of each control (distilled water), prepared glucose standards, and samples in the triplicate cuvette (this assay can be done directly in the spectrophotometer cuvettes).

- Add 1 ml of Trinder reagent to all tubes sequentially at timed intervals (i.e.: 15 seconds or less between each tube). The first cuvette should be read after an incubation of 25 minutes at room temperature, followed sequentially by the rest of the cuvettes (each tube has incubated for 25 minutes).

Results

- Provide a table of OD readings, average OD reading with standard deviation for each of the Glucose standard solutions
- Provide tables for each individual of OD readings, average OD readings, glucose concentrations (in mg/dl and in mmole/L)
- Graph 1: Plot the standard curve (average OD readings vs. glucose standard concentration in mmole/L)
- Graph 2: Plot the glucose concentrations (mmole/L) of sera A and B versus time (minutes)