PRACTICAL 4: RAPID ANALYTICAL TECHNIQUES FOR DIAGNOSING AND MANAGING DIABETES

4.1   Introduction

The **aims** of this practical are:

- to demonstrate the biochemical basis, specificity and use of rapid "sideroom" tests for the detection of urinary sugars, ketone bodies and protein

- to demonstrate the use of high performance liquid chromatography (HPLC) — an important analytical technique — for the detection of glycated haemoglobin in diabetic sera.

4.2   Theory and background

A detailed discussion of diabetes is beyond the scope of this short introduction. A good account can be found in the "ABC of Diabetes" by P.J. Watkins (4th edition, 1998), BMJ Publishing Group and "Diabetes: Pocket Picture Guides" by H.J. Bodansky (1989) (J.B. Lippincot).

Primary diabetes mellitus is divided into two forms: insulin-dependent diabetes (Type 1, or **IDDM**) and non-insulin-dependent diabetes (Type 2, or **NIDDM**). The former results from the destruction of pancreatic B cells in the islets of Langerhans with the consequent loss of insulin production while the latter has many possible causes leading either to lowered insulin secretion or increased resistance to the action of insulin — mostly due to a loss of **insulin receptors** in normally insulin-responsive tissues. Differential diagnosis is important for the proper treatment and management of the disease. In all cases, however, **hyperglycaemia** is present and an elevated glucose concentration can be detected in both blood and urine (**glucosuria**) due to the inability of the tissues to take up glucose (an insulin-dependent process). Typically, an elevated fasting blood glucose (>7 mM) or a randomly taken blood glucose > 11 mM indicates diabetes. Measurement of blood glucose in a glucose tolerance test may be done in equivocal cases. However, measurement of urinary glucose is also a good indication of diabetes and can be combined with other urine tests.

Tests for sugars

For proper diagnosis, it is important that the method used is specific for glucose, since an elevated level of other sugars in the urine (**glycosuria**) may indicate quite different disorders e.g. **fructose intolerance** or **galactosaemia**.

- ❖ **CLINISTIX**^{TM}: As you should know by now, nothing matches the specificity of enzymes and antibodies. **CLINISTIX**^{TM} sticks contain the enzyme **glucose oxidase** dried onto the paper pad at the end of the stick. This oxidises <u>only</u> glucose (and no other sugar) to yield

gluconic acid and **hydrogen peroxide**. A second enzyme on the pad, **peroxidase**, uses the hydrogen peroxide to oxidise a coloured dye to a different colour (different manufacturers use different colours). The degree of colour change reflects the amount of glucose. This is an example of a "**coupled reaction**", many of which are used in analytical assays. The colour can either be estimated by eye or, more quantitatively, using a simple colorimeter.

❖ **CLINITEST**™ tablets detect the presence of **reducing sugars**. These are sugars which possess a free aldehyde group in their linear form and include glucose, galactose, fructose and lactose (but not sucrose). Under alkaline conditions, this reactive aldehyde can reduce the Cu^{2+} **cupric** ion in copper sulphate to the Cu^+ **cuprous** ion. Hydrated cupric sulphate is blue whereas the insoluble cuprous oxide formed by reduction in alkali is red. Depending on the amount of sugar, an overall colour ranging from blue through green to brown is produced. This is the basis of **Benedict's test** for reducing sugars which is incorporated in tablet form in **CLINITEST**™ tablets (which are <u>white</u> due to the use of <u>anhydrous</u> cupric sulphate).

➢ *In Experiment A you will examine Benedict's test for reducing sugars and use CLINISTIX*™ *and CLINITEST*™ *tablets to examine urine samples for the presence of sugars*

Ketone bodies

Most of the **acetyl-CoA** produced by fatty acid oxidation in the liver is fully oxidised in the mitochondrial **citric acid cycle** (also known as **TCA** or **Kreb's** cycle) to CO_2 to yield energy. Some, however, is converted to **acetoacetate** in a process known as **ketogenesis** . This acetoacetate can then be enzymically reduced to **β-hydroxybutyrate** or non-enzymically decarboxylated to **acetone** and CO_2 (Figure 1) Collectively, these three compounds are known as **ketone bodies** and are the water-soluble equivalent of fatty acids. Acetoacetate and β-hydroxybutyrate are released from the liver and carried in the bloodstream to peripheral tissues such as cardiac and skeletal muscle where they serve as important alternative fuels.

acetoacetate   β-hydroxybutyrate   acetone
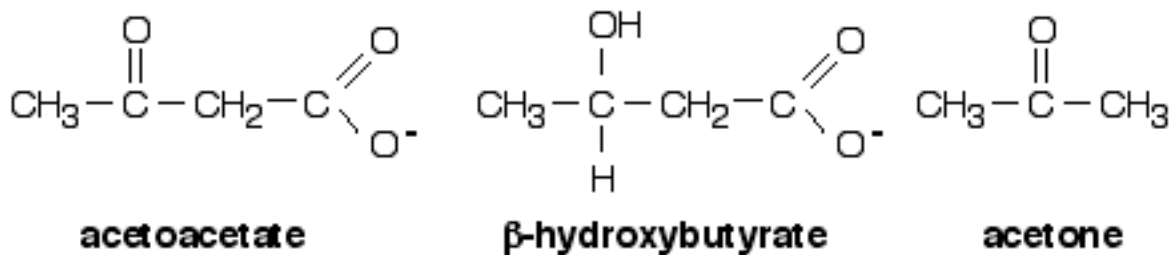
Another symptom of uncontrolled diabetes is **ketosis**, where acetoacetate is produced faster than it can be metabolised. This is due to the greater than normal reliance on the breakdown of lipid stores to provide energy due to the inadequate supply of absorbed carbohydrate. Ketosis also occurs during fasting (starvation), and under these conditions ketone bodies become the brain's major fuel source. However, as ketone bodies are water soluble, significant amounts are lost in the urine and represent a loss of calories in addition to those lost as glucose. If IDDM is left undiagnosed and untreated, or if insulin is withdrawn for some reason, then **diabetic**

ketoacidosis (DKA) can occur. Since acetoacetate and β-hydroxybutyrate are acids, elevated blood concentrations overload the buffering capacity of the blood and the kidneys, which control blood pH by excreting the excess H^+ into the urine. This H^+ excretion is accompanied by Na^+, K^+, phosphate and water loss. This adds to the already severe water loss due to osmotic **diuresis** driven by the high glucose concentration in the blood (**hyperosmolarity**) and results in "**diabetic coma**" and a potentially fatal reduction in blood volume.

Tests for ketone bodies

Measurement of ketone bodies is useful in distinguishing between different types of DKA which require different treatment. Acetoacetate, usually the major ketone body, is easily detected by the maroon colour produced when it reacts with **sodium nitroprusside** at alkaline pH. This chemical is dried onto the paper pad at the end of **KETOSTIX**™ used for urine testing. However, about 10-15% of patients with DKA appear <u>negative</u> or only <u>mildly positive</u> when tested with **KETOSTIX**™. This is because some individuals convert nearly all their acetoacetate to β-hydroxybutyrate, which does not react with nitroprusside. If this is suspected, an alternative assay (immuno- or enzyme) for β-hydroxybutyrate must be carried out to confirm the diagnosis. Another group (typically older patients) presenting with coma and acidosis may also test negative for ketone bodies. They are described as **hyperosmolar non-ketotic (HONK)** as they have high glucose but no ketone bodies. In this case, acidosis is due mainly to lactic acid. All DKA cases require rehydration, but with HONK this must be done slowly. Hence, correct diagnosis is important.

> ➢ *In Experiment B, you will use examine the reaction between acetoacetate and nitroprusside and use KETOSTIX™ to examine urine samples for the presence of ketone bodies*

Test for proteinuria

The incidence of renal complications increases with the duration of diabetes. Normally, urine contains no protein but as renal function deteriorates, **protein** can be detected. Since **albumin** is the predominant serum protein, detection of this protein indicates general **proteinuria**..
At pH values below its **isoelectric point** (the pH at which its overall charge due to surface ionisable amino acids is zero — about 5), albumin is positively charged and so it will bind **anions** (negatively charged ions). Indicator dyes, such as **tetrabromophenol blue (TBB)**, change colour when they ionise.

$$HTBB \rightleftharpoons H^+ + TBB^-$$
(yellow) (blue, anion)
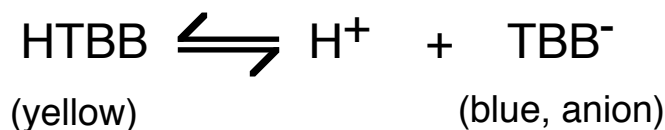
Both coloured forms are in equilibrium in solution, with the yellow form predominating at a pH just below the pK for the dissociation (3.5 in this case). However, if albumin is present, it binds

the small amount of the blue anion and removes it from free solution, thus shifting the equilibrium to the right. More anion is produced and so on. The degree of colour shift from yellow (through green) to blue depends on the amount of protein bound blue-anion generated i.e. on the amount of albumin. This is the basis of the **ALBUSTIX**™ test for proteinuria.

> ➢ *In Experiment C, you will examine the binding of tetrabromophenol blue to albumin and use ALBUSTIX*™ *to examine urine samples for the presence of protein*

Test for glycated haemoglobin

Glucose and some other sugars have the ability to react chemically with some of the amino acid side chains in proteins to form **glycated** proteins. Small amounts of these are present normally in blood. However, a persistent elevated blood glucose will lead to the accumulation of higher levels of glycated proteins such as albumin and haemoglobin. A particular modified haemoglobin called **haemoglobin A1$_c$ (HbA1$_c$)** is quite stable with a half-life of 60-90 days. Therefore, the level of HbA1$_c$ is a good indicator of how well a patient has been managing his or her diabetes over a period of several weeks. HbA1$_c$ is normally 4-6% of total Hb. A high level (>7%) indicates persistent hyperglycaemia over that period and, therefore, poor management. An errant patient attending the diabetic clinic may try to fool the doctor by taking a dose of insulin just before the consultation to lower the blood glucose and by falsifying the **glucose control diary**. However, high HbA1$_c$ will identify the fraud. Therefore, a rapid assay that can be performed in a few minutes in the clinic while the patient is present is extremely valuable. A simple dipstick test that would differentiate between normal haemoglobin and HbA1$_c$ is not available. However, HbA1c can be rapidly separated from normal haemoglobin and measured by **high performance liquid chromatography (HPLC)**.

Liquid chromatography

Liquid chromatography encompasses a variety of techniques that utilise as their basis the relative partition of solutes between a stationary phase and a mobile phase that passes through or across the stationary phase (Figure 2). A solute that has a high affinity for the stationary phase (black sphere) will be retarded relative to a solute that remains in the mobile phase [i.e. has a low affinity for the stationary phase - (grey sphere)]. Thus, different solutes may be separated on the basis of their **partition** between the two phases of the chromatographic system.

Mobile phase

stationary phase

solutes
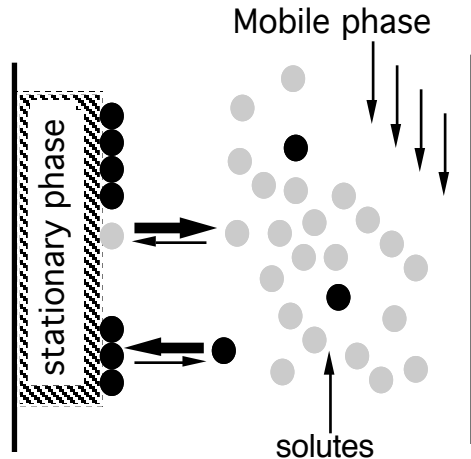
The different forms of liquid chromatography differ in the nature of the stationary and mobile phases. In the most common forms of chromatography, the stationary phase consists of an insoluble material (usually in beaded or granular form, e.g. cellulose or polystyrene) that is packed into a column and through which the mobile phase (containing the solutes) can flow.

❖ In **ion-exchange chromatography**, the stationary phase carries a charge (positive or negative) and charged solutes dissolved in the mobile phase (e.g. proteins) are differentially retarded depending on the degree of charge they carry (due to charged amino acid side chains on their surface, e.g. glutamate, lysine etc.). Elution of the column usually involves passage through it of a gradient of increasing salt concentration that progressively disrupts the stronger interactions of the more highly charged solutes. pH gradients are also used.

❖ In **gel permeation chromatography** (gel filtration), solutes (e.g. proteins) are separated on the basis of their size. The material comprising the stationary phase contains pores through which solutes below a certain size may pass. Larger solutes are excluded and quickly flow unimpeded through the column.

❖ In **affinity chromatography**, the stationary phase has attached to it a ligand (e.g. an enzyme substrate or an antibody) that will specifically bind the appropriate enzyme or antigen from a crude mixture dissolved in the mobile phase. Elution may involve use of the free (competing) ligand, salt or pH.

❖ In **reversed phase chromatography**, the stationary phase is rendered **hydrophobic**, usually by the attachment of alkyl chains [e.g. butyl (C_4), octyl (C8) or stearyl (C18)]. Solutes (e.g. proteins) separate on the basis of their own hydrophobicity (due to hydrophobic amino acid side chains on their surface, e.g. leucine, valine, phenylalanine etc.). Elution may require the use of polar and water-miscible organic solvents (e.g. methanol or acetonitrile) in the mobile phase to disrupt the hydrophobic interactions.

High performance liquid chromatography

The degree of resolution achievable in liquid chromatography is in part dependent on the surface area of the stationary phase to which the mobile solutes are exposed. An increase in surface area can be achieved by decreasing the size of the particles comprising the stationary phase. This is turn leads to closer and tighter packing of the particles, so high pressure is required to pump the sample and the mobile phase through the column.. The tubing and columns used in **HPLC** (**h**igh **p**erformance or **h**igh **p**ressure) **l**iquid **c**hromatography are usually made of stainless steel to withstand the pressure produced by the pumps. In the case of proteins, the highly resolved peaks from a complex mixture are normally visualised in the **eluate** coming out of the column by passing this though a continuous flow UV detector that measures the **absorbance** of the proteins in the ultraviolet range (due to the UV-absorbing amino acids **tyrosine** and **tryptophan**). Coloured proteins (e.g. haemoglobin) may also be detected by their absorbance in the visible region of the spectrum.

> ➢ *In Experiment D, you will see a demonstration of reversed phase HPLC for the measurement of haemoglobin A1$_c$ in sera from well-, moderately- and poorly-controlled diabetics.*

The system to be demonstrated employs combined reversed-phase and cation exchange chromatography followed by **bichromatic spectrophotometric** measurement (i.e. measurement of absorbance at a primary wavelength of 415 nm and a secondary wavelength 500 nm).
The urine samples

1. In experiments A, B and C, there are five urine samples to be tested for glucose, reducing sugars, ketone bodies and protein. These represent the following clinical cases:

❖ **Normal** (no sugar, no ketones, no protein).
❖ **Early diabetic at diagnosis** (10-15 mM glucose, no ketones, no protein).
❖ **Fructose intolerance** (5-10 mM fructose, no glucose, no ketones, no protein).
❖ **DKA** (high glucose 15-20 mM, ketones >5 mM, protein 5-20 mg/l).
❖ **HONK** (very high glucose >30 mM, no ketones, protein 20-40 mg/l)

On the basis of the tests, you will determine which urine sample (A, B, C, D or E) corresponds to which case.

2. The samples to be tested for glycated haemoglobin are separate from those above.

4.3 Experiment A: Determination of urinary sugars using Benedict's solution, CLINISTIX™ and CLINITEST™ tablets

Reagents: Benedict's solution (copper sulphate, sodium citrate , sodium carbonate)
2% (w/v) glucose
CLINISTIX™
CLINITEST™ tablets
Urine samples A, B, C, D and E

Procedure (label all tubes with a marker pen to avoid confusion):

1. First, you will set up a series of six standard glucose solutions of known concentration to mimic the test that can be done with CLINITEST™ tablets. Do NOT use the tablets at this stage (they are too expensive to "waste" on a set of standards). Using the 2% glucose solution and distilled water, set up a series of 6 **large glass** test tubes containing respectively 2%, 1%, 0.75%. 0.5%. 0.25% and 0% glucose, each in a final volume of 200 µl.

2. Add 400 µl Benedict's solution to each tube, mix, and place in a boiling water bath for exactly 90 seconds.

3. Remove and compare the colours in these tubes to the chart that comes with the CLINITEST™ tablets. How do your test and the commercial test compare for sensitivity?

4. Now you will use the CLINITEST™ tablets to measure the reducing sugar content of the five urine samples. Place 200 µl of each of the five urines in the bottom of five separate **large glass** test tubes. Next, add 400 µl of water to each tube.

5. Add one CLINITEST™ tablet to each tube and watch while the complete boiling reaction takes place. Do NOT shake the tubes during boiling or during the 15 seconds after boiling has stopped. Once 15 seconds have elapsed after boiling has finished, shake the tubes and compare the final colour to the colours on the chart provided with the tablets. Record the % reducing sugar in the first row of Table below.

6. Finally, test fresh samples of each of the urines with the CLINISTIX™ strips. Dip the reagent end of the strip in a urine sample (directly into the flask), remove, wait **EXACTLY 10** seconds, then compare the colour to the chart on the side of the reagent strip bottle and record the result in the second row of the Table below.

	A	B	C	D	E
% Reducing sugar (CLINITEST)					
Glucose (CLINISTIX)					

4.4 Experiment B: Determination of acetoacetate using sodium nitroprusside and KETOSTIX™

Reagents: **Colour reagent** (3% (w/v) sodium nitroprusside in 0.2M glycine-NaOH buffer, pH 10)
32 mM lithium acetoacetate
KETOSTIX™
Urine samples A, B, C, D and E

Procedure (label all tubes with a marker pen to avoid confusion):

1. Using 32 mM lithium acetoacetate and distilled water, set up a series of six **small plastic** test tubes containing respectively 32, 16, 8, 3.2, 1.6 and 0 mM acetoacetate, each in a final volume of 200 μl.

2. Add 200 μl colour reagent to each tube, mix and leave for 10 minutes.

3. Compare the colours in these tubes to the chart that comes with the KETOSTIX™. How does your test and the commercial test compare for sensitivity? (**Remember that your acetoacetate concentrations will have been halved by mixing with an equal volume of colour reagent**). This simple experiment illustrates the basis of KETOSTIX™. You are now finished with these tubes. Do **NOT** use them for step 4.

4. Finally, measure the apparent ketone content of the five urine samples using the KETOSTIX™ reagent strips. Dip the reagent end of the strip in a urine sample (directly into the flask) , remove, wait **EXACTLY 15** seconds, then compare the colour to the chart on the side of the reagent strip bottle and record the result in the Table below.

	A	B	C	D	E
Ketones					

4.5 Experiment C: Determination of urinary albumin using tetrabromophenol blue and ALBUSTIX™

Reagents: **Tetrabromophenol blue** (0.2 mg/ml in 0.1 M citrate buffer pH 3.5)
4% (w/v) serum albumin
ALBUSTIX™
Urine samples A, B, C, D and E

Procedure (label all tubes with a marker pen to avoid confusion):

1. Using 4% albumin and distilled water, set up a series of six **small plastic** test tubes containing respectively 4.0, 0.6, 0.2, 0.06, 0.02 and 0% albumin, each in a final volume of 200 μl. (**Hint**: to make the 0.06% and 0.02%, first make 200 μl of a 1 in 10 dilution of the 4% (becomes 0.4%) and use this, rather than the 4% albumin).

2. Add 200 µl tetrabromophenol blue solution to each tube and mix.

3. Compare the colours in these tubes to the chart that comes with the ALBUSTIX™. How does your test and the commercial test compare for sensitivity? (**Remember that your albumin concentrations will have been halved by mixing with an equal volume of dye solution**). This simple experiment illustrates the basis of ALBUSTIX™. You are now finished with these tubes. Do **NOT** use them for step 4.

4. Following the instruction sheet enclosed with the ALBUSTIX™, measure the protein content of the urine samples. Dip the reagent end of the strip in a urine sample (directly into the flask) , remove, wait **EXACTLY 60** seconds, then compare the colour to the chart on the side of the reagent strip bottle and record the result in the Table below.

	A	B	C	D	E
Protein					

Results

On the basis of the results of experiments A, B and C, which urine sample corresponds to which case?

Normal	
Early diabetic at diagnosis	
Fructose intolerance	
DKA	
HONK	

4.6   Experiment D: Determination of haemoglobin A1$_c$ by HPLC

This part of the practical will be described and demonstrated to you in groups. You can take notes for future reference on this page.