Section 1

Automated analysers - CB-1-C-4

Whilst spending one week in the general laboratory (w/c 08.12.14) I used the automated analyser cobas® 8000 (Roche). This is a modular analyser comprising an indirect ion selective electrode (ISE) module, a c702 high-throughput clinical chemistry module (spectrophotometry) and two e602 immunoassay modules. The scientific principles for these methods are detailed in Section 2. Standard operating procedures are available for the correct running and maintenance of analysers in the general laboratory.

Software

The data management system allows monitoring and control of all modules in one place. The software displays procedures for daily, weekly and monthly maintenance. These must be signed off by the laboratory worker as each task is performed. The system also manages quality control data (see below) and tracks the progress of each specimen through the analyser.

Quality control

Quality control provides the laboratory staff and clinicians with confidence in the results produced.

Internal quality controls (IQCs) are commercially produced samples, ideally sourced from human blood with the same matrix as specimens to be run on the assay. Some IQCs contain multiple analytes so can be used as the quality control for several assays. IQCs are performed for each assay and are run every 4-6 h to ensure that the assay is producing the correct results for a patient sample. This allows the performance of the assay to be monitored in real-time. Usually two IQCs are run per assay, at high and low concentrations to check accuracy across the measureable range. The data management system displays statistical data based on the IQCs, including Levy-Jennings plots and Westgard Rules. These are used to monitor the performance of the analyser for accuracy (how close the IQC runs to the mean) and precision (how much the IQCs differ over time). The data management system will highlight IQCs which violate the Westguard rules. Generally, patient results are released if the IQCs run within 2SD of the mean. Assays which have IQCs that run between 2 and 3SD are flagged by the data management system and are investigated. The IQC may be re-run and if the IQC is unchanged the assay may be re-calibrated. Calibration is performed daily for assays which are known to drift (e.g. creatinine, urea, magnesium). If the IQC runs at greater than 3SD then the run is rejected, the assay is recalibrated and any specimens in that run are re-tested.

External Quality Assessments (EQAs) are performed retrospectively every one to two months and enable comparison of an individual laboratory's performance with other laboratories across the country. EQA samples are sent from an external organisation, NEQAS, to each laboratory and the sample is treated as a routine specimen. Thus EQA allows the whole testing process to be assessed. EQA reports summarise the performance of the laboratory over the last 6 months, displaying results as a graph and therefore allowing the time-point when drift began to be identified. This is useful when investigating the cause of drift, e.g. due to a change of reagent lot at that point. Accuracy, bias and consistency of bias are reported as ABC scores, which should be minimised. EQA reports also group results according to the method used, allowing comparison within and between method groups.

Analysis

Serum, plasma, urine, CSF specimens are loaded onto the analyser in racks. The barcode on each specimen is read which informs the analyser which tests are to be performed. There is random access for the racks: specimens are transported between the necessary modules via a conveyer belt system, so that samples can go from everywhere to everywhere. The software has been programmed to prioritise faster tests to ensure results are generated most efficiently (e.g. electrolytes will be measured on ISE before thyroid hormones are measured by immunoassay). Samples can be continuously loaded onto the analyser, providing high throughput.

The ISE module contains an Ag/AgCl reference electrode and solid state ISEs for the measurement of sodium, potassium and chloride. The module incorporates a clot detector to identify specimens which need to be filtered and re-centrifuged. The chemistry module uses robotic arms to take an aliquot from the specimen, which is mixed with reagent via ultrasound in cuvettes. The reagents required for the test are selected and aliquoted appropriately for the analyte being measured (as specified by the barcode). Probes are automatically washed after sampling. The concentration of the analyte is measured by spectrophotometry. Analytes measured on the chemistry module include albumin, ALP, ALT, creatinine, glucose, HDL, urea and bilirubin. The immunoassay module utilises a chemiluminescence detection system. Disposable tips are used to prevent cross-contamination, and there is a detection system for clots and air bubbles. Analytes measured by immunoassay include TSH, T4 and PTH.

Interferences

In the chemistry module, the specimen integrity is monitored via serum indices to detect haemolysis, lipaemia and icterus, as well as detection systems for clots and liquid level. Serum indices are calculations of absorbance measurements which offer semi-quanititative measurements for haemolysis, icterus or lipaemia. The Haemolysis Index (H) correlates well with the haemoglobin concentration, the Icterus Index (I) with the bilirubin concentration and the Lipaemic Index (L) with estimates the sample turbidity. H, I and L indices are automatically added to chemistry tests. Certain or all test results will not be reported if haemolysis, icteria or lipaemia exceed certain values (Table 1).

Haemolysis	H index > 90 Potassium and ALT results not reported	H index > 200 No results reported.
Icterus	Bilirubin concentration > 171 µmol/L Repeat creatinine via enzymatic method.	Bilirubin concentration > 342 µmol/L Perform 1:3 dilution of specimen to measure creatinine via enzymatic method.
Lipaemia	L index > 11 No spectrophotometric results reported. Potassium and sodium can be measured on direct ISE. Triglyceride level can be reported. Request a repeat fasting specimen.	

Table 1 Serum indices and action taken on reporting

Haemolysis, icterus and lipaemia can interfere with assays as summarised in Table 2 and Figure 1.

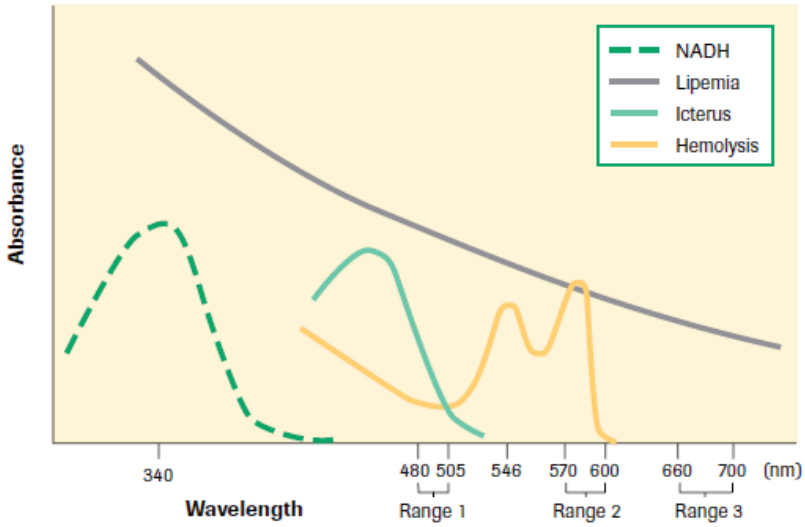

Figure 1 Wavelengths at which there is spectrophotometric interference by haemolytic, icteric and lipaemic samples. From Roche, 2007, https://www.rochediagnostics.fr/Htdocs/media/pdf/actualites/2b_SI_Brochure_2007.pdf

	Effect on spectrophotometric tests	**Effect on chemistry tests**	**Other interference mechanisms**
Haemolysis	Strong absorbance by haemoglobin at 415 nm can interfere with measurement of analytes with absorbance in this range, e.g. the activity of ALP can be measured by the absorbance at 405 nm of the product of its enzymatic reaction, ρ-Nitrophenol (http://www.isu.edu/~galisusa/alp_sop.html).	Blood constituents can interfere with assays e.g. adenylate kinase released from red blood cells may cause an increase in CK.	Release of contents of intracellular analytes, thus falsely increasing the normal serum concentration, e.g. potassium, LDH, ALT.
Icterus	Strong absorbance by bilirubin between 340 and 500 nm which can exceed the linear range of the spectrophotometer. Under acidic conditions, bilirubin absorbance shifts towards the UV and can interfere with measurement of analytes with absorbance in this range e.g. ACE (340 nm), paracetamol (257 nm).	Bilirubin is a reducing agent and reacts with H2O2, interfering with assays which use H2O2 generation to measure analyte concentration (e.g. glucose, triglycerides, cholesterol, urate).	-
Lipaemia	Light scattering by triglycerides interferes with all spectrophotometric assays.	Lipids can sequester lipid-soluble analytes to	Volume depletion effect of lipids decreases the apparent concentration of

		give a falsely decreased result (e.g. cholesterol, vitamins, lipid-soluble drugs such as antidepressants).	analytes dissolved in the water component of serum (e.g. sodium).

Table 1 Summary of the mechanisms behind interferences
https://www.rochediagnostics.fr/Htdocs/media/pdf/actualites/2b_SI_Brochure_2007.pdf

Biological variation, reference ranges and interpretation

Rules bases programmed into the result display software (APEX) highlight in green results which fall outside of the reference range. Reference ranges are generated by measuring the concentration of the analyte in a population of healthy people. The lower and upper limits of the reference range are the mean ± 2 standard deviations, which encompasses 95% of the population. This means that 5% of the 'healthy' population have analyte levels which lie outside of the reference range and therefore an 'abnormal' result is not necessarily pathological. Similarly, a test result which is in the reference range is not necessarily 'normal': for example, a TSH level of 2.5 g/L with a free T4 level of 6 g/L would be inappropriately normal. Therefore test results should be interpreted within the context of that patient's clinical details, previous results and other test results. Biomedical scientists and clinical biochemists validate results which fall outside of the reference range. Automated, written and telephoned comments may be added by the biochemist to aid interpretation of the results by the requesting clinician.

Action limits

A standard operating procedure is available which outlines the action limits for results to be telephoned or faxed to the requesting clinician. These are summarised in Table 3 and are highlighted in yellow on APEX.

Analyte	Action limits
Sodium	Less than 120 mmol/L Greater than 150 mmol/L Has increased/decreased by more than 5 mmol/L within 24h
Potassium	Less than 2.8 mmol/L Greater than 6.0 mmol/L (6.5 mmol/L for patients under the nephrologists)
Urea	Greater than 30.0 mmol/L
Creatinine	Greater than 400 µmol/L Increase of greater than 20% for patients under the nephrologists
Corrected calcium	Less than 1.8 mmol/L Greater than 3.0 mmol/L
Magnesium	Less than 0.5 mmol/L Greater than 2.0 mmol/L

Phosphate	Less than 0.35 mmol/L Greater than 2.00 mmol/L
Glucose (random)	Less than 2.5 mmol/L Greater than 25.0 mmol/L
Glucose (fasting)	Less than 2.5 mmol/L Greater than 20.0 mmol/L
Creatinine kinase	Greater than 1000 IU/L
Theophyline	Greater than 20.0 mg/L
Triglycerides	Greater than 11.0 mmol/L
Amylase	Greater than 500 IU/L

Table 3 Action limits for telephoning/faxing results to the requesting clinician

Archiving

After a specimen comes off the analyser, its barcode is scanned by a BMS to manually check if all of the tests have been performed. If not, the specimen is returned for the further tests to be completed, either on the same analyser or on a different automated or manual analyser. When all tests have been completed, the specimen is sent for archiving. The post-analytical platform (RSD) scans the barcode of each specimen to double-check that all of the tests have been done. If not, the specimen is placed into the appropriate rack for the outstanding test. If all tests have been performed, the RSD assigns the specimen to a position in the archive rack. This position is logged on the data management system to enable to specimen to be retrieved if a further test is requested. Specimens are capped with a foil lid and stored in the fridge for three days. After this the specimens are disposed of in clinical waste and incinerated.

Section 2

Scientific principles behind methods used in clinical biochemistry

Ion-selective electrodes

An ion-selective electrode (ISE) is an electrochemical sensor which responds to a specific ion. It is used in clinical laboratories and POCT devices to measure ions (Na+, K+, Cl-, Li+), pH and blood gases (O2, CO2). ISEs measure the potential difference generated by the diffusion of ions from higher to lower concentration across a semi-permeable membrane. The difference in potential between the measuring and reference electrodes, measured with a voltmeter, is used to calculate the activity (≈ concentration) of the ion in the specimen.

Potentiometric methods such as ISEs are based on the measurement of potential in the absence of any other appreciable currents, i.e. at equilibrium. Therefore the Nernst equation applies. For the reaction $aA + bB \leftrightarrow cC + dD$,

$$\Delta E = \Delta E^{\circ} - \frac{RT}{nF} \ln \frac{[C]^{c} +}{[A]^{a} + [B]^{b}}$$

Where ΔE = the change in potential difference, ΔEo = the difference of the standard potentials of the half-cells involved, R = gas constant (8.314 J mol-1 K-1), T = temperature (in K), n = number of electrons transferred in the reaction and F = Faraday constant (96485 C).

Figure 2 shows a schematic of a typical electrochemical cell.

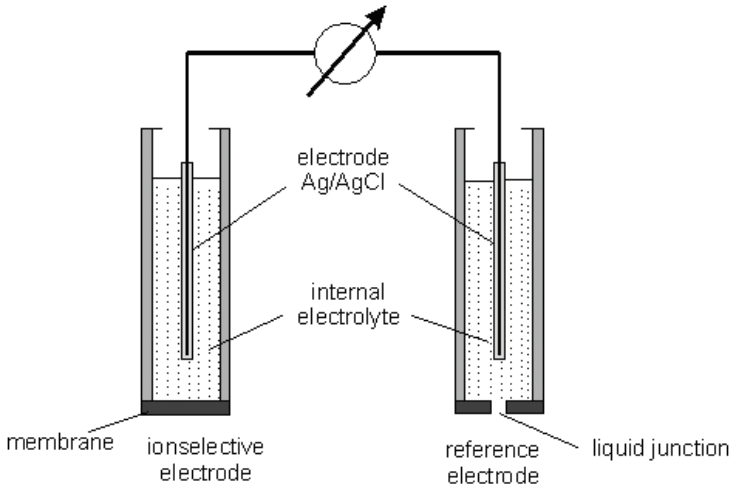

Figure 2 Electrochemical cell schematic, with the ISE (left) and reference electrode (right) connected via a voltmeter. Acknowledgement: Chris Chaloner, Consultant Biochemist, Manchester

Each half-cell (the ISE and the reference electrode) contains an internal electrolyte solution. In the ISE, the internal electrolyte comprises a fixed concentration of ion X (being measured in the specimen). In the reference electrode is the internal electrolyte, an inert solution containing the anion to which the electrode is reversible (i.e. Cl- for an Ag/AgCl electrode).

The reference electrode provides an invariable potential difference between the internal electrolyte and the specimen solution. A liquid junction is any junction between two electrolyte solutions of different concentration, across which arises a liquid junction potential (LJP). The LJP must be minimised to ensure the reference electrode provides an invariable potential difference. The LJP can be minimised by using a high concentration of internal electrolyte in the reference electrode. This keeps the LJP small and approximately constant upon substitution of standard solution for test specimen. Alternatively, a 'bridge solution' may be used (not shown in Figure 1). This is a solution of high concentration of inert salt, comprising cations and anions of equal mobility such as KCl. The bridge solution is placed between the internal electrolyte of the reference electrode and the test specimen and negates the LJP.

The electrochemical half-cells (ISE and reference electrode) are placed into a specimen solution. The membrane on the ISE is selective for an ion, for example Na+. Membranes used in ISEs can be solid-state crystal membranes, liquid ion-exchange membranes or neutral carrier membranes. Selectivity is imparted by the exact composition of the membrane; for example, K+ ISEs use a valinomycin membrane and Na+ ISEs use a membrane containing SiO2, Na2O and Al2O3. A potential difference develops across the ISE membrane according to the difference in concentration

of Na+ in the internal electrolyte and in the specimen. The reference electrode provides an invariant potential difference, allowing the potential difference between the ISE and reference electrode (ΔEISE) to be measured by the voltmeter.

The response of ISEs (EISE) over their activity range is compatible with the Nernst equation, i.e. it is proportional to the log of the activity of the ion being measured. The activity of an ion is the number of atoms that act as true ions in a defined volume of water.

$\alpha = \alpha i\ c$

where α = activity of ion x, αi = activity coefficient of ion x and c = concentration of ion x.

The activity coefficient, αi = 1 at infinite dilution. So the Nernst equation for the response of an ISE (EISE) is:

$EISE = 2.303Ei\ (RT/ZiF) \log \alpha i$

where 2.303Ei is a constant and Zi is the charge of ion x.

There are two types of ISE: indirect and direct. Indirect ISEs are most often used in automated analysers in clinical biochemistry laboratories. In indirect ISEs, the sample is diluted such that the activity coefficient ≈ 1, so activity measured ≈ concentration. The water content of plasma is ≈ 93% and activity is measured for the whole volume of plasma. This is suitably accurate for clinical purposes, except in hyperlipidaemia or hyperproteinaemia due to the displacement of water from plasma by excess lipids or protein. This decreases the percentage of plasma which is water, therefore giving a falsely low measurement of ionic activity. This is a relatively common cause of pseudohyponatraemia.

In direct ISE the sample is not diluted and activity is measured specifically for the water content of plasma. Therefore direct ISEs are not affected by elevated lipid or protein levels and are often used to check the sodium concentration in suspected pseudohyponatraemia caused by hyperlipidaemia or hyperproteinaemia. However, direct ISEs are less accurate than indirect ISEs because the activity coefficients are assumed to be constant for samples, calibration and QCs. See competency CB-1-C-5 for more an example of using direct ISE in the general laboratory.

Blood gas analysers use ISEs based on the principle that gases such as CO2 and NH3 can diffuse across a selective membrane and cause a change in pH, which is detected by a potentiometric pH meter.

Enzyme electrodes involve enveloping the electrode in an enzyme which catalyses a reaction that produces a gas. For example, urease ISEs can be used to generate NH3 from urea. NH3 can diffuse across a selective membrane and cause a pH change.

Limitations of ISEs

Variations in the liquid junction potential, which can be difficult to predict and can be a major source of drift during measurements.

Except for pH electrodes, membranes are ion-selective (rather than ion-specific) and similarly charged ions can cause interference resulting in a ≈ 1 mV error. This results in a 4% error for a monovalent cation and an 8% error for a divalent cation.

Electrodes can be fouled by serum proteins. This can be cleared by deproteination using a NaOH solution, or be changing the membrane.

Quality control

ISEs can be monitored by between-run precision. The high and low IQCs for an analyte (e.g. sodium) are measured every day for 5 days. The mean, standard deviation and coefficient of varience are calculated. The COV should be <5% for a reliable electrolyte measurement. This is a stringent cut-off because in health, electrolytes are kept within a tight concentration range and therefore seemingly small drifts from the normal range are often clinically significant. The between-run precision shows the fluctuation that can be expected from the analyser over time, which is important for repeated tests.

Spectrophotometry

Light which interacts with matter may be absorbed, transmitted or scattered. Most spectrophotometers measure absorbance and transmittance.

Absorbance

Chromophores are molecules which absorb light. The energy from absorption causes excitation of electrons in the molecule to a higher energy level. The electrons then return to the ground state, with loss of the energy via fluorescence (radiatively) or vibration (non-radiative). Absorbance (A) is calculated from the intensity of the incidence light (I0) compared to the emergent light (I) at any given wavelength:

$A = \log (I/I0)$.

The degree of absorbance depends on the path length of the light (l), the molar concentration of the chromophore (c) and the amount of light the chromophore will absorb at that wavelength (ε, the molar extinction coefficient). This relationship is expressed in the Beer-Lambert law:

$A = \varepsilon c l$

Absorbance is linear with concentration, but only at low concentrations. Therefore samples must be diluted into the linear range for spectrophotometric analysis. Consideration must be taken of matrix effects when diluting specimens, with the maximum dilution for a serum sample being no more than 1 in 3 to ensure the results are clinically relevant.

Transmittance

Transmittance is the ratio of the intensities of the emergent and incident lights:

$T(\%) = (I/I0) \times 100$

Transmittance is the detection method for turbidimetry (see the section on immunochemical techniques).

Light sources

Tungsten lamps are commonly used in clinical laboratories since they are inexpensive, have a long lifetime and cover the visible range reasonably well. Deuterium lamps emit light of the ultraviolet range, and are less frequently used because they are more expensive. They also have a shorter lifespan than tungsten bulbs. The light source of light depends on the wavelength at which the chromophore absorbs light.

Detectors

There are two main types of detector: photo-multiplier tubes and photo-diodes. Photo-multiplier tubes (PMTs) exponentially amplify the light signal and consequently are highly sensitive. They convert light to electron beams and subsequently to an electrical signal. PMTs are relatively expensive so typically are usually only found in fluorimeters and some spectrophotometers. Photo-diodes are semiconductive components which convert light into an electrical current and are less expensive than PMTs. They can be combined into a diode array to simultaneously measure multiple wavelengths of light, making them a rapid detection device. However, photo-diodes are less sensitive than PMTs.

Calibration

Holmium oxide (Ho2O3) is used in the calibration of spectrophotometers since it has complex absorbance spectrum, with well-defined peaks across the UV/visible range.

Clinical use

Spectrophotometry is used for a large number of clinical assays, often coupled to enzymology and immunoassays as a method for detecting a coloured product or to monitor changes in transmission upon immune complex formation. See the next enzymology and immunoassay sections for specific examples.

Enzymology

Enzymes increase the rate of a reaction without being changed or consumed and without changing the position of the equilibrium. They are usually specific for their substrates so are useful for use in the laboratory for measuring a specific analyte in a complex biological mixture. Analytes commonly measured using enzymes as reagents are: glucose, urate, creatinine, cholesterol, triglycerides, lactate, urea, ammonia and total CO2.

Enzymes can be used as reagents to produce measureable products, to remove interfering substances or as signal amplifiers (labels) in immunoassay. For example, creatinine kinase (CK) is used in the assay for determining creatinine concentration, which is proportional to the concentration of NADPH produced in the following scheme. NADPH formation is measured by spectrophotometry.

Creatine-Pi + ADP → Creatine + ATP (Creatine kinase)

ATP + Glucose → Glucose6Pi + ADP (Hexokinase)

Glucose6Pi + NADP → 6-phosphogluconate + NADPH (G6PiDHase)

Ascorbic acid can interfere in reactions which use peroxidase in the final step to generate a coloured product from H2O2 + a dye. Ascorbic acid re-reduces the coloured product as fast as it is formed so ascorbate oxidase is included in these assays to remove this interference.

Enzymes can be used as labels in immunoassay. For example horseradish peroxidase can be coupled to the secondary antibody and H2O2 added to generate a chemiluminescent signal.

Assays

End point assays

The concentration of product at the end of a reaction is proportional to the analyte according to the stoichiometry of the reaction. For example, lactate dehydrogenase is a reagent used in the determination of lactate concentration:

[LDH]

Lactate + NAD ⇌ Pyruvate + NADH

The formation of NADH is measured by absorbance and is directly proportional to the lactate concentration. In this reaction, the equilibrium is unfavourable towards NADH, so a 'trapping' reagent may be used, such as hydrazine. Hydrazine reacts with pyruvate, pulling the equilibrium towards the right according Le Chatelier's principle. The reaction continues until all of the substrate (lactate) is used up.

End-point assays require large concentrations of enzyme and an excess of other substrates (NAD in this example), which increases the cost of the assay. Enzymes with a low Km (high affinity) for the analyte of interest are preferable. End-point assays are also relatively slow since the end-point of the reaction must be reached.

Kinetic assays

The activity of an enzyme can be measured by the formation of product or the consumption of substrate. Measurement of rate can be fixed point, in which measurements (e.g. of product concentration) are taken at two fixed time points and the rate calculated by dividing the difference by time. However, the timings of measurement are crucial since the rate of product formation displays sigmoidal kinetics (fig 3). Measurements need to be taken during the steepest section of the curve to avoid underestimation of rate.

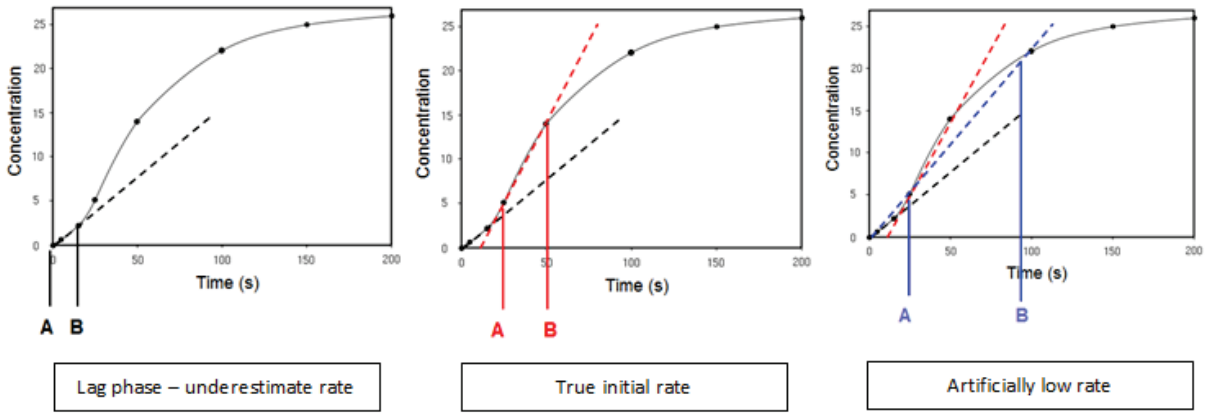

Figure 3 Rate of product formation is underestimated if both measurements are taken during the lag phase or when the substrate is limiting towards the end of the reaction. The true initial rate is calculated from two measurements taken in the steepest section of the curve. Acknowledgement: Dr Phillip Monaghan, the Christie Hospital

Alternatively, rate can be monitored by continuously taking a measurement over a designated time period. This has advantages over fixed-point measurement: lag phases are identified, the true initial rate can be determined and the progress of the reaction can be assessed. However, in order for the rate to be proportional to the analyte, all other factors which may alter the rate of reaction (e.g. temperature, limiting concentrations of reagents) must be carefully controlled to yield precise results.

Enzyme activity is expressed in international units (U), where 1U is the amount of enzyme which will convert 1 µmol of substrate in 1 min. The rate of change in absorbance (ΔA/min) is converted to enzyme activity using the Beer-Lambert law:

$$\text{Enzyme activity (U/L)} = \frac{\Delta A/\text{min} \times 106 \times \text{Total vol (mL)}}{\varepsilon\ (\text{L/mol/cm}) \times l\ (\text{cm}) \times \text{Sample vol (mL)}}$$

Immunochemical techniques

Immunochemical techniques are based on the formation of immune complexes between antibodies and the immunodeterminant regions (epitopes) of antigens. The antibody-antigen interaction is highly specific and sensitive, making immunochemistry a simple, rapid and robust technique that can often be automated. Immunochemical techniques are used in the clinical laboratory in tests involving immunogenic molecules, such as for hormones, tumour markers, viral antigens and also for some drugs. Molecules such as steroids need to be conjugated to a larger molecule to become immunogenic and measurable by immunoassay. Immunochemical techniques can be qualitative or quantitative, and they include immunoassays, immunohistochemical techniques and immunochromatographic techniques (e.g. dipstick analysis).

Standards

The standard used should contain the analyte in an identical form to that which is in the specimen, or similar to the extent that the behaviour of the analyte in the assay is the same for the standard as for the specimen. There should be no difference in matrix between the standard and the sample. Where possible, international standards should be used: these are available for growth hormone (GH), parathyroid hormone (PTH) and insulin-like growth factor 1 (IGF-1).

Immunoassay

The main immunochemical technique used in the biochemistry laboratory is immunoassay. IgG is the antibody is used in the majority of immunoassays, since it is available at the highest yield, it has the highest affinity for antigen binding and it has several sites available for the coupling of reporter molecules without interfering with immune complex formation. The affinity and avidity of the antibody for the antigen determines the sensitivity and specificity of the assay. Immunoassays can be performed on blood, serum, plasma, urine, saliva and CSF specimens.

There are two main types of immunoassay design: homogenous, in which the immune complex is not separated from the reaction mixture, and heterologous immunoassay which involves removal of unbound reagent and sample. Homogenous assays are simple and easy to automate because no separation step is required; however they are less sensitive than heterogenous assays.

Homogenous immunoassays

The formation of immune complexes between multivalent antibodies and antigens with more than one epitope cause scattering of light, which can be measured spectrophotometrically. This is the basis for nephelometry and turbidimetry. In nephelometry, immune complex formation is detected by measuring the scattering of light by immune complexes with detectors placed at 30o and 90o to the incidence beam. Scattering is directly proportional to the concentration of immune complexes. An excess of antibody is required to minimise precipitation: in the absence of precipitation, there is a stoichiometric relationship between the number of complexes formed and the concentration of antigen. Nephelometry is best performed with dilute solutions because at higher concentrations, destructive light scattering reduces the sensitivity.

Tubidimetry detects the reduction in transmittance of light through the sample due scatter, with the detector placed opposite the incidence beam. Transmittance is inversely proportional to the concentration of immune complex. Turbidimetry requires more concentrated specimens sample to achieve sufficient density of solution for precise measurement; for this reason direct turbidimetry lacks sensitivity for analytes at low concentration. Sensitivity can be increased by using particle-enhanced immunoassay using antibody-coated microspheres, which increase the relative light-scattering signal.

Heterogenous immunoassays

The separation of immune complex from the reaction mixture can be done using primary antibodies which are coated onto the surface solid phase plate and unbound reagent can be washed off. Alternatively, separation can be performed by liquid phase through using a tertiary antibody to precipitate the immune complex, using salts to separate the immune complex or using primary antibodies fixed to a magnetic solid phase. The two main types of heterogenous assay are competitive (reagent limited) and two-site immunometric assays (reagent excess).

Competitive immunoassays

Antibodies against the antigen of interest are fixed to a solid phase. The sample and a limited amount of a labelled molecule are incubated with the antibody. The antigen in the sample is measured by its ability to compete with the labelled molecule. Unbound sample and competitor are washed off. The amount of labelled molecule bound is measured (which could give a fluorescent, chemiluminescent or radioactive signal) (Figure 4).

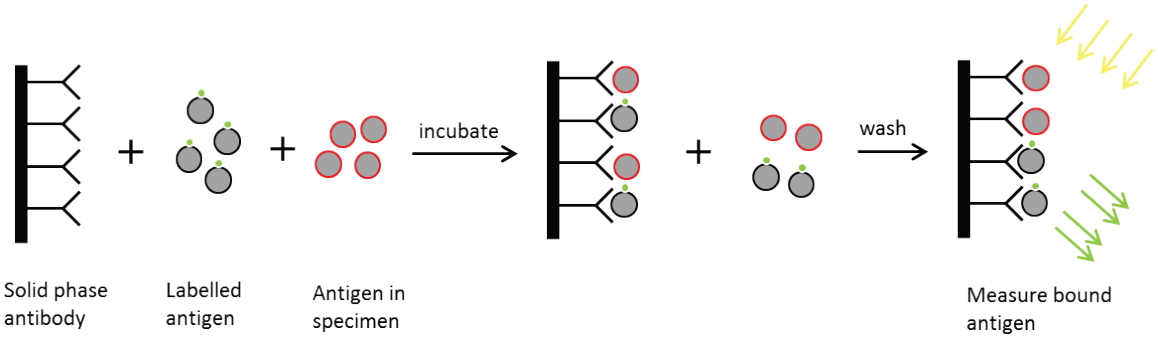

Figure 4 Schematic for competitive immunoassay. The signal of bound labelled antigen is inversely proportional to the amount of antigen of interest in the sample

Two-site immunometric assays (sandwich assay)

Antibodies against the antigen of interest are fixed to a solid phase. The specimen is incubated with the antibodies and unbound specimen is washed off. In a second incubation step, a labelled antibody against a different epitope on the specimen antigen is added; unbound secondary antibody is washed off. The amount of sample antigen bound is measured by measuring the label (figure 5).

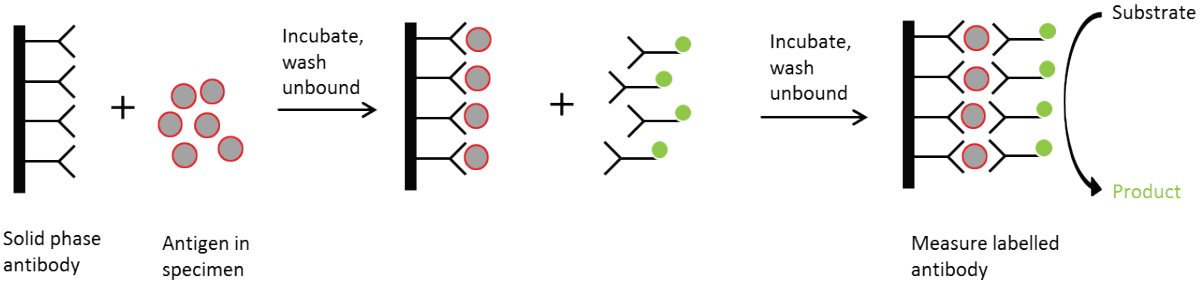

Figure 5 Two-site immunometric assay. The signal intensity is directly proportional to the amount of antigen of interest in the sample

Two-site immunometric assays have greater sensitivity, specificity and assay range than competitive assays. However, they can only be used to test for antigens which have two distinct immunogenic sites and there is often non-specific interference from heterophilic antibodies. It is also a more expensive assay as it requires a large quantity of high purity (usually monoclonal) antibody.

Detection

Labels used can be radioactive (e.g. 125I substituted onto the aromatic ring of tyrosine), fluorescent, photometric or luminescent. Secondary antibodies can be directly labelled or enzyme linked (such as to alkaline phosphatase or horseradish peroxidase) and the product of the enzymatic reaction is the signal generated. The intensity of the signal is proportional to the concentration of analyte in the specimen and can be measured by gamma counter (for radioactive labels), fluorometry, luminometry or spectrophotometry

Clinical use

The Roche chemistry analysers in the automated laboratory use turbidimetry for analysis of microalbumin. A random urine sample is mixed with a buffer containing anti-human albumin. If very small amounts of albumin are present in the urine, insoluble immune complexes will form, which cause scattering of light passing through the measurement cuvette proportional to the microalbumin concentration in the specimen. Creatinine is measured by an automated colorimetric method at the same time so that the albumin creatinine ratio (ACR) can be calculated. This corrects the albumin concentration for the urine concentration, allowing determination of the degree of microalbuminuria regardless of how concentrated the urine sample is.

Microparticule immunoturbidimetric assays can be used for the quantitative measurement of drugs in body fluids. The assay is based on competition between free drug in the sample and drug coated on a microparticle. In the absence of drug in the specimen, the microparticle agglutinates in the presence of antibody. If free drug is present in the specimen it competes with the microparticle-bound drug and therefore slows the rate of agglutination. Thus higher drug concentrations in the specimen cause a lower agglutination rate and therefore a slower rate of change in absorbance. The same principle can be used for measuring biomarkers such as serum ferritin.

http://www.bangslabs.com/sites/default/files/imce/docs/TechNote%20304%20Web.pdf

http://labmed.ascpjournals.org/content/37/8/490.full.pdf+html

Solid phase chemistry

Solid phase or ‘dry slide’ chemistry involves applying a thin layer of serum onto a slide. The slides are dry, multi-layered analytical elements on a polyester support and contain the enzymes and reagents needed for the reaction. The analyte in the specimen catalyses reagents to generate products with absorbance in the range 340 – 680 nm. This is monitored by spectrophotometry; the amount of coloured complex formed is proportional to the concentration of analyte.

Clinical use

Solid phase chemistry is used in point-of-care devices such as the Johnson & Johnson Vitros DT60 II Chemistry System, typically for small samples from geriatric and paediatric patients. A range of analytes can be measured, including electrolytes, enzymes and the lipid profile.

Electrophoresis

Electrophoresis is the movement of charged molecules (usually DNA or proteins) upon application of an electrical current. Molecules are separated according to their size, shape and charge. In the clinical biochemistry laboratory, protein electrophoresis is used on urine and serum samples. Agarose or cellulose acetate supports are used in an alkaline buffer (pH 8.6) and serum proteins are separated into six major bands according to their electrophoretic mobility (Figure 6). Serum specimens are used rather than plasma because the fibrinogen present in plasma produces a band in the β-zone which can be mistaken for a paraprotein. The separated proteins are fixed by hot air flow or in an acid/alcohol mix and stained with amidoblack solution. Protein electrophoresis is performed on urine samples primarily to detect paraproteinuria such as Bence-Jones proteins, indicative of myeloma.

Figure 6 Major bands in serum protein electrophoresis. Image from Graeme Wild, Protein Reference Units

Capillary zone electrophoresis (CZE) is also commonly used in clinical laboratories. Narrow capillaries are used to separate molecules with high resolution whilst minimising reagent use and volume of sample required. High voltages are applied which generate electrophoretic flow of the buffer in the opposite direction to the movement of proteins, facilitating improved resolution. Detection of separated proteins is automated and generates an electropherogram with peaks for each protein band which would be present on the corresponding gel (figure 7).

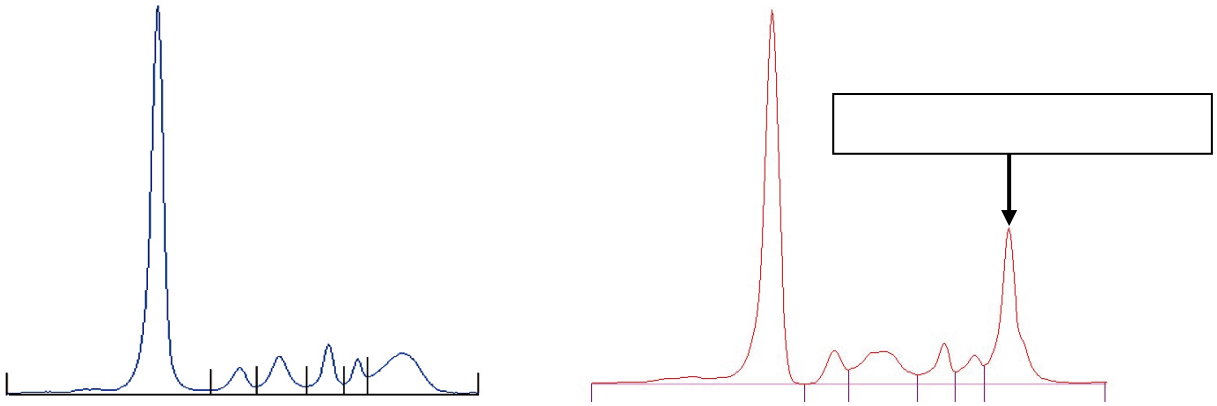

Figure 7 Capillary zone electropherograms. 1. Albumin, 2. α-1 zone, 3. α-2 zone, 4. β-1 zone, 5. β-2 zone, 6. γ zone. A. A normal trace for serum proteins. B. A monoclonal peak is present in the γ zone, indicative of a paraprotein. Acknowledgement for electropherograms: Graeme Wild, Protein Reference Units

Quality control

A serum control is run with each gel which identifies the normal position of the six zones. Ideally, fresh serum specimens are used to maximise stability of the proteins in the specimen. The β-2 fraction (C3 complement) disappears after 3 days. Samples which have been frozen can be used although there may be application marks due to protein or lipid denaturation. Freezing samples after refrigeration is not recommended because the β-lipoproteins shift from the β-2 zone to the α-1 or α-2 zones. It is not recommended to use haemolysed samples because haemolysis increases the α-2 and β zones. Common shifts in migration pattern are summarised in Figure 8. The electrophoretic mobility of lipoproteins is very sensitive to storage conditions, lipoprotein concentration, drug treatment (such as heparin) and variations in the gel hydration level or raw materials. Therefore an

anionic shift α and β lipoproteins may be observed, with corresponding band splitting. Band splitting may also occur due to variations in genotype for certain proteins. For example, α-1 anti-trypsin variants (M, S and Z) each have a slightly different electrophoretic mobility.

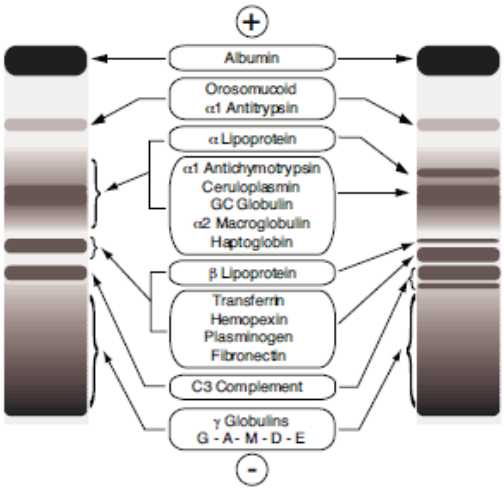

Figure 8 Common shifts in migration pattern, from HYDRAGEL PROTEIN(E) K20 – 2012/03 (Serbia Instructions and Data sheets)