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HANDOUT FOR PRACTICAL WORK

Medical Biochemistry

intended for the

4th year Veterinary Doctor

Edited by the Module Manager

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PRACTICAL WORK PROGRAM

1st session: Spectrophotometric dosing

- Protein
- Glucose

2nd session: Enzyme activity measurement

- Transaminases

3rd session: Calcium dosage

- Atomic spectrophotometry

4th session: Electrophoresis

- Serum proteins

5th session: Chromatography on thin layer

6th session: radio-immunological methods

- Dosage T3, T4

7th Session: Urine Analysis

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Some concepts in Clinical Biochemistry

Unitage and Conversions

The International System of Units (System International: SI) was adopted in 1960 by the General Conference of Weights and Measures as a coherent system based on seven basic units: the meter, kilogram, second, ampere, kelvin, candela, and mole. In human medicine, the system has not been adopted universally. Some of the common clinical chemistry units are provided here with factors to convert from the traditional non-SI units to SI unitage in plasma or serum.

International System Of Units

The International System of Units (Système Internationale d'Unités [SI units]) has standardized the reporting of data for improved comparison of results throughout most the world, with the exception of the United States, Brazil, and a few other countries. Units used for serum enzyme activity were particularly inconsistent in the past, when many enzyme procedures had results reported in

units named after the author of the procedure. Now enzyme activity is reported as international units per liter (IU/L) in the United States or ukat/L in many other countries.

Note that IU/L for enzyme activity is not an SI unit! The SI unit for enzyme activity is ukat/L. U.S. laboratories still use "traditional" units such as mg/dl. Appendix II includes common conversion factors to convert a result from one unit of measure (e.g., mg/dl) to another unit of measure (e.g., mmol/L). Unfortunately, laboratories in the same hospital may report results using different units of measure, causing confusion for clinicians when interpreting those results.

Units for Volumes, Masses, and Moles

fl or fL = femtoliter (10–15/L) fg = femtogram fmol = femtomole

pl or pL = picoliter (10–12/L) pg = picogram pmol = picomole

nl or nL = nanoliter (10–9/L) ng = nanogram pmol = picomole

µl or µL = microliter (10–6/L) µg = microgram µmol = micromole

mL = milliliter (10–3/L) mg = milligram mmol = millimole

dl or dL = deciliter (10–1/L)

kDa = kilodalton

Molarity and Molality

The mole is a unit of mass and is expressed as the molecular weight of substance in grams (g). A molar solution will contain one mole solute per liter of solution. A molal solution contains one mole solute per 1000 g of solvent.

pH/Hydrogen ion

The recommended SI unit is nmol/L. This relationship is expressed by the formula:

$pH = -\log10[H+]$, where [H+] is expressed in mol/L.

Blood urea nitrogen

To convert mg/dL to mmol/L, multiply by 0.357.

To convert urea nitrogen values to mg/dL, multiply by 2.14 to obtain urea mg/dL values.

Osmolarity and Osmolality

Osmolarity refers to the molar concentration (mol/L), whereas osmolality refers to molal concentrations and is usually expressed as moOsmol/kg body water.

Enzymes

Enzyme values vary considerably between laboratories, and these values are dependent on measurement temperature, substrate concentration, pH, buffer, activators, and other methodological variations. Enzyme activities may be expressed by various units; these units include:

International unit

defined as the enzyme activity that will convert one micromole of substrate per minute under defined conditions. It may be expressed as IU/L, or sometimes as U/L or mIU/mL. One IU/L corresponds to 16.67 nanokatals per liter.

Katal

defined as the catalytic activity that will convert one mole of substrate per second under defined conditions. One nanokatal (nKat) equals 109 mol/s or 0.06 IU/L.

If the reaction conditions vary (e.g., using different substrates for the same enzyme), then using these conversion factors may be inappropriate for comparison. Even for common enzymes such as the aminotransferases, there are several different national and international recommended methods for use with human sera or plasma, and these yield differing values despite conformity in units. There are no internationally recommended conditions for samples obtained from laboratory animals.

Some Common Biochemical Abbreviations

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

CBP: Cortisol binding protein

CRF: Corticotrophin releasing factor

Da: Dalton, unit of mass

DNA: Deoxyribose nucleic acid

DPG: Diphosphoglycerate or diphosphoglyceric acid

EDTA: Ethylenediaminetetra-acetic acid

ELISA: Enzyme linked immunosorbent assay

EM pathway: Emden Meyerhof pathway

EPO: Erythropoietin

ESF: Erythropoietin stimulating factor

F6P: Fructose 6 phosphate

FSH: Foliotrophin (follicle stimulating hormone)

G6PD: Glucose 6 phosphate dehydrogenase

GH: Somatotrophin (growth hormone)

GHRH: Growth hormone releasing hormone

GnRF: Gonadotropin releasing factor

GSH: Reduced glutathione

GSSG: Oxidized glutathione

HMGCoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A

IG or Ig: Immunoglobulin

IL: Interleukin

LCAT: Phosphatidylcholine sterol acyltransferase

LDH: Lactic dehydrogenase

LH: Luteotrophin (lutrophin; luteinizing hormone)

LPS: Lipopolysaccharide

NAD: Nicotinamide adenine dinucleotide

NADH: Reduced nicotinamide adenine dinucleotide

NADPH: Reduced nicotinamide adenine dinucleotide phosphate

PPAR: Peroxisome proliferator-activated receptor

PTH: Parathyrin (parathyroid hormone)

RT-PCR: Real-time polymerase chain reaction

T3: Triiodothyronine

T4: Thyroxine

TBG: Thyroxine binding globulin

Tg: Thyroglobulin

TH: Thyroid hormone

TRH: Thyrotropin-releasing hormone

TSH: Thyroid stimulating hormone (thyrotropin)

TTR: Transthyretin

Laboratory Equipment

A variety of general laboratory equipment is needed for the clinical laboratory. the tests that are routinely performed in the laboratory determine the equipment and instrumentation needed. Minimal equipment includes a microscope, a refractometer, a microhematocrit, and centrifuge. Additional instrumentation that may be needed including blood chemistry analyzers, cell counters, water baths, and incubators. test tubes, pipettes, heat blocks, and aliquot mixers are also commonly found in veterinary practices. The proper use and maintenance of this equipment are essential to ensure accurate test results and safety of personnel.

The Microscope

Different types of microscopes are available for clinical use, but the veterinary laboratory generally has just one type.

- Electron microscopes, which use an electron beam to create magnified images of objects, are primarily found in research settings and large human medical facilities.
- Light microscopes are those that utilize a visible, ultraviolet, or laser light source and include compound light microscopes, fluorescent microscopes, phase-contrast microscopes, and dark field microscopes.
- Phase-contrast, fluorescent, and dark field microscopes are used primarily in reference laboratories, especially for viewing of unstained specimens.

In the veterinary practice laboratory, a high-quality binocular compound light microscope is essential (Picture1). This microscope may be used to evaluate blood, urine, semen, exudates, and transudates; other body fluids; feces; and other miscellaneous specimens. It may also be used to detect internal and external parasites and to initially characterize bacteria. The practice should ideally maintain two microscopes. One should be used for performing routine parasite studies and procedures that involve the use of corrosive or damaging materials. The second microscope should be reserved for use with cytology and hematology evaluations.

A compound light microscope is so named because it generates an image by using a combination of lenses. Compound light microscopes have many components and a light path. The optical tube length is the distance between the objective lens and the eyepiece. In most microscopes, this distance is 160 mm. The mechanical stage holds a glass slide to be evaluated. The microscope should have a smoothly operating mechanical stage to allow easier

manipulation of the sample . Left- or right-handed stages are generally available. Coarse and fine focus knobs are used to focus the image of the object being viewed.

The compound light microscope consists of two separate lens systems: the **ocular** system and the objective system. The ocular lenses are located in the eyepieces and most often have a magnification of 10×. This means that the ocular lens magnifies an object 10 times. A monocular microscope has one eyepiece, whereas a binocular microscope, which is the most commonly used type, has two eyepieces. The two eyepieces can be adjusted to match the interpupillary distance of the user.

Most compound light microscopes have three or four objective lenses, each with a different magnification power. The most common objective lenses are 4× (scanning), 10× (low power), 40× (high dry), and 100× (oil immersion). The scanning lens is not found on all microscopes. An optional fifth lens, a 50× (low oil immersion), is found on some microscopes. Some microscopes may also have phase-contrast lenses. It is important that only immersion oil designed for microscopy be used on the microscope. Other oils may be damaging to the optics.

Total magnification of the object being viewed is calculated by multiplying the ocular magnification power and the objective magnification power. For example, an object viewed through the 40× objective lens and the 10× ocular lens is 400 times larger in diameter than the unmagnified object:

10 x (ocular lens)x 40 xobjective lens total magnification)

= 400 x (total magnification)

Picture 1: light microscope

Centrifuges

are vital instruments with many uses in the veterinary practice laboratory (Picture 2). The centrifuge is used to separate substances of different densities that are in a solution. The centrifuge spins samples at high speeds, which pushes the most dense components in the sample to the bottom of the tube. Liquid components are layered above the solid components, also according to their densities. When solid and liquid components are present in the sample, the liquid portion is referred to as **the supernatant,** and the solid component is referred to as **the sediment**. The supernatant (e.g., plasma or serum from a blood sample) can be removed from the sediment and stored, shipped, or analyzed. Centrifuges vary in size, capacity (i.e., the number of tubes that can be spun at one time), and speed capabilities. Veterinary practice laboratories often have more than one type of centrifuge.

A microhematocrit centrifuge is designed to hold capillary tubes, whereas a clinical centrifuge accommodates test tubes of varying sizes. Larger referral practices and reference laboratories may have additional types of centrifuges. A refrigerated centrifuge is used when materials must be kept cool during centrifugation (e.g., processing of blood components for transfusion therapy).

Clinical centrifuges that are used in veterinary laboratories are one of two types, depending on the style of the centrifuge head. A horizontal centrifuge head, which is also known as the "swinging-arm" type, has specimen cups that hang vertically when the centrifuge is at rest. During centrifugation, the cups swing out to the horizontal position. As the specimen is centrifuged, centrifugal force drives the particles through the liquid to the bottom of the tube. When the centrifuge stops, the specimen cups fall back to the vertical position.

Picture 2 : Laboratory centrifuge

Microhematocrit centrifuge

Microhematocrit centrifuges are a type of angled centrifuge. The microhematocrit centrifuge (Picture3) is configured to accommodate capillary tubes. In veterinary practice, the microhematocrit centrifuge is used exclusively for spinning down microhematocrit tubes. This centrifuge is used for. This process is used for determining a patient's PCV (packed cell volume) in a whole blood sample and can also provide a plasma sample for protein analysis.

Picture 3: Microhematocrit centrifuges

Refractometer

The purpose of a refractometer is to measure the refractive index of a solution (Picture 4). When measuring a solution (i.e., urine), light passes through the sample and bends. The angle of this refraction is visualized as a shadow and correlates to the concentration of the solution. Veterinary specific refractometers are now on the market allowing for minor differences between dog and cat urine specific gravity and total protein values.

The most common use of a refractometer in veterinary laboratories is to measure urine specific gravity and plasma total protein. Refractometers have built-in scales to measure both of these, and some brands of refractometers will also possess a refractive index scale. This scale, with the use of an appropriate conversion chart, can be used to measure the concentration of many other solutions.

Picture 4: Refractometers measurement

Chemistry analyzers

There are a wide variety of chemistry analyzers available for veterinary use. Most use the principles of photometry to quantify analytes, such as enzymes, proteins, and other constituents in the blood. Electrochemical methods are used to analyze ionic compounds such as electrolytes. These two methods may require the use of two separate analyzers, or they may be combined into one.

Another variation among analyzers is the way they facilitate the photometry testing procedure. A sample needs to be added to a substrate to initialize the test. Examples include slides, rotors, or cartridges . Depending upon the analyzer or the analyte being tested, a serum or plasma sample is required. Some analyzers have the ability to process whole blood sample as well. The type of anticoagulant recommended should be confirmed by reviewing the manufacturer recommendations. Regardless of the analyzer type chosen, it is important to maintain the equipment according to the manufacturer's recommendations. With such a wide variety of analyzers available.

Photometry

Photometry is a general term used to describe an analytical chemistry technique in which the concentrations of substances and the activities of enzymes are determined by measuring the intensity of light passing through or emitted from a test chamber (Figure1) . This test chamber contains the substance to be detected and, in most cases, reagents intended to react with that substance to produce a color reaction. Strictly speaking, the term spectrophotometry should be applied when the instrument being used has the ability to produce light of a variety of

wavelengths through some type of light-fractionating device, such as filters, prisms, or diffraction gratings

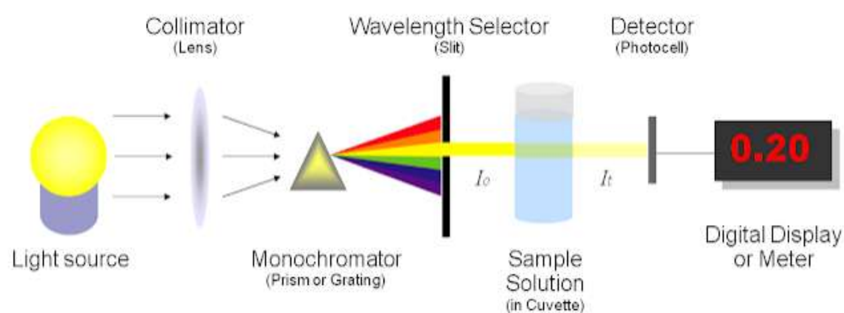


Figure 1: Components of spectrophotometer

Absorbance spectrophotometry

Absorbance spectrophotometry is an analytic technique in which concentrations of substances are determined by directing a beam of light through a solution containing the substance to be detected (or a product of that substance) and then measuring the amount of light that either of these absorb (Picture 5) . The principles described here are incorporated into automated and semiautomated processes on today’s chemistry analyzers. Automation, from sample and reagent addition management to calculation of test results to generation of a patient diagnostic report, is made possible by computer control and information processing integral to these systems.

Picture 5: UV – VIS Spectrophotometer

To understand absorbance spectrophotometry, some basic knowledge regarding light is necessary. Typically, light is classified by its wavelength, which is measured in nanometers (nm). Light with the shortest wavelengths (<380 nm) is termed ultraviolet (UV) light (Table 1). Light in the visible spectrum has wavelengths of 380–750 nm.

Table 1. Wavelengths resulting in ultraviolet light, various colors of visible light, and infrared light.

Wavelength (nm)	Color
<380	None (ultraviolet)
380–440	Violet
440–500	Blue
500–580	Green
580–600	Yellow
600–620	Orange
620–750	Red
750–2000	None (infrared)

Atomic absorption spectrophotometry

Atomic absorption spectrophotometry (AA) is used for measuring the concentrations of many elements. Advantages of AA include its superior sensitivity (i.e., it can detect smaller concentrations) and its ability to measure the concentrations of various elements (Figure 2). AA is typically limited to toxicology laboratories for clinical purposes. Applications include measurement of concentrations of elements such as lead, copper, and selenium in fluids or tissues. As the name implies, AA involves measuring absorption of energy by atoms. This technique involves heating a sample in a flame that is hot enough to cause the element in question to dissociate from its chemical bonds and form neutral atoms – but not hot enough to cause large numbers of electrons to jump to the excited state. These atoms then are in a low-energy (i.e., ground) state and can absorb light of a narrow wavelength that is specific for that element. If a light of this wavelength is projected through the flame, the amount of light absorbed is proportional to the concentration of the element in the sample. Measurement of

the amount of light absorbed, therefore, allows the concentration of that element in the sample to be calculated. Focusing devices, photodetectors, meters, and readout devices serve the same purposes in AA as in other types of spectrophotometry.

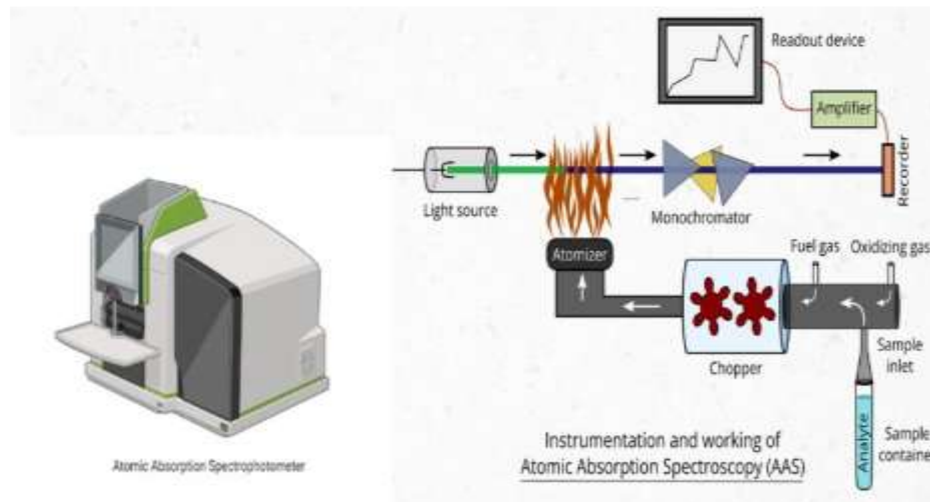


Figure 2: Instrumentation of atomic absorption spectroscopy

Protein electrophoresis

Electrophoresis is an analytic technique based on the movement of charged particles through a solution under the influence of an electrical field (Figure 2) . In clinical chemistry, electrophoretic techniques most commonly are used to separate and analyze serum proteins. When serum is placed on or in a supporting substance that allows migration of these proteins and can carry an electrical charge, these proteins move through this material just as other charged particles do. The movement of proteins through such a substance depends on the net charge on the protein molecule, the size and shape of the protein molecule, the strength of the electrical field applied, the type of supporting medium, and the temperature. In a given electrophoresis application, the latter three items are held constant. Therefore, the migration of protein molecules depends on the net charge and on the size and shape of the molecules. As a result, different serum proteins migrate at different rates and, possibly, in different directions in the supporting substance.

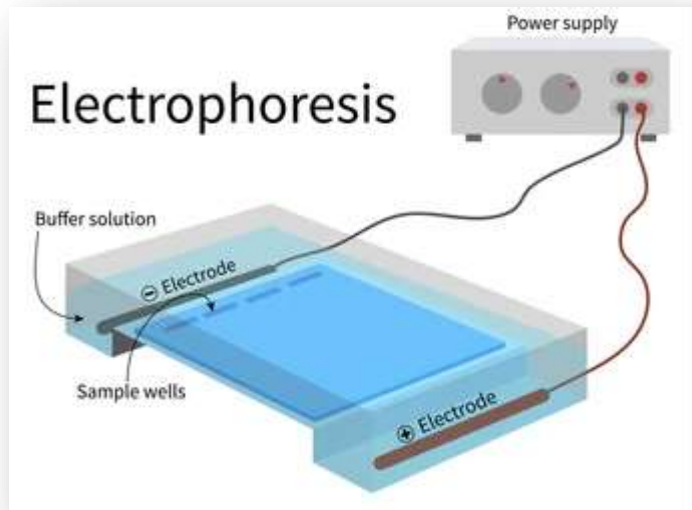


Figure 3: Electrophoresis Equipments

Temperature-controlling equipment

Incubators

A variety of microbiology tests require the use of an **incubator**. Incubators for the in-house veterinary practice laboratory are available in a variety of sizes and configurations. The incubator must be capable of sustaining a constant 37° C, which is the temperature at which the majority of pathogenic organisms grow. The incubator should be fitted with a thermometer, or one should be placed inside the chamber to monitor the temperature. Heat should be provided by a thermostatically controlled element. A small dish of water should also be placed inside to maintain proper humidity. Some incubators have built-in humidity controls, but this type of equipment tends to be expensive.

Refrigerators

Many reagents and test kits that are used in the veterinary clinical laboratory require refrigeration, and some may require storage in the freezer. Samples such as blood and urine may also require refrigeration. A basic tabletop refrigerator can be used for most items. This refrigerator may not contain any food for human consumption.

Water Baths

Some clinical chemistry assays, coagulation tests may require the use of a water bath that is capable of maintaining a constant temperature of 37° C. A variety of types of water baths are available, including simple standard water baths, circulating water baths, and waterless bead baths.

Containers for sample collection

A variety of commercially available tubes are used for blood collection. These tubes contain the appropriate anticoagulant for the various diagnostic procedures and a vacuum for drawing in the appropriate volume of blood. These tubes are commonly known as vacutainer tubes. The following commonly used vacuum tubes are described in the approximate order of their frequency of use. Tubes are commonly referred to by their stopper color, which is used to identify the type of anticoagulation system the tube contains.

Picture 6: Blood collection tubes

Table 2. Collection Tubes

Tube top color	Content	Function
Purple top	EDTA	Complete blood cell counts
Red top	No additive	Serum studies
Blue top	Sodium citrate	Coagulation studies
Green top	Heparin	Blood smears

EDTA, ethylamine diamine tetraacetic acid

Purple top or lavender-top tube

Contains the anticoagulant ethylenediaminetetraacetic acid (EDTA) salt. This tube is used to collect blood for hematologic determinations. The EDTA anticoagulant results in the most consistent preservation of cell volume and morphologic features.

Red-top or serum collection tube

The red-top or serum collection tube contains no anticoagulant. Blood that is placed in this tube is expected to clot so that serum may be harvested. This tube is used to collect serum for common biochemical determinations, such as those tests used in creating biochemical profiles.

Green-top or heparin tube

contains lithium heparin. This anticoagulant is used for certain special biochemistry tests, particularly those that require a whole-blood aliquot for determination and that might be influenced by the presence of other chemical anticoagulation systems.
Some systems recommend use of lithium heparin for all common clinical chemistry determinations. The advantage is that time is not required for clotting to completion to yield serum. The plasma may be separated immediately for testing, and results for most analytes are equivalent for serum and plasma. There are two exceptions. Total protein will be slightly higher for plasma because it includes fibrinogen. Potassium averages about 0.5 mmol/L higher for serum because of platelet release during clotting. Lithium heparin is also used for electrochemical determinations.

Blue-top or citrate tube

The blue-top tube contains sodium citrate. It is used for coagulation biochemistry determinations.

Gray-top or fluoride tube

The gray-top tube contains sodium fluoride. Fluoride is not an anticoagulant, however. Rather, it inhibits enzymes in the glycolytic pathway and prevents erythrocytes from metabolizing glucose while whole blood is transported to the laboratory. It is not commonly used.

Table 3. Advantages and disadvantages of blood tubes for biochemical analysis

	Advantages	Disadvantages
Serum tube (red top)	No interfering substances, easy to use	After centrifugation, the serum must be removed from the cells; otherwise, the cells will continue to metabolize glucose and may leach potassium into the serum
Heparin (green top)	Do not have to wait for the sample to clot to centrifuge the sample	Use of sodium heparin tubes can interfere with accurate sodium measurements, not all tests validated for plasma

Pipettes

Some pipettes and pipetting devices may be needed in the veterinary practice laboratory (Picture7). The primary types of pipettes used in the practice laboratory are transfer pipettes and graduated pipettes. Transfer pipettes are used when critical volume measurements are not needed. These pipettes may be plastic or glass, and some can deliver volumes by drops. Graduated pipettes may contain a single volume designation or have multiple gradations. Pipettes with single gradations are referred to as volumetric pipettes and are the most accurate of the measuring pipettes. It is important that the pipette be used correctly to ensure that the desired volume is measured.

Picture 7: Micropipettes

Blood sample collection and handling techniques

Blood is collected from animals in order to conduct many tests, ranging from hematology parameters to clinical chemistries, and even to evaluate coagulation disorders. Good technique when obtaining blood samples is important to maximize the reliability of the results. Blood can be collected from many sites on animals; however, the most common for mammals is the jugular, cephalic, and lateral and medial saphenous veins, avian have different locations for blood collection. It is important to collect a sufficient quantity of blood for all the tests to be conducted.

Blood can be drawn utilizing a syringe and needle or a vacutainer system. The vacutainer technique is preferred, as this reduces the chance for a clotted sample, draws the accurate amount into the tube, and is considered sterile. Anticoagulant blood tubes should be gently inverted a number of times immediately after collection. Utilizing a blood rocking machine may be useful; however, overmixing or prolonged mixing of a sample can cause cellular damage.

Some tests require an anticoagulated sample (whole blood) or plasma, while others require serum. Whole blood samples are a representation of all the cells, liquid, and compounds within the blood mixed in a solution that will not allow the sample to clot. This can be spun down in a centrifuge to separate the cells (white blood cell [WBC], red blood cell [RBC], etc.) from the liquid (plasma); but, even when spun down, the sample has not clotted, it is just separated. Serum is often used for chemistries and is the liquid portion of a blood sample after it has clotted. The difference between plasma and serum is the clotting factors, primarily fibrinogen. Plasma has them, but serum does not, because those clotting factors are caught up in the clot.

1st session: Spectrophotometric dosage

Protein measurement

Indications

Serum and plasma protein concentrations are commonly evaluated as a part of routine laboratory diagnostics. Understanding the basic mechanisms that drive changes in these values is important to the practitioner. Proteins in the serum and plasma consist of albumin and globulins. Albumin and the majority of globulins are produced by hepatocytes with a major exception being immunoglobulins that are produced by B cells and plasma cells. Plasma differs from serum in that plasma contains fibrinogen and clotting factors, whereas serum does not. Thus, total protein concentration derived from plasma will be slightly higher than that from serum.

The measurement of total serum protein is not directly indicative of the presence of any disease but indirect correlation with disease process is extremely helpful. The protein examination is indicated in liver diseases, immunosuppression, parasitic diseases, chronic wasting diseases, pregnancy, starvation and edematous swelling of dependent parts of the body. In serum, total proteins, albumin, globulin, albumin and globulin ratio and gammaglobulins are measured.

Method (Biuret Reaction and BCG Dye-Binding Reaction)

These methods are used to measure total protein (Biuret) and albumin (BCG dye-binding) concentrations in serum samples and are the values generally reported as part of a serum chemistry panel. Both are based on spectrophotometric measurement of color change with binding of reagents directed at peptide bonds (Biuret) or albumin (BCG dye-binding). The globulin concentration reported on most serum chemistry panels is a calculated value derived from subtracting the albumin concentration from the total protein concentration.

Principle of the method

Proteins give an intensive violet-blue complex with copper salts in an alkaline medium. Iodide is included as an antioxidant. The intensity of the color formed is proportional to the total protein concentration in the sample.

Procedure

1. Take 3 test tubes and place them on a test tube stand. Mark the tubes as ‘T’ for test, ‘S’ for standard, and ‘B’ for blank. Pipette 4.9 ml normal saline solution in tube ‘T’ and ‘S’ and 5.0 ml in tube ‘B’.
2. Add 0.1 ml test serum in tube ‘T’ and 0.1 ml standard in tube ‘S’.
3. Add 5 ml biuret reagent in all tubes and mix well.
4. Incubate the tubes in water bath at 37°C for 30 minutes.
5. Read the absorbance or transmittance in a spectrophotometer at 454 nm wavelength. Set zero with blank and calculate the total serum protein by using following equation :

$$\underset{\text{(gram/100 ml serum)}}{\textbf{Total serum protein}} = \frac{\text{OD of the test}}{\text{OD of standard}} \textbf{ X } \underset{\text{(gram / 100 ml)}}{\textbf{Concentration of the standard}}$$

Interpretation

A decrease in protein content is known as hypoproteinemia which is observed in maldigestion, malabsorption, starvation, burns, lactation, renal disease, liver disease, chronic wasting diseases, proteinuria, pregnancy, parasitic diseases, diarrhea and dysentry. An increase in total serum protein (hyperproteinemia) is observed in shock, dehydration, neoplasms like lymphosarcoma and plasmacytoma. The normal values of total serum proteins, albumin and globulins is given in Table 4.

Table 4: Serum proteins in animals

Species	Total serum protein (gm/100 ml)	Albumin (gm/100 ml)	Globulin (gm/100 ml)		
			Alpha	Beta	Gamma
Bovine	7.56	3.4	0.85	1.08	2.16
Sheep	5.81	2.96	1.10	0.45	1.30
Goat	6.25	3.95	0.42	1.24	0.91
Horse	6.72	2.60	2.63	0.81	0.68
Dog	6.1-7.8	3.1- 4.0	1.20	1.30	0.80

Serum albumin

Albumin is one of the most important proteins in plasma or serum. It makes up 35% to 50% of the total plasma protein in most animals, and any significant state of hypoproteinemia is most likely caused by albumin loss. Hepatocytes synthesize albumin, and any diffuse liver disease may result in decreased albumin synthesis. Renal disease, dietary intake, and intestinal protein absorption may also influence the plasma albumin level. Albumin is the major binding and transport protein in the blood, and it is responsible for maintaining the osmotic pressure of the plasma. The primary photometric test for albumin in veterinary patients is the bromocresol green dye-binding method.

Indications

The albumin is measured in serum in such conditions in which globulin level is to be calculated. The globulin content is calculated by subtracting the albumin values from total serum proteins. The measurement of albumin is indicated in all such situations in which total serum protein is determined.

Procedure

1. Take 6" test tube and pipette 3.0 ml sulfatesulfite reagent and mix 0.2 ml serum to be tested. Add 3.0 ml ethyl ether and mix thoroughly by shaking the tube 40 times in about 20 seconds. Centrifuge the tube at 2000-3000 rpm in a clinical centrifuge for 5-10 minutes. After centrifugation, suck 2.0 ml clear solution from lower layer by pipette and place in another test tube and mark the tube as ‘T’ for test.

2. In another tube, take 2.0 ml sulfate-sulfite reagent and mark it as ‘B’ for blank.

3. In tube ‘S’, place 2 ml of standard solution prepared in total serum protein estimation as 4% bovine serum albumin.

4. Add 2.0 ml sodium hydroxide (1.5 N NaOH) in each tube and mix.

5. Add 0.8 ml Biuret reagent to each tube and mix properly. Keep the tubes for 20 minutes. Read the absorbance or transmittance against a reagent blank at zero and calculate the albumin content by following equation:

$$\underset{\text{(gram/100 ml serum)}}{\textbf{Albumin in serum}} = \frac{\text{OD of the test}}{\text{OD of standard}} \textbf{ X } \underset{\text{(gram / 100 ml)}}{\textbf{Concentration of the standard}}$$

Interpretation

The increase in serum albumin content is termed as hyperalbuminemia which is observed in shock and acute dehydration. The hypoalbuminemia, decrease in albumin content in serum, is met in starvation, maldigestion, malabsorption, chronic hepatic diseases, prolonged fever, diabetes mellitus, acute nephritis, nephrosis, ascites and parasitic diseases.

The globulins

are a complex group of proteins. Alpha globulins are synthesized in the liver and primarily transport and bind proteins. Two important proteins in this fraction are high-density lipoproteins and very-low-density lipoproteins.

- ✓ Beta globulins include complement (C3, C4), transferrin, and ferritin. They are responsible for iron transport, heme binding, and fibrin formation and lysis.
- ✓ Gamma globulins (immunoglobulins) are synthesized by plasma cells, and they are responsible for antibody production (immunity). The immunoglobulins (Ig) that have been identified in animals are IgG, IgD, IgE, IgA, and IgM.

Direct chemical measurements of globulin are rarely performed.

Calculated globulin concentration

The serum total protein and albumin concentrations are measured routinely as part of serum biochemical profiles. The globulin concentration as reported on these profiles is not measured, however, but rather is calculated by subtracting the serum albumin concentration from the total protein concentration.

Albumin / Globulin Ratio

An alteration in the normal ratio of albumin to globulin (A/G) is frequently the first indication of a protein abnormality. The ratio is analyzed in conjunction with a protein profile. The A/G can be used to detect increased or decreased albumin and globulin concentrations. Many pathologic conditions alter the A/G. However, if the albumin and globulin concentrations are reduced in equal proportions, such as with hemorrhage, no alteration in A/G will be present.

The A/G is determined by dividing the albumin concentration by the globulin concentration. In dogs, horses, sheep, and goats, the albumin concentration is usually greater than the globulin concentration (i.e., the A/G is more than 1.00). In cattle and cats, the albumin concentration is usually equal to or less than the globulin concentration (i.e., the A/G is less than 1.00).

Serum gamma globulin

Indications

Serum gamma globulin level is measured in conditions when specific or non-specific immune status of animal is determined. The evaluation of any vaccine or immunomodulator can be done by measuring the level of serum gamma globulin. It is also measured in immunosuppression, immunopathological reactions including congenital agammaglobulinemia and hypogammaglobulinemia and in determining the prognosis of disease.

Procedure

1. In a test tube take 5.7 ml ammonium sulfate-sodium chloride solution (19.5% ammonium sulfate and 2.03% sodium chloride; pH 6.4); to this overlay 0.3 ml clear serum sample. Mix the contents by gently inverting the tube at least 6 times and place it on ice baths for 15 minutes. Centrifuge this at 3000 rpm for 10 minutes in a clinical centrifuge and discard the supernatant. Repeat the process twice and finally dissolve the contents in 2.0 ml normal saline solution.

2. Add 5.0 ml Biuret reagent and keep it for 10 minutes at room temperature.

3. Make a blank with 2.0 ml normal saline and 5 ml Biuret reagent in tube marked ‘B’.

4. Make a standard by placing 2.0 ml 0.15% bovine serum albumin and add 5.0 ml Biuret reagent in this tube marked as ‘S’.

5. Read the absorbance or transmittance of test and standard against blank set at ‘zero’ at 555 nm wavelength and calculate the gamma globulin by following equation:

$$\textbf{Serum gamma globulin} \text{ (gram/100 ml serum)} = \frac{\text{OD of the test}}{\text{OD of standard}}$$

Interpretation

Increased values of gamma globulins are observed in immunostimulatory reactions such as after vaccination, administration of immunostimulatory drugs, in hypersensitivity reactions and in infectious diseases. The decrease in serum gamma globulin level is seen in congenital hypogammaglobulinemia, immunosuppression, immunodeficiency diseases and due to environmental pollutants or contaminants.

Glucose measurement

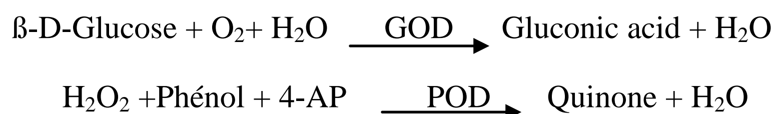
Indications

Glucose measurement is an important metabolic indicator that is highly regulated in all species. Food intake, glycolysis, glycogenolysis, and gluconeogenesis all contribute to supporting blood glucose levels in the normal range across a wide range of nutritional conditions. Glucagon, corticosteroids, catecholamines, and growth hormone all increase plasma glucose concentrations, while the primary hormone lowering glucose is insulin.

The blood glucose examination is performed in animals suspected for diabetes mellitus or convulsions, such as in hunting dogs who show fatigue. In sheep and cattle suspected for ketosis and young chicks suspected for hypoglycemia associated with starvation. In animals suspected for pancreatitis, the glucose examination in blood is indicated. In aged animals, it is performed as routine check-up.

Principle of the method

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H2O2), is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD):



$$\text{ß-D-Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O$$

$$H_2O_2 + \text{Phénol} + \text{4-AP} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the glucose concentration in the sample.

Procedure

1. In a clean, dry centrifuge tube, take 3.4 ml distilled water and 0.2 ml blood (sodium fluoride used as anticoagulant). Add 0.2 ml 2/3 N sulfuric acid and mix well.

2. Add 0.2 ml 10% sodium tungstate solution slowly with constant agitation in tube, mix properly and keep the tube on stand for 5 minutes.

3. Centrifuge the tube at 2000-3000 rpm in a clinical centrifuge and take out the supernatant.

4. Take 4 Folin sugar tubes and mark them as T for test, S1 and S2, for standard and B for blank. In tube T place 1 ml of supernatant as produced in step 3. In tubes S1 and S2, place 1 ml standard in each containing 0.1 mg/ml and 0.2 mg/ml; these can be prepared by dissolving

10 mg and 20 mg glucose in 100 ml saturated benzoic acid solutions respectively. The 0.1 mg/ ml and 0.2 mg/ml standard are equivalent to 200 mg/100 ml and 400 mg/100 ml blood glucose level.

5. Add 1 ml distilled water to each tube and mix well. Take 2 ml distilled water in tube 'B'.

6. Add 2 ml alkaline copper sulfate in each tube and mix; place the tubes in boiling water bath for 8 minutes.

7. Cool the tubes in running tap water for 2-3 minutes and add 2 ml phosphomolybdic acid solution to each tube, mix properly and place in boiling water bath for 2 minutes.

8. Cool the tubes in running tap water and keep them on stand for 3-5 minutes and then dilute to 25 ml mark with distilled water. Apply stopper and mix the contents.

9. Take 3-5 ml from each tube in cuvette and read the absorbance/transmittance at 420 nm wavelength by setting zero with blank and calculate the glucose level in blood by following equation.

$$\textbf{mg glucose/100 ml blood} = \frac{\text{OD of the test}}{\text{OD of standard}} \ \textbf{X mg value of standard}$$

Interpretation

The blood glucose level reflects the nutritional, emotional and endocrinal conditions of animal. Increased blood sugar values are called as hyperglycemia which is observed in diabetes mellitus, hyperpituitarism, shock, urinary obstruction, hyperthyroidism, chronic nephritis, burns, increased intracranial pressure, epilepsy, tetany, convulsions, anoxia, terminal stage of pregnancy and toxemia. The decreased blood sugar level is known as hypoglycemia and is met in hyperinsulinism, acetonemia, pregnancy toxemia, hepatic insufficiency, starvation, neoplasms of pancreas, hypothyroidism and hypopituitarism. Normal values of blood glucose in different animal species are given in Table 5.

Table 5: Blood glucose level in animals

Species	Blood glucose (mg/100 ml blood)
Bovines	35-55 mg
Sheep	35-60 mg
Goat	45-60 mg
Horse	60-100mg
Dog	55-90 mg
Cat	60-100mg

Measurement of enzymatic activity

Transaminases (Aminotransferases)

Principle

The measurement of enzyme activities is a critical component of the routine serum biochemical profile. While low values are rarely diagnostically significant, elevations can be used to determine the spectrum of tissues involved in a pathologic process and to estimate the severity of the condition. Serial assessments can aid in evaluating progression of pathology or response to therapy. The presence of most diagnostic enzymes in the blood is incidental and reflects release due to routine cell turnover in tissues. Because these enzymes rarely serve any biological purpose in the blood, their concentrations are not regulated, thus reference intervals tend to be wide. Therefore, elevations are often expressed in fold elevations above the upper limit of the reference interval. While some enzymes originate from a single tissue, many have multiple potential sources.

Serum Alanine and Aspartate Aminotransferases

The serum activity of the aminotransferases, AST and ALT, are measured to detect hepatocellular injury.

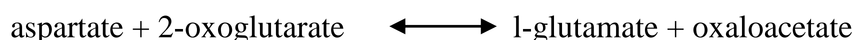
➪ Alanine aminotransferase (ALT)

ALT also known as glutamic pyruvic transaminase (GPT) is often investigated in clinical biochemistry in association with ASAT catalyzes the reaction :

ALT is measured in serum (heparinized plasma in selected assays) by spectrophotometric and dry reagent methods. ALT is stable in separated serum for approximately 1 (at 22° C) to 7 (at 4° C) days.

➪ Aspartate aminotransferase (AST)

AST also known as glutamic oxaloacetate transaminase (GOT) or serum glutamic oxaloacetic transaminase (SGOT) catalyzes the reaction :



Indications

- ALT is used as a screening procedure for hepatic disease in patients with undiagnosed illness. Most patients with known chronic hepatitis should undergo periodic ALT determinations to monitor the problem**.**

- The determination of AST is not specific for any organ or tissue. However, its value is determined in liver or muscle diseases or when a widespread damage is suspected in body. It is particularly indicated in muscular degeneration and necrotic conditions.

Procedure

1. A standard curve is prepared by making a series of dilution of sodium pyruvate solution in following manner.

a. Stock standard solution is prepared by dissolving sodium pyruvate 0.0222 gm in 100 ml phosphate buffer (0.1M, pH 7.5).

b. Take 6 tubes in a test tube stand and place 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml stock standard of sodium pyruvate in these tubes; Add 1.0 ml, 0.9 ml, 0.8 ml 0.7 ml, 0.6 ml and 0.5 ml aspartateglutamate substrate in the tubes respectively so that each tube should have one ml content. Add 0.2 ml distilled water in each tube.
c. Add 1.0 ml of 2,4-dinitrophenylhydrazine reagent in each tube, mix properly and keep for 20 minutes. Add 10.0 ml 0.4 N sodium hydroxide and mix vigorously.
d. After keeping the tubes for 5 minutes, read the OD in spectrophotometer at 505 nm wave length and plot the curve. In tubes of sodium pyruvate solution.
2. Take 0.5 ml aspartate-glutamate substrate into a test tube and add 0.1 ml serum and incubate at 37°C for 60 minutes.
3. Add 0.5 ml of 2,4-dinitrophenylhydrazine reagent and mix properly. Keep the tubes at room temperature for 15 minutes and add 5 ml of 0.4 N sodium hydroxide and mix well. After 20 minutes, read the OD in spectrophotometer at 505 nm wave length using water blank.
The activity of equivalent AST and ALT is given in Table 6.

Table 6: AST and ALT values in standard solution

Tube no.	Sod. pyruvate	Equivalent AST (Frankel unit)	Equivalent ALT (Frankel unit)
1	0 ml	00	00
2	0.1 ml	20	23
3	0.2 ml	55	50
4	0.3 ml	95	83
5	0.4 ml	148	125
6	0.5 ml	216	--

Interpretation

In normal animals the AST and ALT values are given in Table 7.

Table 7: ALT and AST values in animals

Species	ALT(U/L)	AST(U/L)
Bovine	5−35	46−176
Ovine	10−44	49−90
Equine	2.7−21	160−595
Canine	8.2−109	9−49
Feline	25−97	7−40
Caprine	15−52	43−230

Increased level of serum AST levels are observed in association with cell necrosis like, hepatic necrosis, myocardial infarction, muscular necrosis, azoturia, starvation and white muscle disease or stiff lamb disease.

3rd session: Calcium dosage
Atomic Spectrophotometry

Calcium

More than 99% of the calcium in the body is found in the bones. The remaining 1% or less has major functions in the body, which include the maintenance of neuromuscular excitability and tone (decreased calcium can result in muscular tetany), the maintenance of activity of many enzymes, the facilitation of blood coagulation, and the maintenance of inorganic ion transfer across cell membranes. Calcium in whole blood is found almost entirely in plasma or serum. Erythrocytes contain little calcium.

Calcium concentrations are usually inversely related to inorganic phosphorus concentrations. As a general rule, if the calcium concentration rises, the inorganic phosphorus concentration falls. Hypercalcemia is an elevated blood calcium concentration. Hypocalcemia is a decreased blood calcium concentration.

Samples for calcium testing should not be collected using EDTA or oxalate or citrate anticoagulants, because these substances bind with calcium and make it unavailable for assay. Hemolysis results in a slight decrease in calcium concentration in samples as the fluid from the ruptured erythrocytes dilutes the plasma.

Procedure

1. Take 3 centrifuge tubes and mark them as ‘T’ for test, ‘S’ for standard and ‘B’ for blank. Pipette 2 ml serum in tube ‘T’, 2 ml standard calcium (5 mg/100 ml) in tube ‘S’ and 2 ml distilled water in tube ‘B’.

2. Add 1 ml chloranilic acid slowly and agitating constantly. Keep tubes on stand for 30 minutes and then centrifuge at 2000 rpm for 10 minutes in a clinical centrifuge; discard the supernatant and collect the precipitate on filter paper.

4. Transfer the precipitate into another tubes by washing with 6-7 ml isopropyl alcohol and resuspend in test tubes.

5. Centrifuge and discard the supernatant, suspend the precipitate in 0.1 ml distilled water.

6. Add 6 ml EDTA solution to each tube, apply stopper and shake vigorously.

7. Read the optical density at 520 nm in spectrophotometer by adjusting zero with blank; and calculate the calcium level in serum by following equation:

$$\text{Serum calcium (mg/100 ml)} = \frac{\text{OD of the test}}{\text{OD of standard}} \times \text{Concentration of the standard}$$

Interpretations

The increased serum calcium levels are known as hypercalcemia which is observed in hyperproteinemia, hyperthyroidism and after administration of vitamin D. The hypocalcemia, decreased serum calcium levels, is met with hypothyroidism, starvation, ketosis, milk fever, eclampsia and rickets.

In animals the normal serum calcium levels are given in Table 8.

Table 8: Serum calcium level in animals

Species	Calcium mg/dl
Sheep	10.4-13
Bovine	7.9-10.0
Horse	11.0-13.9
Cat	8.8-11.7
Dog	9.4-11.8

Determination of calcium by
Atomic Absorption Spectrometry

Principle

The calcium is directly determined in the liquid oenological product (or in the mineralisation solution) suitably diluted by atomic absorption spectrometry by air-acetylene flame after the addition of spectral buffer.

Apparatus

Instrumental parameters (given as an example)

- Atomic absorption spectrophotometer
- Reducing air-acetylene flame
- Hollow-cathode lamp (calcium)
- wave length: 422.7 nm
- width of slit: 0.2 nm
- intensity of the lamp: 5 mA
- No correction of non specific absorption.

Reagents

1. demineralised water

2. calcium reference solution at 1 g/l, commercial or prepared as follows: dissolve 5.8919 g of $Ca(NO_3)_2.4H_2O$ in a solution of HNO_3 0.5 M, adjust at 1 l with HNO_3 0.5 M.

3. calcium solution at 100 mg/l:

- place 10 ml of the reference solution in a 100 ml graduated flask and 1 ml of pure nitric acid.
- complete to volume with demineralised water

4. concentrated hydrochloric acid (R): 35% minimum

5. lanthanum solution at 25 g/l:

- weigh 65.9 g lanthanum chloride ($LaCl_3.6H_2O$) in a 250 ml cylindrical vase, transfer to a 1000 ml graduated flask with demineralised water; add to the test tube 50 ml of

concentrated hydrochloric acid (R); after solubilisation, allow to cool, complete to volume with demineralised water.

6. set of calibration solutions: 0, 2, 4, 6, 8 mg/l of calcium

- place successively 0, 1,0, 2,0, 3,0 and 4.0 ml of the solution at 100 mg/l of calcium in 5, 50 ml graduated flasks, add 10 ml of lanthanum solution at 25 g/l, complete to volume with demineralised water.

Preparation of samples

1. Case of liquid or solution oenological products

In a 50 ml graduated flask place 10 ml of the lanthanum solution and a volume of sample as after having being completed to volume with demineralised water; the concentration is below 8 mg/l.

2. Case of solid oenological products

Proceed with mineralisation by dry process;

Put in each solution of the set the same quantity of acid used for putting cinders in solution or mineralisation .

Take up cinders and 2 ml of concentrated hydrochloric acid (35% minimum) in a 100 ml flask; add 20 ml of lanthanum solution at 25 g/l and complete to volume with demineralised water.

Perform a blank test in the same conditions.

Procedure

Pass each solution of the set in ascending order of the concentration of calcium.
For each solution, perform 2 absorbance readings when they are perfectly stabilised integration time of signal: 10 seconds).
Pass each sample twice and calculate the calcium content.

4th session: Electrophoresis

Serum proteins

Principle

Serum protein electrophoresis (SPE) allows for separation of serum proteins into albumin, α1-globulin, α2-globulin, β1-globulin, β2-globulin, and γ-globulin fractions due to differential migration of these proteins through a matrix to which an electrical field is applied. Migration occurs at different rates based on the size and charge of individual proteins. Following electrophoretic separation, proteins are stained and proportions of each fraction can be determined from a densitometer tracing. SPE is most often performed to investigate an abnormal (generally elevated) globulin concentration.

Storage and Stability

If storage is necessary, samples may be stored covered at 2 to 8°C for 48 hours. Cerebrospinal fluid and urine specimens may be used after proper concentration with a concentrator.

Materials of protein electrophoresis

- Buffer: barbital with an ionic strength of 0.05 and pH 8.6
- Sample volume: 3 to 5 µl
- Power supply: 1.5 mA per 2-cm width of cellulose acetate medium; 10mA per 1-cm width of agarose medium
- Run time:40 to 60 min producing a 5- to 6-cm migration distance for albumin

Procedure

Fresh serum is the preferred specimen. The use of plasma should be avoided, as fibrinogen will appear as a distinct narrow band between the beta and gamma fractions. Cerebrospinal fluid may be used if concentrated approximately 100 times; urine may be used if concentrated up to 300 times, depending on original protein concentration.

- Patient's specimen is placed into a sample trough within agarose gel, is placed in an alkaline buffer solution
- a standardized voltage is applied and allowed to run for 1hr
- the agarose gel is processed in acetic acid and alcohol washes to fix the proteins in the agarose.

- the protein fractions are stained with Coomassie Brilliant Blue protein stain.
- After a second wash, fixed protein bands can be visualized and quantified with densitometry.
- In normal serum electrophoresis 5-6 bands are visible:
  - Albumin
  - Globulins: α1-, α2-, β-, and γ-

Visualization of the Protein Bands

1. At the end of the electrophoresis time, remove the plate(s) from the chamber. Place them in 40-50 mL of Ponceau S stain (sufficient volume to cover the plate(s) for 6 minutes. When staining 2 or more plates, carry out the protocol vertically in a rack. The stain may be reused until the plate background contains stain precipitate.

2. Destain in 3 successive 2 minute washes of 5% acetic acid or until the plate background is white. The plates may be dried and stored as a permanent record at this point if stored in a plastic envelope to protect the surface. If a transparent background is desired (i.e. for densitometry), proceed to the next step.

If using Clear Aid Solution:

3. Dehydrate by rinsing the plate in two absolute methanol washes for two minutes each wash. Allow the plate to drain for 5-10 seconds before placing in the next solution.

4 Place the plate into the clearing solution for 5-10 minutes.

5. Drain off excess solution. Then place the plate, acetate side up, onto a blotter, and into a drying oven at 50-60°C for 15 minutes or until dry.

If using PermaClear Solution:

3. Place the plate(s) into the diluted PermaClear clearingsolution for 2 minutes.

4. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetate side up, onto a blotter, and into a drying oven at 50-60°C for 15 minutes or until dry.

Evaluation of the Protein Bands

Scan the plates on QuickScan using a slit size of 5.

Stability of End Product

The completed, dried serum protein plate is stable for an indefinite period of time and may be stored in Titan Plastic Envelopes

Results

The fastest moving band, and normally the most prominent, is the albumin band found closest to the anodic edge of the plate. The faint band next to this is alpha1 globulin, followed by alpha2 globulin, beta, and gamma globulins. Prealbumin is seldom visible with this system.

5th session: Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is an important technique used for identification and separation of mixture of chemical compounds into its individual components.

TLC is a form of liquid chromatography consisting of two phases: **A mobile phase (liquid)** and **A stationary phase (solid)**. Differences in the interactions between the solutes and stationary and mobile phases enable separation.

Principle

TLC technique involves the distribution of components of a mixture to be separated between two phases.

• The components of the mixture are partitioned between an adsorbent (stationary phase), and a solvent (mobile phase).

• Different compounds will have different solubility and adsorption to the two phases between which they are to be partitioned.

• In TLC separation of the individual substances is based on their relative affinities towards stationary and mobile phases.

- The stationary phase: is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.

- The mobile phase: is a developing liquid which flows through the stationary phase, carrying the samples with it.

• Components with more affinity towards stationary phase travels slower.

• Components with less affinity towards stationary phase travels faster

Method

Adsorbents used as Stationary Phase:

- **Inorganic**: Silica Gel, Kieselguhr, Aluminium Silicate, Bentonite.
- **Organic:** Cellulose & its acetylates, Charcoal & activated Charcoal, Dextran Gel, Polyamides.

- **Solvents used as Mobile Phase:** - Petroleum ether, Benzene, Carbon tetrachloride.
- **Selection of Adsorbents and Solvents:**
  - ✓ Adsorbent should not adhere to glass plate.
  - ✓ Solvents should be of high purity.
  - ✓ Selected based on the nature of the compound to be separated (polar or non polar.)

Rf value

Rf value indicates the position of migrated spots on chromatogram.

In TLC the results are represented by Rf value which represents the migration of solute relative to the solvent front.

The Rf value is calculated as:

$$\text{Rf Value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

TLC - Technique

Step 1: Preparation of Slurry

- ✓ A plastic, glass or aluminum sheet is coated with a thin layer of silica gel (adsorbent).
- ✓ Plates must be dried, activated and stored in desiccator until used.

Step 2: Preparation of Tank

- ✓ Solvent mixtures should be freshly prepared for analysis.
- ✓ Solvent is poured down side of the tank (1.5cm depth).
- ✓ Tank is covered with the glass lid and kept for saturation.

Step 3: Application of Sample (Spot)

- ✓ A very small amount of sample (solution) to be analyzed is applied in a small spot with a capillary tube, 1cm from the bottom of the TLC plate.
- ✓ The TLC is developed in a chamber which contains the mobile phase (solvent).

- ✓ When the mobile phase rises up the plate up by capillary action, the components dissolve in the solvent and move.

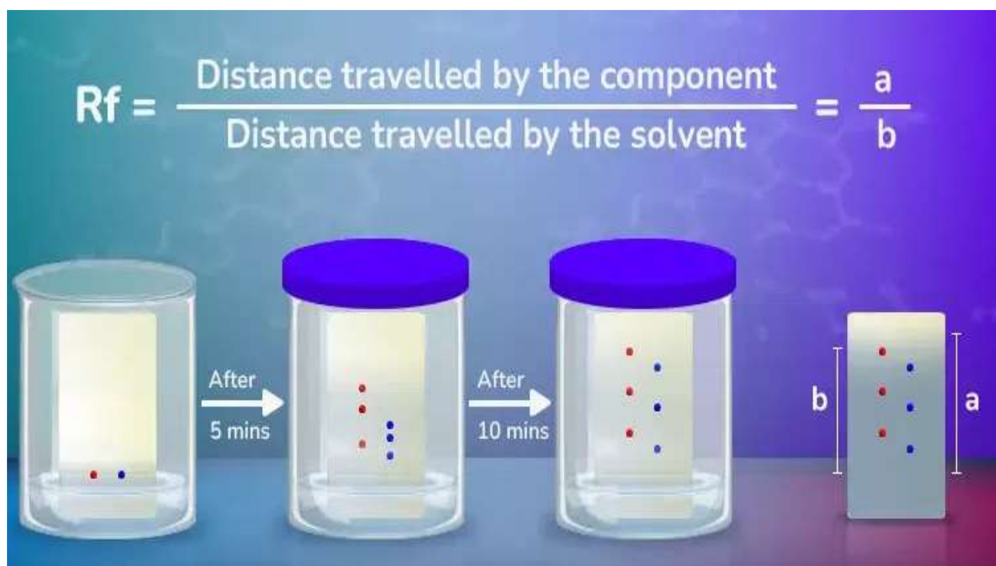
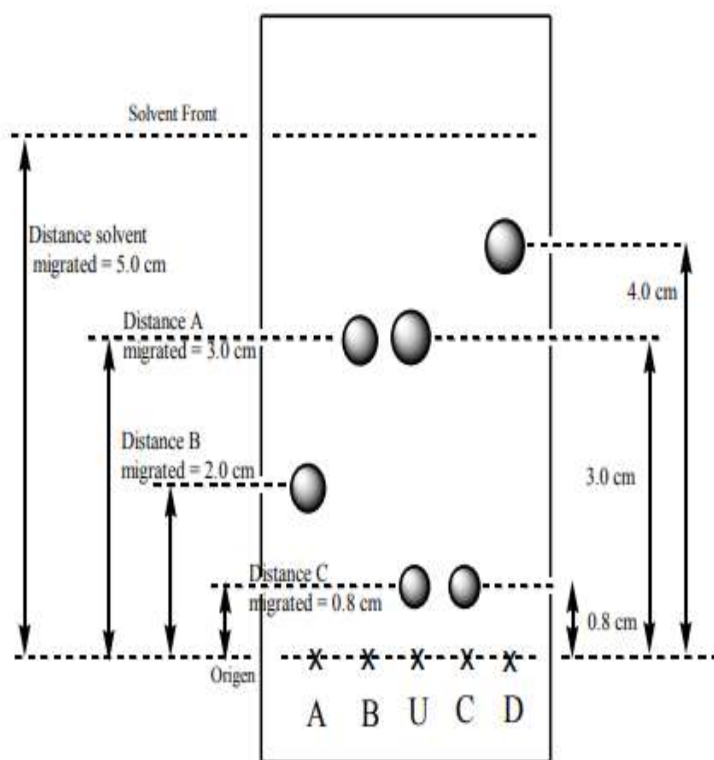


Figure 4: Steps of Thin Layer Chromatography (TLC)

- ✓ Individual components in the sample move up at different rates.
- ✓ More polar analytes interact more strongly with the stationary phase move very slowly up.
- ✓ More nonpolar analytes interact less strongly with the polar silica gel and more strongly with the less polar mobile phase move higher up.
- ✓ Once the solvent reaches the top (below 1-2 cm) of the TLC sheet the plate is removed from the developing chamber and position of solvent front is marked
- ✓ The solvent is allowed to evaporate from the TLC sheet.
- ✓ As the compound is colorless, it can be visualized by suitable methods.
- ✓ Lipids - Iodine vapors • Amino acids - Ninhydrin reagent.
- ✓ Also, manganese-activated zinc silicate (fluorescent compound), is added to the adsorbent that allows the visualization of spots under a black light (UV254 lamp).
- ✓ Once visible, the Rf value of each spot can be determined.

Calculation of Rf value

$$R_f = \frac{\text{Distance from centre of solute spot (cm) to the baseline}}{\text{Distance from solvent front to baseline (cm)}}$$



$$R_f(A) = \frac{2.0\text{ cm}}{5.0\text{ cm}} = 0.40$$

$$R_f(B) = \frac{3.0\text{ cm}}{5.0\text{ cm}} = 0.60$$

$$R_f(C) = \frac{0.8\text{ cm}}{5.0\text{ cm}} = 0.16$$

$$R_f(D) = \frac{4.0\text{ cm}}{5.0\text{ cm}} = 0.80$$

$$R_f(U_1) = \frac{3.0\text{ cm}}{5.0\text{ cm}} = 0.60$$

$$R_f(U_2) = \frac{0.8\text{ cm}}{5.0\text{ cm}} = 0.16$$

Results of TLC - Rf value

Qualitative results of TLC

- Expressed As Fractions Of 1.0
- Can Be Expressed From Rf Values (Ex: Rf X 100)
- No More Than Two Decimal Places

☞ Rf values can be used to aid in the identification of a substance by comparison to standards.

☞ Comparison should be made only between spots on the same sheet, run at the same time.

☞ Identical substances will have the same Rf value, whereas non- identical compounds will differ in their Rf values.

6th session: Radio Immunological Assays

Triiodothyronine (T3) & Thyroxine(T4)

An RIA requires the following: a sample containing the antigen of interest, a complementary antibody, and a radiolabelled version of the antigen. The sample antigen and antibody are incubated together, allowing the sample antigen to bind with the antibody. The radiolabelled antigen is then added. The radiolabelled antigen competes with the sample antigen and displaces it from the antibody. The more sample antigen present, the less the radiolabelled antigen is able to bind to the antibody. A secondantibody thatbinds theprimaryantibodycan thenbeadded, along with serum from the species of the primary antibody, to cause the solution to flocculate and allow for separation of the primary antibody from solution. Since solution containing antigen –antibody complex ismore dense than that containing free-antigen, centrifuging this mixture allows separation, resulting in a pellet containing the bound sample antigen/radiolabelled antigen. By measuring the radioactivity of the pellet, it is possible to determine the amount of radiolabelled antigen that has bound to antibody, and therefore the concentration of antigen in the sample . The drawbacks of RIA relate to the use of a radiolabel (usually [125I]) andhence short shelflife. These assaysdonot use enzymes and thus reduces the risk of interference from the sample itself.

Principle of Radioimmunoassay

It involves a combination of three principles.

- An immune reaction i.e. antigen, antibody binding.
- Acompetitive binding or competitive displacement reaction. (It givesv specificity).
- Measurement of radio emission. (It gives sensitivity).

The classical RIA methods are based on the principle ofv competitive binding:

- An unlabeled antigen competes with a radiolabeled antigen for bindingv to an antibody with the appropriate specificity.

- The amount of free (not bound to antibody) radiolabeled antigen isv directly proportional to the quantity of unlabeled antigen in the mixture.

Technique of Radioimmunoassay

- A mixture is prepared ofν radioactive antigen (“hot")
- Antibodies ("First" antibody) against that antigen.
- Known amounts of unlabeled ("cold") antigen are added to samples ofν the mixture. These compete for the binding sites of the antibodies.
- At increasing concentrations of unlabeled antigen, an increasingν amount of radioactive antigen is displaced from the antibody molecules.
- The antibody-bound antigen is separated from the free antigen in theν supernatant fluid, and the radioactivity of each is measured.
- From these data, a standard binding curve can be drawn.
- The samples to be assayed (the unknowns) are run in parallel.
- After determining the ratio of bound to free antigen ("cpm Bound/cpmν Free") in each unknown, the antigen concentrations can be read directly from the standard curve.

Separating Bound from free Antigen

- Precipitate the antigen-antibody complexes by adding a "second" antibody directed against the first. For example, rabbit IgG and anti rabbit-IgG.
- The antigen-specific antibodies can be coupled to the inner walls of a test tube and after incubation:
    - the contents ("free") are removed;
    - the tube is washed ("bound"), and
    - the radioactive of both is measured.
- The antigen-specific antibodies can be coupled to particles,

like Sephadex. Centrifugation of the reaction mixture separates the bound counts (in the pellet) from the free counts in the supernatant fluid.

Measurement of serum thyroxine (T4)

T4 concentration is measured in serum by RIA or enzyme, chemiluminescent, particle-enhanced, turbidimetric, or dry chemistry immunoassay systems. T4 concentrationin serum is stable for days at room temperature. Hemolysis, freezing, and thawing does not affect serum T4 concentrations. Despite stability, serum samples should be frozen and sent to the laboratory on cool packs. It must use an assay validated for the species being tested.

Measurement of serum 3,5,3-triiodothyronine (T3)

concentration is done during the T3 suppression test for hyperthyroidism in cats. Although theoretical indications for the measurement of serum T3 concentration are the same as those for serum T4, baseline serum T3 concentration offers little additional diagnostic information beyond that obtained with serum T4 concentration in identifying feline hyperthyroidism. It offers minimal to no value in assessing canine thyroid gland function, in part because the majority of circulating T3 comes from conversion of T4 to T3 in peripheral tissues. Measurement of serum T3 concentration is not currently recommended for assessment of thyroid gland function**.**

Table 9 : Serum T3 and T4 level in animals

	Units	**Dog**	**Cat**
T3	ng/dl	85-250	85-250
T4	µg/dl	1.2-3.0	1.2-3.0

7th Session: Urinalysis

A urinalysis (UA) is a basic diagnostic screening test that is easy to perform, relatively inexpensive, and can be useful in evaluating renal/urologic and non renal conditions.

The first step in performing a urinalysis is the proper collection of a urine sample, which must be carefully obtained to ensure accurate results. The analysis of urine samples should be performed only on samples taken before the administration of therapeutic agents. Urine specimens may be obtained via the natural voiding of urine, **bladder expression**, **catheterization**, or **cystocentesis**. The two preferred methods are cystocentesis and catheterization. These methods provide optimal samples for all aspects of urinalysis by avoiding contamination from the distal genital tract and external areas. Collecting samples by voiding or the expression of the bladder may be easier, but urine collected in these ways may be of limited diagnostic value. Except for cytologic examination, performing a urinalysis on preprandial morning samples is best, although not always practical, in veterinary patients. Morning samples tend to be the most concentrated and least affected by dietary factors, thereby increasing the chances of finding formed elements.

Physical properties of urine include all of the observations that may be made without the aid of a microscope or chemical reagents. The volume, color, odor, transparency, and specific gravity of urine are evaluated.

Testing for various chemical constituents of urine is usually performed with reagent strips that are impregnated with appropriate chemicals or with reagent tablets. There are some automated analyzers that are used for serum chemistry that can also be used for urine testing, although modifications of the procedures may be required. Many of those tests are performed concurrent with electrolyte testing. The container of reagent strips must be stored at room temperature with the lid tightly closed, and the expiration date should also be noted. Some reagent strips simultaneously test for numerous constituents, whereas other strips exist for individual tests. The reagent strip should be dipped into the sample so that it is fully immersed; it should then be removed and the long edge tilted on a paper towel to allow excess urine to be wicked away. Alternatively, urine can be added to the reagent strip from a pipette, making sure that each reagent pad is fully saturated. Color changes on each reagent pad are noted at specific time intervals. The concentration of various constituents is determined by comparing the colors on the strip with the color chart on the label of the strip container. The

manufacturer's directions must be carefully followed. It is important to note that a large number of conditions (e.g., medications, dietary factors, environmental factors) can affect urinalysis test results .

Equipment

Examination of urine's volume, color, clarity, and odor requires the least "supplies." You need only:

- exam gloves for handling biofluids
- a fresh sample of urine within a clean, clear collection container
- a clear conical centrifuge tube or alternate means of measuring urine volume.
- eyes to see
- nose to smell
- a white background against which to assess urine color
- graph paper or text on a white background against which to assess turbidity.

Volume

An accurate representation of urine volume should be assessed over a period of 24 hours. All voided urine is collected and measured to determine if the quantity produced is appropriate. When assessing urine volume, ensure consistency with the patient's regular routine. (i.e., regular diet and access to water; regular medications and/or supplements). Normal daily urine output can be calculated using a rate of 1mL/kg/h.

Terminology related to urine volume

Polyuria: Increased amounts of urine volume

Oliguria: Decreased amounts of urine volume

Anuria: No urine production.

Color

Normal urine is described as being straw colored. The pigment of the urine often correlates with the concentration of the sample, but this should still be confirmed with a SG reading (Table10)

Table 10 : Common urine colors and associated causes.

Straw	Normal
Colorless-pale	Dilute urine
Deep yellow	Concentrated urine; bilirubinuria
Orange-red	Hematuria; hemoglobinuria
Red-brown	Myoglobinuria
Milky white	Pyuria

Turbidity

Turbidity refers to the "cloudiness" of urine. An increase in turbidity in the urine can be caused by cellular elements, crystals, microorganisms, or mucous. When examining the turbidity of a sample, there are a few key points to remember :

- Ensure your sample is well mixed by inverting the container several times prior to examination. Items listed above that can cause an increase in turbidity will settle to the bottom of a standing sample.
- Assess turbidity on a room temperature sample.
- Examine the turbidity in a clear container of a standard depth. A good choice is a urine tube or sterile red top blood collection tube.
- Horses and rabbits typically have turbid urine. This is due to the presence of mucous and calcium carbonate crystals. Turbid urine is considered normal in these species.

To examine turbidity:

- With the sample in a clear tube, invert several times to ensure the sample is well mixed.
- Hold the sample up in front of a piece of paper with text on it.
- Assess how clear the lettering can be viewed through the sample.
- Record as clear (0), slightly cloudy (1), cloudy (2), or turbid (3).

Odor

The odor of urine can provide insight into possible conditions that should later be confirmed through chemical and sediment analysis.

Again, it is important to use standard terminology to minimize discrepancies among technicians:

Strong odor: The urine of intact male animals, especially cats and goats, is often quite

strong and should be described as such.

Ammonia: Ammonia-scented urine can be associated with cystitis. Some species of bacteria metabolize urea in the urine to ammonia, producing a distinguishing odor

The presence of bacteria should be confirmed during the sediment examination or with a urine culture.

Putrid: Putrid (or foul) smelling urine is associated with the degradation of protein. This can occur during infections, and should be confirmed with further testing.

Fruity: Increased ketones present in the urine give off a fruity odor. This odor may also be noticed on the patient's breath when they are in a state of ketosis.

Specific gravity (SG)

SG is determined by assessing the density of a liquid (in this case, urine) compared with the density of distilled water.

Measuring SG requires the use of a refractometer .

In a correctly calibrated refractometer, distilled water should read 1.000. Calibration should be checked regularly to ensure accuracy.

If the urine sample that is being tested exceeds the maximum limit of the refractometer, an accurate SG can be achieved by following these steps:

- Mix equal portions of urine with distilled water.
- Place a drop of this mixture on the refractometer and obtain the reading.
- Multiply the last two digits of the reading by 2 in order to obtain the estimated SG.

Terminology related to SG

Hypersthenuria: The SG of the urine is higher than the glomerular filtrate (dogs: >1.030; cats: >1.035).

Hyposthenuria: The SG of the urine is lower than the glomerular filtrate (<1.008)

Isosthenuria: The SG of the urine is equal to that of the glomerular filtrate (1.008–1.012).

Tips for accurate SG readings

- Ensure the sample is well mixed before reading.
- Ensure the sample is at room temperature if it was previously refrigerated.
- Practice good equipment maintenance.

Specific gravity readings using reagent strips

Many commercial reagent strips have a pad allotted to measuring the SG of a urine sample. This method of measurement differs from the refract meter method and has some significant drawbacks that make it unreliable for the veterinary practice.

- ➔ The principle of the test involves the use of a pH indicator to produce a degree of color change associated with a SG value. Urine samples with a pH of 6.5 or higher could result in erroneous findings.
- ➔ The highest SG reading the strip can measure is 1.030, which is below the capacity of dogs and cats.

Urine Chemistry

The chemical examination of urine is usually performed by the use of reagent strips. These are multiparameter strips that contain a number of pads, each designed to test for a specific urine constituent. There are individual tests available that can be used as well. Depending upon the constituent tested, these alternative methods may be more/less specific or may simply be useful as a confirmatory test. Whichever method is chosen, it is important to review the package insert and make note of the testing time required and any possible false positives or false negatives that can occur.

Collection

5–10 mL of urine collected by free catch of avoided sample, manual expression of the urinary bladder, catheterization, or cystocentesis

Handling

- Collect urine into a clean, dry container with a tight-fitting lid and free of potential contaminants (e.g., detergents, disinfectants). It should be labeled with appropriate information (e.g., patient and owner identification; date, time, and method of collection).
- Results are most accurate if urine is analyzed within 1–2 h after collection.
- The sample should be refrigerated if a delay in analysis is unavoidable.
- Refrigerated samples should be warmed to room temperature before analysis.

Storage

- Refrigeration can slow sample deterioration. The container should protect the sample from light and have a tight-fitting lid.
- Refrigeration may alter some results (e.g., increased crystal formation).
- Avoid freezing the urine because this will affect certain tests (e.g., urine sediment examination).
- Preservatives generally adversely affect chemical assays. No single preservative suits all testing requirements.

Stability

See specific chapters for information on individual components.

Protocol

- Mix the sample and transfer it to a centrifuge tube.
- Evaluate the physical features of the urine (i.e., color, turbidity).
- Determine the USG by using a refractometer.
- Measure urine analytes by using a reagent strip.
- Immerse a reagent strip into well-mixed, room-temperature urine and turn the strip on its side, tapping away excess urine.
- Examine the color change of each analyte at the time indicated by the manufacturer and compare the color change to the reference chart.
- Confirmatory tests are affected less by urine color and may be warranted to confirm specific dipstick analyte results. Such tests include the Ictotest (Bayer, Leverkusen, Germany) for bilirubin, the Acetest (Bayer, Leverkusen, Germany) for ketones, and sulfosalicylic acid test (SSA) for protein.
- Centrifuge the sample for 5–10 min at low RPM in a conical tube.
- Ideally, the same volume of urine should be centrifuged each time, and reference values are based on 5 mL of urine. Analysis of smaller urine volume should be noted on the final report because that can result in less sediment.
- The recommended RPM is quite variable (500–3,000 RPM) because it is actually gravitation force (g-force) that is important. A g-force of ≈ 450 is recommended.
- The g-force generated depends on the length of the centrifuge arms (radius of rotation), determined by measuring the radius of the centrifuge arm from center to outermost portion (bottom) of a test-tube cup (in the horizontal position if the cup

swings). See the Internet Resources section for a calculator that can be used to figure out the g-forces generated by your centrifuge at various RPM.

- Excessively high-speed centrifugation can distort cells or fragment fragile elements such as casts.
- Use urine supernatant to measure protein by the SSA method. If urine is bloody or extremely cloudy, double check the USG by using supernatant.
- Decant the supernatant, leaving ≈0.25 mL of urine sediment.
- Resuspend the sediment and transfer a drop to a microscope slide.
- Coverslip and examine the sample microscopically at 10× and 40×.

Interpretation

Normal findings or range

- Fresh urine should be relatively clear, yellow, and have a slight odor (Intact male cats may have a strong urine odor).
- The USG should be evaluated based on the hydration status. In general, the urine concentration from a random urine sample is considered adequate if the USG is >1.030 and >1.035 in dogs and cats, respectively.
- Normal urine should have a pH between 6.0 and 7.5, and should be negative for protein, ketones, blood, bilirubin, and glucose. Some dogs with highly concentrated urine may show a trace to 1+ reaction for bilirubin and/or protein.
- Urine sediment should contain few cells and crystals and no casts (lipid is seen occasionally in cat urine).

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- **PEDAGOGIE : POLYCOPIE**

Auteur : **ROUABAH Zahra**

Intitulé du polcopié : **Handout for Practical Work : Medical Biochimisty**

Vu les rapports favorables des expertrs chargés de l'évaluation du polycopié sus-cité, en l'ocurence :

-Dr. HAFIDHE Nadia - Université Batna 1
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Le conseil scientifique donne **un avis favorable** à l'utilisation de ce polycopié comme support pédagogique destiné aux étudiants de 4eme année Vétérinaire.

Le directeur adjoint chargé de la post graduation, de la recherche scientifique et des relations extérieurs

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والبحث العلمي والعلاقات الخارجية