UNIT: Minerals (Calcium, Magnesium, and Phosphorus)

Purpose

Discussion of clinical significance of calcium, phosphorus, and magnesium and serum determinations of calcium and phosphorus.

Objectives

Upon completion of this exercise, the student will be able to:

1. Determine calcium and phosphorus concentrations by colorimetric methods.
2. Explain the clinical significance of abnormal calcium, phosphorus, and magnesium values.
3. Discuss the most commonly employed automated and manual methods for determining calcium, phosphorus, and magnesium and be able to explain the principle of each method.

Introduction

By definition, electrolytes are ions capable of carrying an electric charge. Mineral ions such as calcium, phosphorus, magnesium, copper, zinc, etc., are also capable of carrying an electric charge and are therefore considered to be electrolytes.

Physiologically, these ions play a tremendous role in the metabolic processes of the human body. They can be found in all locations of the body, including tissue, fluid, and bone. In each area they function to promote and maintain normal body function with a wide range of activities including assisting in body hydration, enzyme activation, maintenance of pH, as cofactors in blood coagulation, controlling neuromuscular excitability, as well as promoting the stability of bones and teeth, and many other functions.

CALCIUM

1. Significance

Calcium is the most abundant mineral in the human body, most of which (98%) is present in the skeleton. One half of the remaining calcium is found in extracellular fluid and the rest in a variety of tissues. Calcium plays a very important role in skeletal mineralization, and it is also vital for basic physiologic processes such as blood coagulation, neuromuscular conduction, and for the maintenance of normal muscle tone.

Serum calcium determination is useful in monitoring certain clinical conditions such as acid-base balance, myeloma, renal failure, cirrhosis, and treatment with thiazide diuretics and hemodialysis.

The extracellular fluid concentration of calcium is controlled by parathyroid hormone (PTH), calcitonin, and vitamin D. PTH increases the rate of reabsorption of calcium in the kidneys and increases the resorption of calcium from bone and absorption from the intestines. Calcitonin decreases the reabsorption of calcium from bone and promotes its excretion in the kidneys.
In serum, calcium is present as:

a. non-diffusible protein-bound
b. diffusible (complexed)
c. physiologically active ionized form) (approximately 60%). Degree of ionization depends on pH.

**Increased serum calcium**

a. deposition of Ca salts in soft tissues (including the kidney)
b. fatigue, weakness, confusion
c. hyperparathyroidism, bone metastases and other malignancies (lung, kidney, bladder), hypervitaminosis D, sarcoidosis, multiple myeloma, acromegaly, and Paget's disease.

**Decreased serum calcium**

a. increases neuromuscular excitability
b. results in tetany or convulsions
c. as seen in hypoparathyroidism, vitamin D deficiency, malabsorption of vitamin D or calcium nephrosis, chronic renal disease, acute pancreatitis.

2. Methods of Determination

a. Ionized Calcium – (Ca^{2+}) – ion-selective electrodes (not practical)

b. Total Calcium

1) Titration (now obsolete) – Clark and Collip

   Ca is precipitated with oxalate and determined by redox titration with permanganate (MnO_4^-) or cesium. Alternatively, complexometric titration with EDTA using a fluorescent end-point indicator.

2) Colorimetric – Calcium in alkaline solution reacts with o-cresolphthalein complexone to form a purple complex proportional to the calcium concentration. Magnesium ions, which also react, are removed by 8-quinolinol or 8-hydroxyquinoline.

3) Atomic Absorption Spectrophotometry – Highly accurate, method of choice. (Flame photometry may also be used.)

**Procedure: Calcium Sigma #587 – Quantitative, Colorimetric, End-point**

**Principle of Reaction**

\[
\text{Calcium + o-Cresolphthalein Complexone} \rightarrow \text{Calcium-Cresolphthalein Complexone Complex}
\]

The reaction of calcium with o-cresolphthalein produces a purple/red complex at pH 10-12 with an absorbance maximum at 575 nm. The intensity of the color, measured at 575 nm, is directly proportional to calcium concentration in the sample.
Specimen Collection and Storage

Serum or heparinized plasma may be used. Prolonged contact with RBC should be avoided. 24 hour urine should be collected into 6N HCl.

Supplies and Reagents

1. Calcium binding reagent (o-cresolphthalein complexone with 8-hydroxyquinoline)
2. Calcium buffer
3. Calcium standard solution(s)
4. Controls and unknown sera
5. Linear and semiautomatic or micro pipets
6. 13 x 100 mm test tubes
7. Spectrophotometer capable of reading absorbance at 575 nm

Interfering Substances

1. Substances known to contain Ca
2. EDTA, citrate, oxalate, fluoride, and calcium heparinate, iodacetate
3. BSP
4. Extreme lipemia or hemolysis gives falsely elevated results

Calcium Procedure Steps

1. Prepare Calcium Assay Solution by mixing equal volumes of Calcium Binding Reagent with Calcium Buffer. (Stable 1 week, 2-6°C) Prepare only what you will use.

2. Appropriately label 13 x 100 mm test tubes for blank, standard(s), controls, and unknown samples.

3. Pipette 2.5 mL of Calcium Assay Solution (prepared in Step 1) to each tube.

4. Add 0.020 mL (20 µl) of each standard, control or serum to the appropriate tube, parafilm, and mix immediately.

5. Zero the spectrophotometer on DI water and read absorbances of the blank, standards, controls, and unknowns at 575 nm. Record results on worksheet provided. Complete readings with ten (10) minutes.

6. Subtract the blank’s absorbance from each of the other readings.

7. Plot the resulting absorbance readings of the standards on graph paper to form a calibration curve. Verify the linearity of the procedure.

8. Use the calibration curve to determine the concentration of the controls and unknowns.

9. Record results on sheet provided.
Procedural Notes

1. If calcium concentration is > 15 mg/dL, dilute specimen with equal volume of water and repeat assay. Multiply result by 2.

2. Urine calcium calculation

\[ \text{urine Ca mg/dL} \times \text{dilution factor} \times \frac{24 \text{ hr. vol. (in mL)}}{100} = \text{urine Ca mg/24 hr} \]

Normal Values

It is recommended that each laboratory establish an expected range characteristics for the local population.

- serum/plasma = 8.0 - 10.8 mg/dL
- urine = 50 - 300 mg/24 hours

PHOSPHORUS

1. Significance

Phosphorus is an essential mineral nutrient occurring as inorganic phosphate (oxidation states -3, +3, +5) \((\text{PO}_4^{3-})\) primarily in the bones and teeth, and as organic phosphate in high energy molecules such as ATP in muscle and neural tissues.

*Increased serum phosphorus*
- renal disease (especially failure), hypervitaminosis D, and hypoparathyroidism
- pyloric obstruction, starvation, hyperinsulinism, and during healing of fractures

*Decreased serum phosphorus*
- primary hyperparathyroidism, vitamin D deficiency, intestinal malabsorption, steatorrhea
- rickets, Fanconi syndrome (tubular reabsorption defect)
- myxedema, osteomalacia

2. Methods of Determination

a. Colorimetric – In the determination of inorganic phosphorus in blood, the method of Fiske and Subbarow is almost universally employed. Phosphate reacts with molybdate to produce phosphomolybdic acid. This product is reduced by the addition of sulfonic acid or other reducing agent, to produce a phosphomolybdium blue complex. The intensity of the color is proportional to the phosphate concentration.

b. ACA Phosphorus – The ACA phosphorus (PHOS) method is a modification of the classical phosphomolybdic acid, method introduced by Fiske and Subbarow and uses a mixture of p-aminophenol and bisulfite to reduce the phosphomolybdic acid. The PHOS method measures the absorption of the reduced phosphomolybdic complex at UV wavelengths to improve sensitivity. The 340 nm absorbance of the
reduced phosphomolybdate solution is proportional to the inorganic phosphorus concentration and is measured using a two-filter (340-383 nm) end point technique.

**Phosphorus Procedure**

**Principle of Reaction**

Serum is treated with trichloracetic acid to remove protein and lipid phosphorus. The supernatant fluid is reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulfite, sodium sulfate and 1-amino-2-naphthol-4-sulfonic acid (Fiske and Subbarow Reducer) reduces the phosphomolybdate to form a phosphomolybdenium blue complex as according to the reaction included. The intensity of the color is proportional to the phosphate concentration and is measured at 660 nm.

\[
\text{Phosphorus + Ammonium Molybdate} \rightarrow \text{ammonium phosphomolybdate} \quad \text{acid pH}
\]

\[
\text{Ammonium phosphomolybdate + aminonaphthol-sulfonic acid} \rightarrow \text{heteropolymolybdenum (blue)}
\]

**Supplies and Reagents**

1. Acid molybdate solution
2. Fiske and Subbarow Reducer (NOTE: cannot be make up in its bottle, must transfer to another container.)
3. Trichloracetic acid, 20% (w/v); (made from 100% using the V, C, formula.)
4. 13 x 100 mm test tubes
5. 0.5, 1.0, and 5.0 ml serologic pipets
6. Standard, controls, and unknown samples
7. Spectrophotometer capable of reading at 660 nm

**Specimen Collection and Storage**

Fasting, fresh unhemolyzed serum or heparinized plasma are the specimens of choice. Once separated from RBC, phosphorus is stable at 2-6°C for one week. Plasma collected in oxalate, EDTA and citrate will yield lower results. Urine for 24 hour collection should be preserved with HCl acid.

**Interfering Substances**

1. Hemolysis releases a large amount of organic phosphorus which may convert to inorganic phosphorus

2. Glassware should be thoroughly rinsed to remove traces of phosphorus-containing detergents

3. Grossly lipemic specimens should not be used
Manual Phosphorus Procedure

1. If 24 hour urine is to be tested, a 10 fold dilution must be made prior to testing and results multiplied accordingly.

2. Preparation of a protein free filtrate on control and patient serum samples.
   a. pipet 0.5 mL serum or plasma, control samples, and diluted urine samples into respectively labeled tubes
   b. add 2.5 mL DI H₂O
   c. add 2.0 mL TCA acid (20%)
   d. stopper and shake vigorously
   e. let stand 5-10 minutes

3. Centrifuge until clear (10 minutes).

4. Label an appropriate number of tubes for blank, standards, controls and unknowns.

5. Pipet as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA acid (20%)</td>
<td>2.0 ml</td>
<td>–</td>
</tr>
<tr>
<td>filtrate</td>
<td>–</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>DI H₂O</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>acid molybdate solution</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

6. Mix gently.

7. Add to each tube 0.25 ml Fiske and Subbarow solution. Mix by inversion.

8. Let stand at room temperature for 10 minutes.

9. Read absorbance of tests against the blank at 660 nm. Record readings/results on worksheet provided.

10. Determine the concentration of unknowns using a calibration curve.

Procedural Notes

1. Complete absorbance readings within 10 minutes. Linearity – to 12.5 mg/dL.

2. Urine phosphorus
   a. Make a 10-fold dilution of a 24-hour urine specimen. (1 part urine to 9 parts deionized water)
   b. The diluted urine is handled the same as serum. (It must be deproteinized.)
c. Urine phosphorus calculation.
   1) Value from curve x 10 (dilution factor)
   2) \( \frac{\text{Inorganic } P \ (\text{mg/dL}) \times 24 \ 	ext{hr urine vol. (mL)}}{100,000} \)
      = Urine Inorganic P (g/24 hr)

Normal Values

<table>
<thead>
<tr>
<th></th>
<th>Adults</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum</td>
<td>2.5-4.8 mg/d</td>
<td>4.0-7.0 mg/dl</td>
</tr>
<tr>
<td></td>
<td>0.8-1.6 mmol/L</td>
<td>1.3-2.3 mmol/L</td>
</tr>
<tr>
<td>urine</td>
<td>0.34-1.0 g/24 hr</td>
<td>0.53-0.84 g/24 hr</td>
</tr>
<tr>
<td></td>
<td>11-32 mmol/24 hr</td>
<td>17-27 mmol/24 hr</td>
</tr>
</tbody>
</table>

MAGNESIUM

1. Significance

Magnesium (Mg\(^{2+}\)) is an essential dietary mineral. About 35% of the body's magnesium is in cells, 64% in bones, and approximately 1% in extracellular fluid. Magnesium is an activator in the ATP phosphate transfer reactions and other physiological reactions.

*Increased serum magnesium*

a. after ingestion of magnesium sulfate, epsom salts
b. impaired renal function (uremia)

*Decreased serum magnesium*

a. malabsorption or excessive loss (i.e., chronic renal disease, pancreatitis, LE, hyperthyroidism)
   b. may result in muscular weakness, tremor, vertigo, cardiac arrhythmia, tetany, and convulsive seizures
   c. is seen in alcoholic delirium tremors

2. Methods of Determination

a. Atomic Absorption Spectrophotometry – most accurate and reliable assay.

b. Fluorometry – Mg chelated with 8-hydroxyquinoline-5-sulfonic acid which fluoresces in acid solution. Obsolete.

c. Colorimetry – Mg forms a colored complex with methylthymol blue (MTB). Ca chelators are added to reduce interference from Ca. The amount of Mg-MTB complex formed is proportional to the Mg concentration. Alternately, colored complexes may be formed with calmagite
3. Procedural Notes
   a. Magnesium assays are performed on serum or urine.
   b. Known Interfering Substances
      1) Extremely high calcium concentrations.
      2) EDTA, Na Fluoride, Na oxalate, or Na citrate anticoagulants cause significant negative interference

**Magnesium - Quantitative, Colorimetric Determination**

**Principle of Reaction**

The metallochromic dye, calmagite, 1-(1-hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid offers one of the best approaches of a dye technique used for the determination of magnesium. The unmetallized form of the dye in the presence of magnesium forms a pink complex that can be measured at 520 nm.

\[
\text{Calmagite + Magnesium} \rightarrow \text{Calmagite-Magnesium Complex}
\]

**Reagents**

Check product insert for details.

**Supplies**

The usual controls, unknown sera, linear and semiautomatic or micro pipets, test tubes of appropriate size, and a spectrophotometer capable of reading at the designated wavelength.

**Specimen Collection and Storage**

Serum or heparinized plasma may be used. Hemolysis should be avoided since red cells contain much higher levels of magnesium than serum or plasma. Specimens should be spun and serum/plasma separated within 30 minutes after collection. Magnesium levels remain unchanged for several days when samples are stored refrigerated.

**Interfering Substances**

1. Certain drugs and other substances are known to influence circulating magnesium levels.
2. Hemolysis

**Linearity**

By most products, the magnesium reagent is linear to 3.0 mEq/L. Concentrations greater than 3.0 mEq/L should be diluted with deionized water, test repeated, and calculated accordingly.

**Magnesium Procedure Steps**

Due to frequent changes in products/kits available to this lab, it is necessary to evaluate the current product insert for the following information.

1. Reagent Preparation
2. Number and size of test tubes required
Minerals Worksheet

Name_______________________________

I. Calcium Sigma # 587

Wavelength _____________ Linearity _____________ Spectrophotometer Used ______________________

<table>
<thead>
<tr>
<th>Identification</th>
<th>Absorbance</th>
<th>Abs$<em>\text{test}$ - Abs$</em>\text{blank}$</th>
<th>Concentration (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
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<tr>
<td>Standard</td>
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<td>Control 1</td>
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<tr>
<td>Control 2</td>
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</table>

Calculation formula(s) and examples

<table>
<thead>
<tr>
<th>Quality Control</th>
<th>Your Results</th>
<th>Controls’ range of expected results.</th>
<th>In control? Yes / No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1 ID</td>
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<td></td>
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<tr>
<td>Level 2 ID</td>
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</tbody>
</table>

Accepting Patient Results? Reason

II. Magnesium Procedure _______________

Wavelength _____________ Linearity _____________ Spectrophotometer Used ______________________
### Magnesium calculation formula(s) and examples

<table>
<thead>
<tr>
<th>Identification</th>
<th>Absorbance</th>
<th>$\text{Abs}<em>{\text{test}} - \text{Abs}</em>{\text{blank}}$</th>
<th>Concentration (units)</th>
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<td>Control 2</td>
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### Quality Control

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<th>Controls' range of expected results.</th>
<th>In control?</th>
<th>Reason</th>
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<tbody>
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<tr>
<td>Level 2 ID</td>
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<tr>
<td>Accepting Patient Results?</td>
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### III. Phosphorus Sigma # 670

<table>
<thead>
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<th></th>
<th>Identification</th>
<th>Absorbance</th>
<th>Concentration (units)</th>
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</thead>
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<tr>
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<td>Control 2</td>
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### Phosphorus calculation formula(s) and examples
Quality Control

<table>
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<th>Your Results</th>
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<tr>
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<td>Level 2 ID</td>
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<tr>
<td>Accepting Patient Results?</td>
<td></td>
<td>Reason</td>
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</tbody>
</table>

Grading Notes

Labs are graded on attractiveness and format of required curves as well as actual results obtained. Points are deducted for reporting errors, out of range results, and messy or omitted results. You should show calculations when appropriate.

Name

Date

Study Questions

Instructions: Legibly write your answers in the space provided. Unless otherwise indicated, each question is worth one point. Using lecture notes, reading assignments and information presented in this lab, answer the following questions.

1. Complete the following for 6 points.

<table>
<thead>
<tr>
<th>Hormone involved in Ca(^{2+}) regulation.</th>
<th>Where it is produced.</th>
<th>Role it plays in regulation.</th>
</tr>
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<tbody>
<tr>
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</tbody>
</table>

2. What vitamin is directly involved in the regulation of calcium blood levels?

3. Where is the majority of total calcium deposited in the body?

4. What is the most commonly employed chromagenic agent in calcium determinations?

5. In the above methodology, what is the purpose of adding 8-hydroxyquinoline?

6. List two (2) conditions that often result in low serum calcium levels. What symptom(s) result? (2 points)
7. List at least two (2) conditions that result in high serum calcium levels. State two (2) or more possible side effects.

8. List the three (3) forms of calcium that can be found in the plasma. Indicate which is/are biologically active. (3 points)

9. What are two (2) functions of the biologically active form of calcium? (2 points) Accept 2

10. What are the physiological uses of phosphorus and magnesium? List two (2) for each. (1 point each for A and B)
   A. phosphorus –
   B. magnesium –

11. List the hormones that regulate plasma levels of phosphorus.

12. List the three (3) reagents used in the phosphorus procedure. What is the purpose of each? (3 points)

13. What is the method of choice for magnesium determination?

14. A specimen for magnesium assay is drawn on the floor at 4 a.m. and is delivered to the lab at 6:30 a.m. No hemolysis is noted after spinning the sample. How might the collection and handling affect the test results?