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Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline—Second Edition

This document addresses preanalytical considerations, such as patient condition, specimen choice, collection, and handling—that can influence the accuracy and clinical utility of ionized calcium measurements.

A guideline for global application developed through the NCCLS consensus process.

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Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline—Second Edition

Abstract

Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline—Second Edition (NCCLS document C31-A2) is a guideline for specimen collection for ionized calcium determinations. The primary audience for this publication is personnel responsible for ionized calcium determinations. This document discusses the reasons for *in vivo* (nonpathologic) and *in vitro* changes in ionized calcium concentrations, and it presents recommendations for avoiding or minimizing these effects.

NCCLS. *Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline—Second Edition*. NCCLS document C31-A2 (ISBN 1-56238-436-8). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA 2001

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Contents

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Foreword

Ionized calcium determinations have proven to be clinically useful in the differential diagnosis of calcium disorders of endocrine origin, identification of hypercalcemia in various neoplasias, and managing the critically ill adult and neonatal patient. However, it is the responsibility of the laboratorian to choose which specimen is most appropriate for each clinical situation and how to collect and handle that specimen to ensure accuracy and clinical utility. This choice is complicated by the equilibrium between free (ionized) and bound calcium in blood, which is influenced by alterations in hydrogen ion and/or ligand concentrations. This guideline is designed to aid the laboratorian in determining the most appropriate specimen and its proper handling for each specific purpose.

Specifically, C31-A2 offers guidance in recognizing preanalytical factors that can affect ionized calcium determinations. The influence of patient conditions (e.g., physical activity, posture, meals, ventilation rate, and circadian variation) is considered in Section 5, while the advantages and disadvantages of whole blood, serum, and plasma are discussed in Section 6. The guideline also describes the selection of the collection site and device in Section 7. In Section 8, appropriate transportation, processing, and storage procedures are recommended.

References to pH-adjusted ionized calcium results are found throughout the guideline, and appropriate citations are provided.

Key Words

Ionized calcium, pH, preanalytical conditions, precollection variables, specimen choice, specimen collection, specimen transportation

Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline—Second Edition

1 Introduction

Ionized calcium is widely recognized as a better indicator of physiological calcium status in blood than total calcium. Generally, the reasons for measuring ionized calcium can be divided into three clinical categories: monitoring trends in acute or critical care, routine diagnostic care, and research. Generally, ionized calcium measurements for diagnostic purposes or research purposes require a high degree of accuracy.

This document describes the preanalytical variables for ionized calcium determinations and makes recommendations for minimizing the effects of these variables on the accuracy of ionized calcium measurements. Patient preparation and specimen handling options are presented, as well as the advantages and disadvantages of the various choices for specimen type, collection device, and technique. Recommendations are offered in each section.

2 Scope

This document addresses the preanalytical variables that can influence the accuracy and clinical utility of ionized calcium measurements.

3 Standard Precautions

Because it is often impossible to know which specimens might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80.), [MMWR 1987;36(suppl 2S):2S-18S] and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

4 Definitions[a]

Circadian variation/chronobiological variation, diurnal variation, *n* – Variations in physiological parameters, including blood analyte concentrations, which are related to cyclic events, i.e., time of day, season of the year, and ingestion of meals.

Ionized calcium, *n* – The portion of calcium ions in the plasma water of whole blood that is not bound by protein or other molecules; **NOTE:** This parameter has also been called "free" or "ionic" calcium.

pH-adjusted ionized calcium, *n* – A calculated result empirically based on a measured pH and ionized calcium concentration, with the ionized calcium concentration normalized to a pH of 7.40; **NOTE:** These

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

calculations exclusively compensate for *in vitro* increases in pH due to the loss of CO_2 and, therefore, help compensate for specimen handling errors. The pH-adjusted ionized calcium is included as an option in many of the commercial instruments currently available.

Plasma, *n* – The liquid part {of whole blood} remaining after the separation of the cellular elements ... in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure.

Preanalytical variables, *n* – Events or circumstances that can alter the concentrations of analytes in a blood specimen before the actual measurement; **NOTE:** These can include patient preparation, specimen collection technique, specimen storage and transportation, and specimen handling.

Serum, *n* – The liquid remaining after treated whole blood has coagulated; **NOTE:** Observable after the clot and/or coagulum has retracted and/or has then been spun down in a centrifuge to separate the coagulum and cells from the liquid portion.

Skin puncture, *n* – Collection of capillary blood by producing a break in the skin in an area of the body with a high density of capillaries, e.g., fingertip or heel.

Titrated heparins, *n* – Typically, an aqueous or dry preparation of heparin salt, prepared with a fixed ratio of calcium to heparin, which minimizes changes to the concentration of ionized calcium, typically at 1.25 mmol/L; **NOTE:** When using these preparations to anticoagulate whole blood, the measured ionized calcium is unchanged, provided the calcium ion concentration of the blood specimen is at or near the concentration of calcium used in the preparation. Mixtures of lithium and zinc heparin are also currently used to limit calcium chelation by heparin.

Total calcium, *n* – The entire calcium concentration in plasma, including ionized calcium and calcium bound to proteins or other molecules, such as phosphate, bicarbonate, lactate, and citrate.

5 Precollection Variables: Influences of Physical Activity, Posture, Meals, Ventilation Rate, and Circadian Variation

While these variables can significantly alter the ionized calcium concentration under extreme conditions, they have a modest to insignificant effect when monitoring critically ill patients. However, because a high degree of accuracy is needed for ionized calcium measurements to diagnose a calcium disorder, these variables should be controlled to minimize variation in ionized calcium that is not related to the disease being investigated.

5.1 Effect of Physical Activity

The effect of moderate exercise on ionized calcium has been studied in persons during bicycling[1,2] and stair walking.[3] The mean increases reported in ionized calcium are 0.11 mmol/L after 10 minutes of bicycling,[1] 0.05 mmol/L after 10 to 15 minutes of bicycling,[2] and 0.02 mmol/L after 10 minutes of stair walking.[3] These changes appear to be related to changes in other constituents during exercise, e.g., decreased pH and bicarbonate and increased lactate, albumin, and total calcium.

5.2 Influences of Posture and Prolonged Bed Rest

While a change in posture has a proportionately greater effect on protein and protein-bound molecules, posture also affects the concentration of lower molecular weight ions. For example, as subjects change from a supine (lying) to a standing position, the following increases occur: ionized calcium 1.7%, total calcium 4.6%, hydrogen ion 2.9%, and albumin and total protein 12%.[4] Therefore, posture apparently has

less effect on ionized calcium than on total calcium. These changes are presumably from extravascular shifts of plasma water due to both increased muscle tone and hydrostatic pressure. These acute changes are apparently rapidly reversible when subjects return to their original posture.[4]

Long-term (12-day) bed rest appears to increase ionized calcium by 8% without affecting total calcium.[5] Hypercalciuria results from the kidney's response to the elevated ionized calcium concentration.

5.3 Effect of Meals

Eating a meal is reported to temporarily decrease serum ionized calcium by 5.4%.[6,7] Several factors can play a role in causing this decrease: 1) an increase in pH; 2) an increase in protein concentration; and 3) an increase in bicarbonate and phosphate concentrations. These factors all contribute to increased formation of calcium complexes with albumin and other anions.

5.4 Ventilation Rate

Respiratory alkalosis induced in normal volunteers by hyperventilation is reported to decrease serum-ionized calcium by 0.05 mmol/L for each 0.1 unit increase in pH.[7]

5.5 Circadian Variation

Ionized calcium varies significantly with the time of day, with changes reported ranging from 4 to 10%.[8,9] These changes could be due to the effect of meals,[6,8] daily variation in acid-base balance,[10] and sleep. Because a study has also shown significant differences in the circadian variation of ionized calcium between the sexes,[11] hormonal variations might also have an influence.

5.6 Recommendations

Especially when determining ionized calcium for diagnosing a disorder in calcium metabolism, the following practices are recommended:

- Have the patient relax and breathe normally for at least ten minutes.
- Have the patient sit or lie for at least five minutes before collecting blood.
- Ensure that the patient has not eaten for at least four hours.
- Collect specimens under consistent conditions.

6 Specimen Choice

The patient's clinical status should primarily influence the selection of the specimen type for specimens requiring ionized calcium measurements. *Heparinized whole blood* can be the most appropriate choice in acute and critical care circumstances that require immediate results. *Anaerobically collected serum* can be the best choice for routine diagnostic and research applications. In cases where the patient's circumstances do not dictate the specimen selection, the following information provides guidelines for determining the most appropriate specimen for the clinical application.

6.1 Whole Blood

While most anticoagulants bind calcium, heparin is usually considered acceptable for ionized calcium determinations, because its small degree of calcium binding can be controlled either by using a low

heparin concentration or by using titrated heparins with calcium or other ions such as zinc. See Appendix A for a detailed discussion of heparin anticoagulants.

6.1.1 Advantages of Using Heparinized Whole Blood

Following are some advantages of using heparinized whole blood:

- The entire volume of the specimen is usable for analysis.
- The specimen is available for analysis immediately after collection.
- Rapid analysis minimizes the effects of cellular metabolism on the specimen.
- Other analytes, such as blood gases and potassium, frequently needed at the same time as calcium, may be measured simultaneously on the same sample on the same analyzer.

6.1.2 Disadvantages of Using Heparinized Whole Blood

Following are some disadvantages of using heparinized whole blood:

- The heparin binds calcium ions in proportion to its concentration (see Appendix A), possibly unacceptably reducing the measured concentration of ionized calcium. Because all commercial heparinized specimen collection devices are designed to anticoagulate a nominal volume of blood, incomplete filling of the blood-collection device will result in a higher concentration of heparin in the blood, which may alter the result.
- Whole blood does not store as well as serum because of continued cellular metabolism. Prolonged delays before analysis can change the ionized calcium concentration.
- Hemolysis in whole blood is not readily detectable and can artificially decrease the measured ionized calcium.[12,13] Hemolysis greater than 300 mg/dL hemoglobin causes clinically significant changes in ionized calcium.
- Improperly heparinized and/or mixed specimens can form microclots, which can cause instrument downtime.

6.2 Serum

Serum, anaerobically collected in evacuated tubes, is the most stable specimen for ionized calcium determinations. However, a partially filled evacuated tube will cause pH and subsequent ionized calcium changes due to the loss of CO_2 from the specimen.[12,14] If tubes are at least half full, calcium values are altered by <0.03 mmol/L.[12] The ionized calcium result is stable in half-filled tubes for up to four hours. Concentration of ionized calcium decreases upon exposure to air.

6.2.1 The Advantages of Using Serum

Following are some advantages of using serum:

- The specimen contains no anticoagulants to bind and alter the ionized calcium concentration.
- The specimen is suitable for a variety of tests regularly performed in clinical chemistry laboratories, for example, enzymes and creatinine.

- The specimen is stable for well over 24 hours when anaerobically stored at 4 ºC.[15]

- Hemolysis is readily detected.[12,13]

6.2.2 The Disadvantages of Using Serum

Following are some disadvantages of using serum:

- Analysis is delayed, because serum requires 20 to 30 additional minutes for the blood to clot and for serum exudation, which can be accelerated by centrifugation.

- While cellular metabolism continues during clotting and centrifugation, the effect on ionized calcium is variable in most specimens[16, 17]

- The volume of serum obtained is only about one-half of the amount of blood actually drawn.

- The final ionized calcium concentration and pH are influenced by the temperature of centrifugation.[12,18] Ionized calcium decreases with decreasing centrifugation temperature.

6.3 Plasma

Plasma has no analytical advantages over serum or whole blood. As with whole blood, the binding of calcium, adequacy of mixing, and storage temperature and time should be considered. Plasma derived from evacuated tube collection is unacceptable, because the ionized calcium is influenced by both heparin binding and the completeness of tube filling. As with serum, only half of the volume of the collected specimen is available for analysis, and centrifugation time and temperature can change the final result.

6.4 Recommendations

6.4.1 Whole Blood

When using a whole blood specimen, the following recommendations apply:

- Use modified heparin preparations, such as balanced heparin titrated with calcium or other ions, or, if necessary, use sodium or lithium heparin with concentrations less than 10 IU/mL. (See Appendix A.)

- Although not recommended, if evacuated tubes must be used for ionized calcium determinations, they should be filled to their nominal volume and kept sealed until analysis. This minimizes the heparin concentration and loss of carbon dioxide.

- Thoroughly mix the specimen after collection to distribute the heparin and minimize the formation of microclots.

- Analyze the specimen within 30 minutes after collection or chill the specimen in an ice-water slurry to prevent metabolic changes.

- Handle the specimen anaerobically.

- If samples are received with an unknown heparin source or concentration, either do not accept the specimen or note this in the report.

6.4.2 Serum

When using a serum specimen, the following recommendations apply:

- Fill evacuated tubes completely.
- Handle the specimen anaerobically; if multiple tests are requested, analyze ionized calcium first. If serum has been exposed to air for over ten minutes, the pH-corrected ionized calcium should be reported (see Appendix C).
- Note if hemolysis is present. Severe hemolysis lowers the ionized calcium result.[12,13]

6.4.3 Plasma

Plasma is not recommended.

7 Specimen Collection

The choice of a collection device and technique can have a significant effect on the quality of ionized calcium measurements. It is important that laboratory personnel develop a clear understanding of these variables and define a routine for collecting ionized calcium specimens that will minimize their effect. (Refer to Appendix B for more information on specimen type.)

7.1 Collection Site Selection

The site of blood collection does not appear to influence the ionized calcium result. Recommendations for the selection of the collection site for arterial, venous, and capillary blood are contained in NCCLS documents H3— *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture;* H4— *Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture*; and H11— *Procedures for the Collection of Arterial Blood Specimens.*

7.2 Collection Devices

7.2.1 Syringes

The choice of syringes should be primarily dictated by their ease of use, convenience, and cost. While glass syringes can maintain the integrity of the specimen best, several studies show that no significant change occurs in pH (and, therefore, ionized calcium) during the first three to four hours after specimen collection for blood collected in plastic syringes.[19]

7.2.2 Evacuated Tubes

The difference between plain and "gel separator" serum tubes for ionized calcium and pH are clinically insignificant if manufacturer's instructions for use of tubes are followed; either may be used for ionized calcium measurements.[12,15,20] The possible contamination of a plain evacuated tube with anticoagulants from other tubes makes it necessary that serum or tubes without additives be collected first.[21]

7.2.3 Capillary Tubes

Although the use of capillary tubes for ionized calcium has been questioned because of "microclot" formation,[22] several studies have shown that this specimen is appropriate.[23,24] Many commercially available, heparinized capillary tubes contain too much heparin for ionized calcium measurements.

Capillary tubes with "calcium-titrated" heparin (see Appendix A) minimize this binding. However, in the neonate, because of a certain hypercoagulability, the addition of liquid heparinate is recommended when using these tubes.[25,26] In any case, proper mixing as quickly as possible after the sample is drawn is important when capillary tubes are used.

7.3 Collection Techniques

7.3.1 Using Syringes

Detailed instructions for collecting blood with syringes are contained in NCCLS documents H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* and H11—*Procedures for the Collection of Arterial Blood Specimens*. Special care should be taken when collecting blood from indwelling catheters or other similar devices to avoid contamination with intravenous fluid or heparin solutions.[27] In addition, blood collected in syringes and subsequently dispensed into evacuated tubes can be contaminated with the additives in these tubes if care is not taken. Failure to fill heparinized syringes to their nominal volume results in proportionately higher concentrations of heparin and can alter ionized calcium concentrations.[28]

7.3.2 Using a Tourniquet to Collect Blood

Ionized calcium is reportedly not affected by stasis (up to three minutes), although significant changes occurred with forearm exercise when a tourniquet was in place.[28] If the tourniquet is removed before completely filling the collection device, the ionized calcium will be significantly affected.[29]

7.3.3 Capillary Blood Collection

Skin capillaries are often the only convenient sites for blood collection in the neonate. The amount of blood flow to the capillary bed can vary significantly. This variation is likely to cause differences in the blood pH and, therefore, in the ionized calcium. Arterialization of the capillary bed can avoid this variation. Collect blood from the center of the droplet formed after puncture. See NCCLS document H4—*Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture,* for more detailed instructions. In capillaries, dissolution of heparin is enhanced by the use of a magnetic mixing device ("flea"); however, excessive mixing can cause hemolysis.

7.4 Recommendations

When collecting the blood specimen, consider the following:

Use an appropriate blood collection syringe or tube:

- Use only syringes containing heparin specifically formulated to minimize effects on ionized calcium measurements (see Appendix A).
- Fill syringes to their nominal volume.
- Use either plain or "gel-separator" evacuated tubes for obtaining serum (fill completely).
- If a series of tubes must be collected, fill the serum tube first.
- Use "calcium-titrated" heparin capillary tubes. Mix the blood and heparin well but not excessively.

Use appropriate collection techniques for all specimens:

- When drawing blood from a catheter, first remove and discard between three to seven times the catheter's (and connector's) internal volume.[30]

- When using a syringe and catheter to collect blood for serum measurements, dispense the blood into the plain or serum tube first.

- Leave the tourniquet on until all of the blood required is withdrawn [but not longer than three minutes and do not allow the patient to exercise the forearm (i.e., make a fist)]. Release the tourniquet before removing the needle.

- Arterialize the capillary bed by warming the area to 42 ºC before puncturing the skin. Collect the blood from the center of the formed drop.

8 Specimen Transportation, Processing, and Storage

The stability of ionized calcium in transported or stored specimens is affected by several factors: temperature, time, exposure to air, and cell content. "Transportation" refers to storage of the unprocessed specimen while "storage" refers to the time between processing and analysis. Note that the stability of ionized calcium in stored blood is a dynamic process, with calcium ions being released from albumin by the decrease in pH on one hand, while being chelated by an increase in lactate ions on the other.[15] These changes are the result of continued cellular metabolic activity.

NOTE: This interaction accounts for most of the overcorrection of the pH 7.4 calculations whenever the pH falls due to metabolic activity (see Appendix C) and should be kept in mind when transporting, processing, and storing specimens.

8.1 Anticoagulated Whole Blood in Syringes

One study reports no change in ionized calcium, pH, or pH-adjusted ionized calcium in whole blood kept at 4 ºC for four hours.[31] If ionized calcium is adjusted to pH 7.4, whole blood can be stored at 4 ºC for 24 hours without significant change.[32] However, whole blood kept at either 20 ºC or 37 ºC for four hours showed significant changes due to glycolysis and lactic acid formation.

8.2 Serum

8.2.1 Uncentrifuged Specimens in Evacuated Tubes

One study reports no significant change in ionized calcium, pH, or ionized calcium (pH 7.4) in blood collected in commercially available evacuated tubes when stored at room temperature for up to three hours.[15] With longer room temperature storage, ionized calcium remained unchanged at four hours, but by six hours it showed increases of 0.050 mmol/L or more. The pH-adjusted ionized calcium, however, did not change detectably even at six hours. In addition, a study evaluated plain and heparinized evacuated tubes[12] that remained unprocessed at room temperature for four hours and reported greater changes in ionized calcium in the anticoagulated tubes (+0.030 mmol/L) than in the plain tubes (+0.006 mmol/L).

When uncentrifuged whole blood was stored at 4 ºC in plain tubes, no appreciable change in ionized calcium, pH, or pH-adjusted ionized calcium was seen for up to 70 hours.[15]

The influence of metabolic activity on ionized calcium was also studied.[12] In these experiments, full and half-full, commercially available evacuated tubes were stored (unspun) for 4 hours and 24 hours at 37 ºC.

Clinically significant changes were seen even at four hours. At 24 hours, mean change in ionized calcium was -0.70 mmol/L for full tubes and -0.134 mmol/L for half-full tubes. Adjusting ionized calcium values to pH 7.4 gives erroneous results by overcorrecting the values.

8.2.2 Processing the Serum Specimen

Incorrect processing of the specimen may influence the accuracy of the result:

- If the tube is opened during processing, the subsequent loss of CO_2 will increase the pH and decrease the ionized calcium values.

- The temperature at which the specimen is centrifuged will affect the pH and ionized calcium result of the serum. The temperature coefficient for ionized calcium is 0.006 mmol/L per 1 ºC. Therefore, a centrifugation temperature should be selected and should not vary by more than ±2.5 ºC.

8.2.3 Centrifuged Evacuated Tube (Red-Top or Serum Separator)

Several groups evaluated the stability of ionized calcium, pH, and pH-adjusted ionized calcium in blood collected in red-top evacuated tubes, which were centrifuged and stored unopened. A study reports no change in ionized calcium, pH, pH-adjusted ionized calcium, and lactate when stored for six hours at 4 ºC in either plain red-top or serum separator tubes.[15] In a companion experiment, it was further shown that in serum stored on the cells (centrifuged) at 4 ºC for 24 to 70 hours, ionized calcium remained relatively stable in both plain red-top (range: 0.016 to 0.021 mmol/L) and gel separator (range: 0.025 to 0.030 mmol/L) tubes. However, at 70 hours, pH-adjusted ionized calcium appeared to show a significant negative bias in plain red-top tubes (mean change = -0.032 mmol/L, range = -0.010 to -0.060 mmol/L) indicating that the calculation was overcorrecting ionized calcium (see Appendix C). This was not the case in the serum separator tubes, which showed little change in pH-adjusted ionized calcium at 70 hours (mean change = -0.002 mmol/L).

8.2.4 Long-Term Storage of Serum

A study reports no change in pH-adjusted ionized calcium in serum specimens stored at 4 ºC for seven days, while storage for 14 days at 4 ºC caused a small but significant decrease.[30] Storage of serum at -20 ºC for 45 days did not cause a significant change in pH-adjusted ionized calcium, but after 120 days a significant decrease was found. It has been shown that ionized calcium in serum stored anaerobically in a syringe at -10 ºC was stable for 11 to 20 days.[33]

The pH-adjusted ionized calcium is stable in serum for six to nine months when stored at -70 ºC (MF Burritt, unpublished observations).

8.3 Recommendations for Transporting Specimens

8.3.1 Whole Blood

When transporting a whole blood specimen, the following recommendations apply:

- Transport specimens at 4 ºC, if significant metabolic activity is likely to occur (i.e., large numbers of metabolically active cells and/or if delay before analysis is more than 30 minutes).

- Avoid warming the specimen above room temperature (22 to 24 ºC).

- Store syringes containing "normal" whole blood at 4 ºC no longer than four hours, if ionized calcium (uncorrected) is to be reported. If reporting pH-adjusted ionized calcium, syringes can be stored up to 24 hours.

8.3.2 Serum

When transporting a serum specimen, the following recommendations apply:

- Centrifuge the evacuated tube within four hours of collection. If a delay beyond four hours is likely, store the unprocessed specimen at 4 ºC.

- Maintain the temperature during centrifugation to ±2.5 ºC. (Note that some authors have suggested use of a temperature-controlled centrifuge to avoid the slight pH change caused by variation in centrifugation temperatures.)

- Full, centrifuged plain or gel separator tubes may be stored for up to 70 hours at 4 ºC.

- For storage beyond 70 hours, remove serum anaerobically and freeze at -20 to -70 ºC in a gas-tight container.

- For mailed-in specimens, ship on a cold pack. Do not use dry ice. Shipping on dry ice can cause a supersaturation of CO_2 in the specimen and a significant lowering of the specimen's pH and increase in ionized calcium.

- Do not open the tube before centrifugation.

9 Specimen Handling During Analysis

To ensure that the pH and ionized calcium of the specimen remains stable, whole blood or serum must be maintained anaerobically during analysis.

9.1 Whole Blood

When ionized calcium is measured along with pH/blood gases and other electrolytes, all the preanalytical and analytical considerations associated with all the measurements should be considered. (See NCCLS document C46— *Blood Gas and pH Analysis and Related Measurements*.)

Before analysis, mix the specimen thoroughly to ensure homogeneity. Depending on the instrument design, a portion of the specimen may be injected or aspirated directly from the syringe. Take care to ensure that air is not allowed to bubble into the specimen during aspiration.

9.2 Serum

To maintain the integrity of the specimen, the evacuated tube should remain stoppered until just before the measurement. For those instruments capable of direct sampling through the stopper, the intact evacuated tube is placed in the instrument and the sampling cycle is initiated. Ionized calcium is stable for several minutes in an opened, full (< 1-cm air space) evacuated tube. Attempt to aspirate the specimen from the bottom of the serum column, because changes are less rapid than in the serum layer taken from the top.[34]

9.3 Recommendations

9.3.1 Whole Blood

When handling a whole blood specimen, the following recommendations apply:

- Maintain anaerobic conditions at all times.
- Mix well to maintain full dispersion of heparin and blood cells.
- If necessary, depending on the clinical needs, centrifuge a portion of the blood specimen to determine if the specimen is hemolyzed.

9.3.2 Serum

When handling a serum specimen, the following recommendations apply:

- Take the specimen from the layer above the clot or separator gel.
- Do not remove the stopper until just before the analysis, and keep stoppered after the analysis.

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Appendix A. Anticoagulants

A1 Introduction

Because many anticoagulating agents, such as ethylenediaminetetraacetate (EDTA), oxalate, citrate, and fluoride, bind calcium ions with great affinity, they are not acceptable for use in the determination of ionized calcium.[1] Although ordinary heparin salts (e.g., Na heparin and Li heparin) bind calcium, the effect is relatively small, such that each 5 IU/mL of heparin lowers ionized calcium concentrations by about 0.01mmol/L.[2,3] In addition, heparin is also relatively inexpensive and has shown few effects on other clinical chemistry tests over many years of use. For these reasons, heparin (especially modified heparins as described below) is the recommended anticoagulant for ionized calcium determinations. This appendix describes some of the problems encountered with the use of heparin and some of the heparin products that have been developed to minimize the binding of calcium ions by heparin. Other anticoagulants, such as recombinant hirudin and its derivatives, apparently do not bind calcium ions.[4] However, these anticoagulants are not commercially available, being far too expensive at present.

A1.1 The Problems and Benefits of Liquid Versus Dry Forms of Heparin

Although the use of heparin in liquid form facilitates rapid mixing with blood, ordinary liquid heparin lowers the concentration of ionized calcium and most all other constituents of the blood in proportion to the volume ratio of liquid heparin to blood.[5] Consequently, liquid heparin is now rarely used in blood collection products.

Dry forms of heparin do not dilute the concentration of calcium ions and other constituents of blood. However, because dry heparin requires more time to dissolve in blood collected in a syringe, heparin might not be evenly dispersed in the blood sample, possibly resulting in clot formation. This is especially a problem in capillary collection devices, where either a high concentration of heparin (up to 200 IU/mL), or objects such as magnetic "fleas," are sometimes used to facilitate dissolution and dispersion of sufficient heparin in the blood sample.

A1.2 Amount of Dry Heparin

Because one molecule of heparin catalyzes the binding of many molecules of antithrombin III to thrombin, which prevents the conversion of fibrinogen to fibrin, very little heparin (~1 IU/mL) is required to inhibit coagulation. In reality, however, dry heparin products typically do not dissolve rapidly enough in blood specimens to ensure that such a small amount of heparin would prevent clotting in all samples.

Evacuated blood collection tubes have an air space above the sample that greatly facilitates dissolution and mixing of heparin. Consequently, 10 to 15 IU/mL of (lithium) heparin are adequate in these tubes. In syringes, where no air bubble is present, mixing is more difficult. While 15 IU/mL heparin is often used in syringes, clots occur more frequently in syringes relative to stoppered tubes. Increasing the heparin concentration to 25 IU/mL provides a safer degree of anticoagulation, yet increases the binding of calcium ions.

In capillary blood collection devices, mixing is extremely difficult to ensure. As a result, either magnetic "fleas" or high concentrations of heparin (50 to 200 IU/mL) have been added to the capillary tubes. This amount of heparin would severely lower the concentration of ionized calcium in blood collected in such a capillary.[5]

The modified preparations of heparin described below minimize the binding of calcium, which allows a greater amount of heparin to be added to the syringes (except for the "puff" of heparin).

Appendix A. (Continued)

A1.3 The Effect of Calcium Chelation by Various Preparations of Heparin

Some of the following preparations of heparin are designed to effectively prevent coagulation in the blood sample, while minimizing the effect of ordinary sodium or lithium heparin on ionized calcium results and still be useful for other laboratory tests, such as blood gases, cooximetry, and other electrolytes. These include calcium-titrated heparin, electrolyte-balanced heparin, sodium heparin blended with zinc heparin, and a low concentration of heparin in an inert filler.

A1.3.1 Dissociated Heparin Salts: Sodium, Lithium, Ammonium

These highly dissociated heparin salts bind calcium in proportion to their concentration. However, at heparin concentrations below 10 IU/mL, the ionized calcium will be lowered by less than 0.02 mmol/L for ionized calcium concentrations of 0.5 to 2.0 mmol/L.[3]

A1.3.2 Heparin Titrated with Calcium

To minimize calcium chelation, sodium heparin is titrated with calcium. Initially available as ampuled solutions,[6] titrated heparin is now provided in various dry forms in syringes and capillaries that are ready for use. The concentration of calcium-titrated heparin should not exceed 70 IU/mL.

A1.3.3 Heparin Balanced with Appropriate Amounts of Calcium, Sodium, Potassium, and Hydrogen Ions

At the concentration studied (40 IU/mL), balanced heparin affects ionized calcium values by no more than 0.02 mmol/L for ionized calcium concentrations between 0.9 and 1.6 mmol/L. However, at lower ionized calcium concentrations, balanced heparin increases ionized calcium by 0.03 to 0.04 mmol/L. At higher ionized calcium concentrations, balanced heparin lowers ionized calcium by 0.03 to 0.04 mmol/L.[5,7]

A1.3.4 Heparin with Zinc

A heparin preparation containing zinc with or without cations can effectively eliminate calcium binding by heparin. However, if too much zinc is present, the apparent ionized calcium may be increased by up to 0.03 mmol/L, because calcium ISEs have poor selectivity for zinc.[8,9]

A1.3.5 Low Concentrations of Heparin Dispersed in an Inert Filler

A syringe is available that contains only 2 to 3 IU of heparin per milliliter of blood collected in the syringe.[10] The inert material has two important properties: 1) the filler dissolves rapidly to disperse the heparin throughout the blood; and 2) the filler can precisely deliver a very low mass of heparin into each syringe during production. (Crystals of pure heparin cannot be so precisely dispensed.)[11]

A1.3.6 Heparin in User-Prepared Syringes

In preparing heparinized syringes for collecting specimens for ionized calcium determinations, one may be tempted to use whatever heparin preparation is convenient. However, heparin intended for therapeutic use and found in patient care areas has concentrations in the ampule ranging as high as 10,000 IU/mL. The incorporation of a volume of this material equivalent to the typical dead space of 0.05 to 0.10 mL would result in a dose of heparin per syringe of 500 to 1,000 units. Clearly, specimens collected in this uncontrolled fashion are unacceptable. (Depending on specific concentration and heparin salts, other analytes can also be affected.)

Appendix A. (Continued)

A1.4 Recommendations

- Currently, ionized calcium determinations on whole blood should be performed on specimens transferred into commercially available, ready-to-use syringes or capillaries anticoagulated with one of the modified preparations of heparin designed to minimize the effect on ionized calcium. These products provide ionized calcium results that closely agree with results on uncoagulated whole blood.[9]

- Traditional heparinized syringes designed for blood gas analysis usually contain an unacceptably high amount of sodium heparin (i.e., 30 to 40 IU/mL) and should not be used.

- User-prepared heparinized syringes are neither reproducible nor sterile and should not be used.

- If evacuated tubes containing heparin salts must be used for ionized calcium determinations, they should be filled to their nominal volume. This will keep the binding of calcium ion by heparin at a minimum and consistent level.

References for Appendix A

1. Moore EW. Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes. *J Clin Invest.* 1970;49:318-334.

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4. [†]Sachs C, Rabouine P, Chaneac M, Dechaux M. Hirudin: A calcium non-chelating anticoagulant. In: Burritt MF, Moran RF, eds. Methodology and Clinical Applications of Electrochemical and Fiber Optic Sensors. *Proceedings of the Electrolyte and Blood Gas Division AACC Symposium, Mayo Clinic, September 1989, Vol. 11*. Galveston, TX: University of Texas. 1990:305-309.

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7. Toffaletti J, Ernst P, Hunt P, Abrams B. Dry electrolyte balanced heparinized syringes evaluated for determining ionized calcium and other electrolytes in whole blood. *Clin Chem.* 1991;37:1730-1733.

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[†] The references contained in these proceedings are available through either the International Federation of Clinical Chemistry (IFCC) or the Critical Care Testing Division of the American Association for Clinical Chemistry (AACC), www.aacc.org/divisions/critical/default.stm.

Appendix A. (Continued)

9. Toffaletti J, Thompson T. Effects of blended lithium-zinc heparin on ionized calcium and general clinical chemistry tests (letter). *Clin Chem.* 1995;41:328-329.

10. Swanson JR, Heeter C, Limbocker M, Sullivan M. Bias of ionized calcium results from blood gas syringes. *Clin Chem.* 1994;40(letter):669-670.

11. Toffaletti J. Use of novel preparations of heparin to eliminate interference in ionized calcium measurements: Have all the problems been solved? *Clin Chem.* 1994;40(editorial):508-509.

Appendix B. Specimen Type: Arterial, Venous, or Capillary Blood

Three types of blood may be used for ionized calcium determinations: arterial, venous, and capillary. While major differences in pO_2 and pCO_2 levels exist between arterial, venous, and capillary blood, the differences for ionized calcium concentrations and pH are small. In a study of calcium-heparinized arterial, venous, and capillary whole blood from healthy adult patients undergoing minor surgery, no differences in ionized calcium between capillary blood and arterial blood were found.[1] A small, but insignificant, difference between arterial and venous blood probably resulted from the difference in pH (see the table).

Table B1. Ionized Calcium and pH Differences Between Arterial, Venous, Capillary Blood (mean ± SD). From Swanson JR, Heeter C, Limbocker M, Sullivan M. Bias of ionized calcium results from blood gas syringes. *Clin Chem*. 1994;40(letter):669-670. Reprinted with permission from the American Association for Clinical Chemistry.

Specimen	*n*	Ionized Calcium (mmol/L)	pH
Capillary blood	20	1.28 ± 0.03	7.43 ± 0.03
Arterial blood	20	1.28 ± 0.03	7.42 ± 0.03
Venous blood	20	1.29 ± 0.03	7.39 ± 0.03

Reference for Appendix B

1. Swanson JR, Heeter C, Limbocker M, Sullivan M. Bias of ionized calcium results from blood gas syringes. *Clin Chem*. 1994;40(letter):669-670.

Appendix C. Aerobically Handled Tubes (pH-Adjusted Ionized Calcium)

In a specimen that has been exposed to air, carbon dioxide loss will elevate the pH and decrease the ionized calcium. This type of change can also occur in a previously unopened evacuated tube, if it was not completely filled. In general, the larger the space above the serum, the greater the change.

In an effort to address this problem, many commercially available ionized calcium instruments calculate and report the pH-adjusted ionized calcium result.[1-3] As with any calculation, several assumptions about the specimen have been made. The assumptions, in this case, severely limit the usefulness of this result.[2-5]

Recommendations:

Before reporting a pH-adjusted ionized calcium result, consider the following:

- Preferably, ionized calcium should be reported as the actual measured ionized calcium.
- Use a pH-adjusted ionized calcium only to correct for CO_2 loss.

These recommendations are based on the following information:

- At least five different formulas can be derived from the ionized calcium/pH relationship, with varying accuracy.[1]
- All of the formulas have a limited pH range in which the calculated results are "valid."
- Pitfalls in the pH-adjusted ionized calcium result can be attributable to variations in albumin, types of anions that bind calcium, and a general, interindividual variation in the change in ionized calcium for a given pH change (delta ionized calcium/ delta pH relationship).[6,7]

References for Appendix C

1. Toffaletti J. Use of novel preparations of heparin to eliminate interference in ionized calcium measurements: Have all the problems been solved? *Clin Chem.* 1994;40(editorial):508-509.

2. Buckley BM, Rawson K, Russell LJ, Smith SCH. The influence of protein concentration on the mathematical adjustment of ionized calcium measurements for pH. In: Burritt MF, O'Connell KM, eds. Methodology and Clinical Applications of Ion-Selective Electrodes. *Proceedings of the International Symposium on the Measurements of Blood Electrolytes, Danvers, MA*. Rochester, MN: Davies Printing Co. 1988:121-130.[‡]

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[‡] The references contained in these proceedings are available through either the International Federation of Clinical Chemistry (IFCC) or the Critical Care Testing Division of the American Association for Clinical Chemistry (AACC), www.aacc.org/divisions/critical/default.stm.

Appendix C. (Continued)

Calcium Ion. *Proceedings of the 1980 Meeting of the IFCC Expert Panel on pH and Blood Gases.* Copenhagen: Private Press. 1981:163-190.‡

6. Boink ABTJ, van der Camp RAM, Maas AHJ. Variation in the relationship between pH and ionized calcium concentration in serum. In: Burritt MF, Cormier AD, Maas AHJ, eds. *Methodology and Clinical Applications of Ion-Selective Electrodes*. Rochester, MN: Davies Printing Co. 1988:131-140.‡

7. Sachs C, Chaneac M, Rabouine P, et al. Effects of physiopathological variations of some calcium ligands on the pH standardization of ionized calcium measurements on blood. In: Burritt MF, Cormier AD, Maas AHJ, eds. *Methodology and Clinical Applications of Ion-Selective Electrodes*. Rochester, MN: Davies Printing Co. 1988:146-152. ‡

‡ The references contained in these proceedings are available through either the International Federation of Clinical Chemistry (IFCC) or the Critical Care Testing Division of the American Association for Clinical Chemistry (AACC), www.aacc.org/divisions/critical/default.stm.

NCCLS consensus procedures include an appeals process that is described in detail in Section 9.0 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Committee Responses

C31-A: *Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline.*

General

1. My concern is that the guideline has not addressed the issue of ionized calcium measurements other than those drawn in heparin-balanced syringes. The reality is that there are other circumstances under which ionized calcium may be requested. The problem is that the vacuum tubes that may be used contain different amounts of heparin and I suspect that at a level that will bind calcium, thus producing a less accurate result. In my experience, comparing total calcium in serum versus plasma, I have noticed a negative bias in the plasma samples.

- **Text has been added to Sections 6.4.1 and A1.4 to address this concern. While specimens for ionized calcium analysis will inevitably be drawn in tubes containing unbalanced heparin, the best the committee can recommend is proper sample collection practices to minimize the effect.**

Section 7.2.3

2. In my studies we did about 1,000 newborn capillary samples without major difficulties. It all depends on proper mixing as quickly as possible after the samples have been drawn and no coagulation is seen.

- **The point about rapid and proper mixing of capillary samples is good and has been added to Section 7.2.3.**

Related NCCLS Publications[¶]

C46-P **Blood Gas and pH Analysis and Related Measurements; Proposed Guideline (2000).** This document provides clear definitions of the several quantities in current use, and provides a single source of information on appropriate specimen collection, preanalytical variables, calibration, and quality control for blood pH and gas analysis and related measurements.

H3-A4 **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard — Fourth Edition (1998).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. Includes recommendations on order of draw.

H4-A4 **Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture; Approved Standard —Fourth Edition (1999).** A consolidation of H4-A3 and H14-A2, this standard provides detailed descriptions and explanations of proper collection techniques, as well as hazards to patients from inappropriate specimen collection by skin puncture procedures.

H11-A3 **Procedures for the Collection of Arterial Blood Specimens; Approved Standard—Third Edition (1999).** This standard describes principles for collecting, handling, and transporting arterial blood specimens. The document is aimed at reducing collection hazards and ensuring integrity of the arterial specimen.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** A consolidation of M29-T2 and I17-P, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

[¶] Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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