NBS SPECIAL PUBLICATION 260-67

U.S. DEPARTMENT OF COMMERCE / National Bureau of Standards

Standard Reference Materials:

A REFERENCE METHOD FOR THE DETERMINATION OF CHLORIDE IN SERUM

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Standard Reference Materials:

A REFERENCE METHOD FOR THE DETERMINATION OF CHLORIDE IN SERUM

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George A. Uriano, Chief
Office of Standard Reference Materials

FOREWORD

A fundamental requirement for assuring adequate patient care is the need for the accurate analysis of constituents in body fluids. Two major functions of the National Bureau of Standards (NBS) are to provide certified Standard Reference Materials for the calibration of measurement systems and to develop new or improved analytical methods. The results presented in this NBS Special Publication provide a methodology of known accuracy for the determination of chloride in serum. The evaluation of a reference method by comparison to a definitive method, used for the first time at NBS in the development of reference methods for calcium, sodium, and potassium in serum, also was applied to this work. This hierarchy of analytical procedures has been accepted as a valid format for developing reference methods by the clinical community at a recent Conference on an Understanding for a National Reference System in Clinical Chemistry.

In an undertaking of this magnitude, extensive collaboration with a committee of experts, the Center for Disease Control, the Food and Drug Administration, and a wide spectrum of participating analytical laboratories that included Federal, state, hospital, industrial, and academic laboratories was essential to establish a widely accepted reference method. It is hoped that this work will provide an additional basis for the development of future clinical reference methods through continued collaboration and the concerted efforts of the individual participants.

Philip D. LaFleur, Director
Center for Analytical Chemistry

NOTE

Because of concern for the usability of this chloride reference method, the Center for Disease Control (CDC) management has declined to endorse the method described in this report. NBS supports the evolution of analytical methods and desires to participate in interlaboratory exercises that are aimed toward establishing the transferability of proposed reference procedures. NBS believes it important that the principles of analytical practice delineated in this present report be circulated in a timely manner. Since the method outlined in this report has been shown to satisfy the generally accepted criteria of a reference method, it should function as such until the efficacy of a subsequent method has been demonstrated. NBS will maintain its primary role in supplying SRM's and definitive methods.

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ABSTRACT

Guided by a committee of experts in clinical chemistry, a reference method was established for the determination of serum chloride based on a coulometric titration-amperometric end-point determination (C-A). Its accuracy was evaluated by comparing the values obtained by use of the method in 14 laboratories against the results obtained by a definitive analytical method based on isotope dilution-mass spectrometry (IDMS). Seven serum pools with chloride concentrations in the range 79.2 to 116.8 mmol/L were analyzed. Micro- and macropipetting alternatives were tested using sample sizes of 0.010-0.100 and 5.0 mL, respectively.

The laboratories used several different C-A instruments. The results showed that the standard error for a single laboratory's performance of the procedure was approximately 1.0 mmol/L with a maximum bias of 0.5 mmol/L over the range of concentrations studied. These values are within the accuracy and precision goals that had been set by the committee. The results from the micro- and macropipetting techniques were similar. The calibration curve data showed excellent linearity over the total concentration range, with 12 of 14 curves having standard deviations of fit of less than 0.80 mmol/L.

With appropriate experimental design, the reference method may be used to establish the accuracy of field methods as well as to determine reference chloride values for pooled sera.

Key Words: Amperometry; chloride analysis; clinical analysis; clinical chemistry; coulometry; definitive method; electrolytes; reference method; serum chloride analysis.

I. INTRODUCTION

Chloride, an electrolyte commonly analyzed in serum, plays an integral role in the assessment of acid-base imbalance, in calculating the anion gap, and acts as a secondary indication of changes in other electrolyte concentrations [1][5]. An excess concentration of chloride in perspiration is indicative of the disease cystic fibrosis [2].

Serum chloride has been determined by a wide range of analytical methods; these include gravimetry, photometry, iodometry, titrimetry, electrometry, conductometry, and polarography as well as isotope dilution, indirect flame emission photometry, and atomic absorption [3]. In general, all these procedures suffer from interference from the presence of bromide ion, and care must be taken in interpreting analytical results for patients who may have elevated levels of serum bromide [4].

Three analytical techniques have been suggested as standard procedures. They are: a mercurimetric determination using diphenyl carbazone as an indicator [5]; an argentimetric determination using dichlorofluorescein as an indicator [6]; and a procedure developed by Cotlove based on established principles of coulometric generation of titrant (Ag^+) and amperometric indication of the end point [7]. Whether any of these methods should be considered by clinical laboratories as the clinical reference method for serum chloride has not been proven; the accuracy of none of these methods is known.

Two approaches may be used for establishing the accuracy of analytical methods. In the first, the results obtained from the methods in use for that analyte are compared using typical samples and selected samples containing known interferences for the analyses. Statistical correlations are used

[5]The bracketed numerals refer to the references listed at the end of this paper.

to express the interrelationships of the methods. A technique is then considered to be accurate to the degree established by knowledge of the sources of error and the agreement of results. In the second, a single candidate method is selected (possibly the 'best' of the methods recognized by the first approach) and studied in detail. Each step of the candidate method is optimized and examined so that the systematic and the random errors can be quantitatively expressed.

Studies have been organized using a combination of these approaches to establish the accuracies of clinical chemistry methods for total calcium, sodium, and potassium in serum [8,9,10]. For calcium, the analytical procedure was based on the flame atomic absorption spectrometric method of Pybus, Feldman, and Bowers [11], while for sodium and potassium the method was based on flame atomic emission spectrometry. The accuracies of these methods were assessed by using them on several human serum pools for calcium and bovine serum pools for sodium and potassium in selected clinical laboratories and comparing the results obtained against those obtained for the same pools by isotope dilution-mass spectrometry (IDMS) methods for calcium and potassium and an ion-exchange – gravimetry method for sodium. These analyses were performed at the National Bureau of Standards (NBS) where the high accuracy of those methods[6] were established by determining their systematic and random errors [12].

These studies, carried out with the guidance of clinical laboratory experts, used (a) Standard Reference Materials [13] as the pure, primary reference material to prepare standard solutions of calcium, sodium, and potassium for all the analyses; (b) serum pools prepared at the Hartford Hospital (Hartford) and/or the Center for Disease Control (CDC,

[6]Such methods are referred to as definitive methods because of their high accuracy and utility for evaluating the accuracy of a candidate reference method.

Atlanta); (c) definitive method analyses for calcium, sodium, or potassium at NBS; (d) statistical analysis of the data at NBS; and (e) accuracy and precision goals as performance standards that the methods would have to meet to be recommended as the clinical reference method for total calcium [14], sodium, or potassium [15] in serum.

This same approach was adopted to develop clinical reference methods for a number of other serum electrolytes including chloride, lithium, and magnesium. The work was begun with the cooperation of individuals from the Standards Committees of the American Association for Clinical Chemistry (AACC) and the College of American Pathologists (CAP), the CDC and the NBS. The Food and Drug Administration (FDA) provided major suppport for the NBS work. The progress of this program was reported regularly to the AACC Standards Committee. We present in this report the development of a clinical reference method for serum chloride.

A proposed reference method for both sodium and potassium in serum using similar methodologies but without interlaboratory exercises has been reported recently [16].

II. DEVELOPMENT OF THE SERUM CHLORIDE REFERENCE METHOD

A. Organization

A panel of experts in clinical chemistry was invited to meet at NBS in March 1974 to consider the development of reference methods for five serum electrolytes, namely, chloride, potassium, sodium, lithium, and magnesium. The overall program for the development of these reference methods was organized by Dr. Robert Schaffer (NBS) and Dr. Rance A. Velapoldi (NBS). The invited experts were Dr. George N. Bowers, Jr. (Hartford Hospital), Dr. Bradley E. Copeland (New England Deaconess Hospital),

Dr. Denis O. Rodgerson (Center for Health Sciences, University of California in Los Angeles), and Dr. James M. White[7] (CDC).

Prior to the meeting, several bovine serum pools prepared at the CDC had been analyzed for chloride by a coulometric-amperometric technique and by IDMS. The results, summarized in Table 1, were presented at the meeting as follows: coulometric-amperometric (C-A)[8] as obtained at NBS using commercial instrumentation by Dr. R. A. Durst; and IDMS as obtained at NBS by Mr. T. J. Murphy. On consideration of 1) the quite similar analytical results in Table 1, 2) alternative clinical laboratory procedures, and 3) instrumentation available in most clinical laboratories, it was concluded that the coulometric-amperometric technique using commercial instrumentation for the determination of serum chloride was the appropriate candidate methodology to evaluate as the reference method and that its evaluation should be made using IDMS as the definitive method.

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[7]Dr. James White died after this program was well underway. He was recommended for membership on this Experts Committee on electrolytes by Dr. Joseph H. Boutwell (CDC). Dr. White made significant contributions to the development of the reference methods. His knowledge, advice, and cooperation in all phases of this work contributed greatly to the success of the program.

[8]The end point may be determined potentiometrically; however, for simplicity, the procedure will be represented by the letters C-A.

Table 1. Preliminary results from NBS comparing the coulometric-amperometric (C-A) and IDMS techniques for the determination of serum chloride.

	Cl in Serum, mmol/L	
Pool	IDMS[a]	C-A[b]
1	79.2	79.8
4	101.8	100.8
7	116.8	115.3

[a]Data from T. J. Murphy (NBS).

[b]Data from R. A. Durst (NBS).

The experts agreed to serve as the Committee to oversee the development of the reference method for chloride (as well as for the other electrolytes discussed at the meeting). The Committee chose Dr. Bowers as chairman. Dr. Copeland agreed to serve as the Committee's representative to work with those at NBS who would be involved in writing the protocol for the chloride reference method. The Committee agreed that the coulometric method should use a concentration bracketing technique rather than calibration curves for determining chloride concentrations. However, calibration curve data should be obtained as a general check on the measurement system and to determine which of the primary standard solutions would be used to bracket the chloride levels in the samples being analyzed.

As goals for the candidate reference method, the maximum bias of the method and its one-standard deviation imprecision limit were set by the Committee at 2.0 and 1.5 mmol/L, respectively, for serum chloride at the 100.0 mmol/L level. These goals were to be achieved by controlled, interlaboratory

tests involving a selected group of clinical chemistry laboratories which would perform the analyses by the C-A method according to the written protocol. NBS would provide chloride values by the definitive method.

B. Participating Laboratories, Standards, Serum Samples, and Definitive Method

The laboratories that were asked to participate in the interlaboratory study were chosen to represent a wide spectrum of clinical chemistry interests and included government (federal and state) and hospital laboratories, and laboratories associated with suppliers of instruments and of test and control materials. Two hospitals were located outside the United States. The principal investigators at these laboratories are named in the list below. Other scientists in each of the laboratories who contributed to this study are acknowledged by name in Appendix A. The list includes three laboratories that participated only in the concluding interlaboratory work. They were added to maintain a minimum number of laboratories when some of the original laboratories were unable to continue their participation. In alphabetical order of the principal investigator, the laboratories that participated in the interlaboratory studies are:

Dr. George N. Bowers, Jr.
Dr. Robert W. Burnett
Hartford Hospital
Hartford, CT 06115

Dr. Bradley E. Copeland
New England Deaconess Hospital
Boston, MA 02215

Dr. Richard A. Durst
National Bureau of Standards
Washington, D. C. 20234

Dr. Gordon Edwards
Dade Division
American Hospital Supply Co.
Miami, FL 33152

Dr. Lorentz Eldjarn
Dr. Johan Kofstad
Rikshospitalet, University of Oslo
Oslo, Norway

Dr. Nathan Gochman
Veterans Administration Hospital
San Diego, CA 92161

Mr. Cecil Hassig
American Instrument Co.
Silver Spring, MD 20910

Dr. Denis O. Rodgerson
Center for Health Sciences, University of California
Los Angeles, CA 90025

Mr. William Ryan
Beckman Instruments
Fullerton, CA 92634

Mr. Leonard Sideman
Department of Health
Philadelphia, PA 19130

Dr. Barbara Tejeda
Food and Drug Administration
Washington, D. C. 20250

Dr. Thomas Tracey
Technicon, Inc.
Tarrytown, NY 10591

Dr. James M. White
Dr. Richard Carter
Center for Disease Control
Atlanta, GA 30333

Dr. Charles E. Willis
College of American Pathologists, Cleveland Clinic
Cleveland, OH 44106

NBS Standard Reference Material Sodium Chloride (SRM 919, see Appendix B) was to be used as the pure, primary reference material for all analyses [13]. Seven pools of homogeneous, sterile, bovine serum having different concentrations of chloride, were prepared at the CDC by Dr. David Bayse and Ms. Sue Lewis. Samples of each pool were supplied in approximately 7-mL volumes in sealed vials that were labeled with computer-generated random numbers. The samples, packed in dry ice, were shipped to NBS by air and within 24 h of packing were placed in freezers kept at -50 °C [17]. The pools were numbered in code from 1 to 7 according to increasing chloride concentration.

A definitive method based on IDMS was developed at NBS and is given in Appendix C. The chloride concentrations for the seven serum pools were determined by this procedure and the results obtained are summarized in Table 2.

Table 2. Chloride concentrations for the seven serum pools as determined by IDMS, the definitive method.

Pool	$[Cl^-]$, mmol/L
1	79.2 ± 0.4[a]
2	84.5 ± 0.4
3	94.0 ± 0.5
4	101.8 ± 0.5
5	107.2 ± 0.5
6	112.9 ± 0.6
7	116.8 ± 0.6

[a]Estimated maximum error of 0.5 percent of the value as reported by NBS Analytical Mass Spectrometry Section. This estimated maximum error includes both imprecision and an estimated upper bound for possible systematic errors. The estimated maximum error is believed to be equal to or greater than the true error for the 95 percent confidence limits.

C. Functions of the Various Groups

The interrelationships and functions of the various groups involved in developing C-A as a reference method for serum chloride are represented in figure 1. The Committee, CDC, and NBS provided guidance and technical support for the program and also served as participating laboratories. The Experts Committee selected the candidate reference method, set maximum bias and imprecision goals for an acceptable reference method, assisted NBS in selecting other participating laboratories, and reviewed all analytical results. The CDC provided the serum pools. The participating laboratories provided the interlaboratory test data and critiques of the candidate reference method protocol.

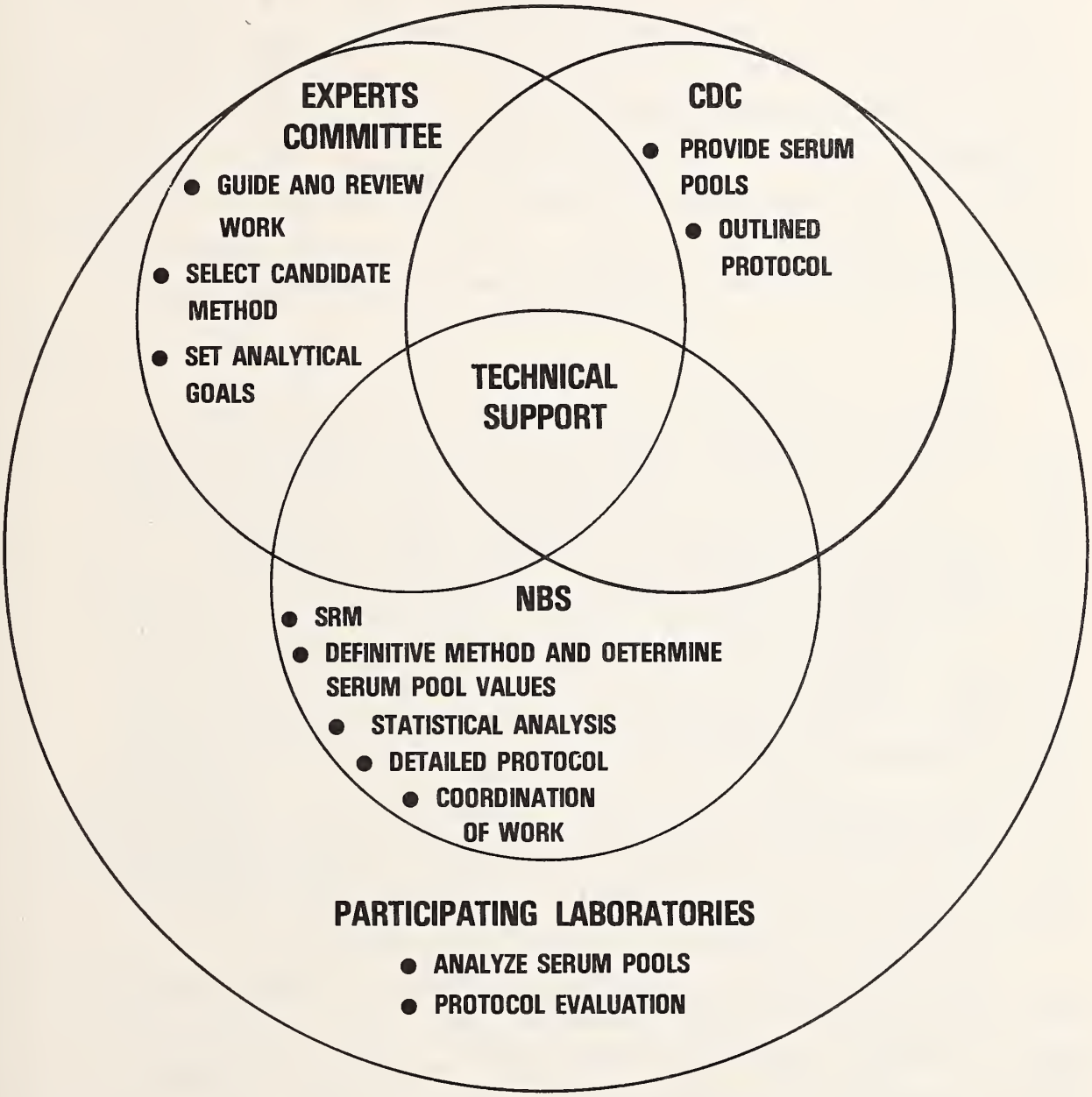

Figure 1. Interrelationships and functions of the various groups in the development of a clinical reference method for the determination of serum chloride.

At NBS, Dr. R. Schaffer served as the Reference Method Program Manager and Dr. R. A. Velapoldi served as the coordinator. The format of the interlaboratory exercises (IE)[9] was established within the constraints imposed by protocol requirements and sample availability by Drs. John Mandel, Robert Paule, and Rance Velapoldi. Dr. Velapoldi wrote the protocol for the candidate reference method from the outline provided by Drs. B. Copeland and D. Grisley. Drs. Mandel and Paule performed the statistical evaluation of the results from the interlaboratory tests. The definitive method was performed by Mr. Thomas Murphy and Dr. John Gramlich.

D. Plan for Testing the Candidate Reference Method

The general plan was to evaluate the candidate reference method by performing a series of interlaboratory exercises, which would consist of a preliminary test (IE-P) followed by successive interlaboratory exercises until the goals for the reference method were reached. A main objective of the IE-P was to allow participating laboratories to become familiar with and comment on the protocol. Since an evaluation of the bias was not sought in the IE-P testing phase, normal bovine serum samples [18] not having definitive method analyses were to be used. However, interlaboratory imprecision was to be measured. If the imprecision of the results in the IE-P was found to be small, IE testing would begin on samples having definitive method chloride values.

In an IE, each participating laboratory would perform the same analyses on two separate days: i.e., analyze a pair of samples from each serum pool on each of two days where a minimum of one day or a maximum of seven days were to elapse between the two series of analyses. The bias and

[9] In previous reports, the Interlaboratory Exercises were called Round Robin Tests.

imprecision values obtained by statistical analysis would then be compared to the goals set by the Committee for the reference method. If the goals were not met, additional IE's using samples from other pools would be conducted by following the protocol or a modified form of it until the bias and imprecision goals were reached. Revisions and modifications to the protocol could be made after an interlaboratory exercise had been completed but would not be made after the final IE.

Three kinds of information were to be supplied by each participating laboratory after finishing an interlaboratory exercise:

1. General Data – a list of the instrumental parameters used and comments on the protocol including problems encountered during the analysis;
2. Calibration Curve Data – a list of the 'instrumentally' measured mmol/L values[10] versus the actual chloride concentrations of the standards calculated from the NaCl used; and
3. Valid Measurement Data – a list of the sets of data that constituted the three measurements on a single sample.

Examples of the data sheets on which the information was collected are shown in Appendix D, Note 8.

[10] These measured values also could have the units of time for coulometric production of the titrant.

III. REFERENCE METHOD PROTOCOL FOR THE DETERMINATION OF SERUM CHLORIDE

A. General

This protocol provides for the analysis of serum chloride by a coulometric-amperometric procedure using a micropipet to introduce the serum samples directly into the titration cup. A macropipetting and diluting procedure is also included as an alternative; however, the same instrumentation is used for the determination. Both procedures are to conform to the requirements of the instrumentation used.

B. Protocol Synopsis

The protocol must be followed exactly. The reference method is used to analyze four samples of a serum or pool: two on one day and the other two on a subsequent day. Approximately 2 mL of sample is required to carry out the micropipetting procedure while 25-40 mL is needed to carry out the macropipetting procedure.

1. Use an analytical balance to weigh the SRM NaCl in the appropriate quantity to prepare a stock standard chloride solution;
2. Use single or multiple micropipets or a single macropipet to transfer aliquots of the sample to the C-A titration cup or to dilute the aliquots to the levels used as working solutions of a) the serum, b) the stock standard chloride solutions, and c) the solution used as a blank;
3. Obtain calibration curve data on the working blank and standards;

4. Measure the C-A values on the working solutions of the serum sample; select the pair of working standards whose C-A values most closely bracket the value for each sample;
5. For each sample to be analyzed, obtain three valid measurement sets by measuring the C-A values obtained from repeated sequential measurements of the working solutions of the low bracketing standard, the aliquot of that sample, and the high bracketing standard;
6. Calculate the chloride concentration of the aliquot for each set by mathematical interpolation;
7. Average the three calculated values to obtain a 'single measurement' of that sample; (in the statistical analysis, each such average is designated a 'single measurement');
8. Perform steps (4) through (7) for each sample to be analyzed on the first day;
9. Repeat steps (1) through (8) on the subsequent day to obtain the second pair of measurements needed for each sample;
10. Average the four values obtained by the replicate determinations to obtain the chloride concentration for each serum pool.

C. Detailed Protocol

The selection of the specific alternatives of the protocol to be used dictates the glassware and diluent volumes needed. These needs are summarized in the protocol or in Appendix D notes. Stock solutions and working solutions are to be prepared at and maintained at a room temperature that is constant within ±2 °C (see Appendix D, Note 1).

1. Reagent Specifications

a. Water: At the time of preparation, the distilled and/or deionized water used should exhibit a specific resistance of at least 10 kΩ·m at 23 ± 2 °C. At the time of use, titration of this water should show a C-A value that is less than 0.1 percent of full scale at the instrumental settings used for the analysis. A large quantity of this water (more than 10 L) must be available for use as diluent and for the final rinsings of all glassware and other apparatus that come in contact with the solutions involved. Unless specified otherwise, the water referred to in this protocol is this tested water.

b. Chloride Standard Solutions: Use Standard Reference Material Sodium Chloride (originally issued as SRM 919, Certificate reproduced in Appendix B) [13] certified by the National Bureau of Standards. The SRM NaCl should be dried at 110 °C for four hours in a loosely capped container and then stored in a desiccator containing $CaSO_4$ or an equivalent desiccant.

c. The supporting electrolyte constituents used are specified in the instruction manual for the particular instrument used.

d. Nitric acid, chloroform, methanol and 95-percent ethanol conforming to ACS [19] (or equivalent) specifications are to be used.

2. Glassware Specifications

a. Volumetric glassware (Appendix D, Note 2) should be of borosilicate material and meet NBS Class A [20] or equivalent specifications.

b. The micropipets should have the following accuracy tolerances:

Volume (μL)	±3 S.D. (μL)
20	0.24
100	1.4

(Examples of appropriate micropipets are: Clay-Adams, Parsippany, New Jersey 07054, Number 4625 for the 100 μL pipet and Number 4618 for the 20 μL, see footnote 11, p. 57.)

All glass or plastic surfaces that come into contact with reagents, water, diluent, sample, or supporting electrolyte must be clean (Appendix D, Note 3).

3. Preparation of Reagents

a. Chloride Standard Stock Solutions: Prepare standard solutions with nominal chloride concentrations of 75-, 90-, 100-, 110-, and 125-mmol/L. Weigh accurately (to 0.1 mg) approximately 1.09, 1.32, 1.46, 1.61, and 1.83 g of dried SRM NaCl (MW = 58.44277, Appendix D, Note 4) and transfer each quantitatively into separate, appropriately labelled, 250-mL volumetric flasks. Dissolve the NaCl using approximately 100 mL of water. Fill flasks to the calibrated volume with water, stopper, and mix by inverting the flask and shaking ten times. Repeat flask inversion and shaking ten times. Calculate the concentration of the NaCl solutions in mmol/L to two decimal places according to the exact weights of sodium chloride used, and record these values on the data sheets; an example of this step is given in Table 3.

Table 3. Sodium chloride standard solutions.

Solution	(NaCl)[a], mmol/L	NaCl, g
1	75.00	1.0958
2	90.07	1.3150
3	100.12	1.4628
4	110.09	1.6085
5	125.10	1.8278

[a]The NaCl concentrations were calculated using atomic weights from the literature reference cited in Appendix D, Note 4.

b. Supporting Electrolyte Solution Follow the instrument manufacturer's instructions for the preparation of the supporting electrolyte.

c. Dilute Nitric Acid (0.77 mol/L): Prepare by making a twenty-fold dilution of concentrated nitric acid (15.4 mol/L) with water.

4. Pipetting and Dilution Procedures

a. For Instruments in Which Microvolumes of Sample are Added Directly to the Titration Cell

(1) Micropipetting of Solutions, General: Several types of micropipets may be used including those in which the solutions are drawn up by capillary action or suction (aspirator or plunger). Micropipets are to be used with a wash-out technique. If all micropipets meet the accuracy specifications given in Section IIIC-2, then different micropipets may be used for solutions of blank, standard, and sample; if they do not meet these specifications, a single micropipet must be used throughout. In

the latter case, the micropipet must be cleaned as described in Appendix D, Note 3, or if this micropipet employs a plunger with a positive displacement that ejects all of the aliquot, the pipet must be rinsed with the solution by filling and expelling three times with some of the solution transferred for this purpose to a separate container.

(2) Micropipetting Technique: Fill the micropipet by immersing the tip just below the surface of the solution and tilting the micropipet so that the solution is drawn up the bore. Stop filling the capillary at the calibrated mark. Withdraw the pipet from the solution. Wipe the exterior wall of the micropipet with clean absorbent paper taking care to avoid contact with the delivery tip. Deliver the aliquot to the sample cell by immersing the tip of the micropipet just below the surface of the supporting electrolyte and gently expel all of the sample from the micropipet by blowing. Alternatively, the solution is carefully ejected by use of the plunger. Check to see that the plunger has ejected all of the solution. Before raising the micropipet tip from the electrolyte solution in the cell, the micropipet is rinsed with the supporting electrolyte-sample mixture by drawing the mixture into the micropipet to the calibrated mark and expelling back into the sample cell. (NOTE: Do not immerse the micropipet deep into the supporting electrolyte-sample mixture and do not wipe off the exterior wall of the micropipet as previously done.) Repeat this rinse operation twice more.

b. For Instruments Which Require Samples that are Prediluted with Supporting Electrolyte: The solution used for measurement must contain a silver chloride dispersant for smooth titration performance. Directions for the preparation and addition of the dispersant are found in the manufacturer's instruction book for the instrument.

(1) Macropipetting and Diluting, General: To prepare working solutions, the blank, stock standard, and serum solutions are diluted fifty-fold with supporting electrolyte solution (Section IIIC-3b) using a 5-mL pipet with a wash-out technique ("to contain" mode). Only one volumetric pipet is used and the wash-out technique is employed throughout to avoid errors that may arise due to differences in pipet volumes and delivery times.

(2) Macropipetting and Diluting Using the Wash-Out Technique: Transfer approximately 200 mL of the supporting electrolyte solution to a 250-mL volumetric flask. Then add 5 mL of the appropriate solution by the following procedure. Fill the 5-mL pipet to approximately 1 cm above its calibration mark, withdraw the pipet from the container, and wipe the delivery tip with clean, absorbent paper. Contact the tip of the pipet to the side of a clean waste container, and allow excess solution to drain until the meniscus is at the calibrated mark on the pipet. Remove the pipet from contact with the container and direct the delivery tip of the pipet into the receiver. Deliver the sample by contact of the pipet tip with the wall inside the volumetric flask and allow the solution to drain fully. After drainage stops, gently expel the residual liquid. Rinse the pipet by lowering the pipet tip into the

solution within the flask and refill the pipet with solution to approximately 1 cm above the mark. Allow the solution to drain fully and gently expel residual liquid. Repeat the pipet rinsing two more times. Finally wash the pipet tip with approximately 4 mL of fresh supporting electrolyte solution using a wash bottle or a clean, disposable Pasteur pipet. (NOTE: New, disposable pipets should be cleaned before use.) Fill the flask to the calibrated volume with the electrolyte solution. Mix thoroughly by inverting the flask and shaking ten times. Repeat the inversion and shaking procedure ten times.

(3) Preparation of Working Solutions:

(a) Working Blank Solution and Working Standard Solutions: Prepare the working solutions of the blank solution and the working 75.0-, 90.0-. 100.0-, 110.0-, and 125.0- mmol/L chloride standard solutions by making dilutions in appropriately labeled volumetric flasks in the order cited. Condition the 5-mL pipet by filling it with the solution to be diluted. Discard this pipetful and repeat filling and discarding twice more. Then refill the pipet with the solution, adjust to the calibrated volume, and deliver into the volumetric flask to be used for the dilution. Rinse the pipet by filling it three times with the supporting electrolyte solution, each time delivering the rinse solution into the volumetric flask. Fill the flask to the calibrated volume with the electrolyte solution. Wash out the pipet three times with water (see Appendix D, Note 5) and expel the residual liquid.

(b) Working Sample Solutions: If at least 10 mL of serum sample is available, condition the 5-mL pipet with some of the sample to be diluted in the

following way: (1) Draw 1-2 mL of the sample into the pipet, (2) withdraw the pipet from the container, (3) wipe off the tip with a clean, absorbent paper, (4) tilt the pipet to a horizontal position, (5) allow a small volume of air to leak in and rotate the pipet so that the conditioning liquid wets all the internal surface to approximately 0.5 cm above the calibration mark, (6) discard this conditioning solution, and (7) repeat steps (1-6). Then prepare the working solutions as described in sections IIIC-4b-(2), fill the 5-mL pipet with the sample, adjust volume to the mark, deliver, rinse three times into the volumetric flask with supporting electrolyte, dilute to the calibrated volume, and mix. Finally, wash out the pipet three times with water (Appendix D, Note 5). For each of the next sample solutions to be diluted, repeat step IIIC-4b-(3)(b).

If between 5 and 10 mL of each sample is available, a dry, 5-mL pipet must be used for each sample since this volume of serum is insufficient for the pipet-conditioning technique described above. In this case, fill the clean, dry, 5-mL pipet with the serum sample, adjust to volume, deliver and rinse into the receiver, and dilute. Clean the pipet for the next sample by washing the pipet with dilute HNO_3, rinse with a minimum of four portions of water and finally rinse twice with 95 percent ethanol. Allow the pipet to drain. Apply minimum suction to the top end of the pipet for one minute to remove any remaining ethanol. The pipet is then ready for use with the next serum sample. (NOTE: If the dilute HNO_3 washings do not drain cleanly from the pipet, wash the pipet as described in Appendix D,

Note 5. Then follow with the ethanol wash as above.) Prepare the working solutions as above.

(4) Solution Transfer to Sample Cell; Macropipetting in the 'To Deliver' Mode, General: Different 4-mL pipets may be used for the transfer of the working low standard, the working sample, and the working high standard solutions to the sample titration cell. Condition the pipet as described in IIIC-4b-(3). Fill the pipet by the procedure described in IIIC-4b-(2). Deliver the solution into the clean sample titration cell by allowing the pipet to drain with the tip against the side of the sample cell. (NOTE: As a minimum, allow the pipet to drain for the delivery time inscribed on the pipet.) After drainage is complete, remove the pipet and proceed with the titration. This pipet may be used for further transfers of the same solution after rinsing once with the solution to be transferred. Keep each of the three pipets used for the transfer of the working low standard, working sample, and the working high standard separate to prevent cross-contamination of solutions. After each titration, clean the sample cell and electrodes by rinsing them three times with water.

5. Coulometric-Amperometric Titration Procedure

It is not possible to provide detailed instructions for each type of instrument to assure necessary instrument stability, linearity, titration conditions, etc. The operator must be familiar with the instrument used. The instrument should meet all the manufacturer's specifications. In general, the accuracy of the method cannot be attained unless the instrument is in optimum operating condition.

a. Determination of Standard Curve for Instruments with Direct Concentration Readout

(1) Calibrate the system with the 100 mmol/L chloride standard according to the instructions for the particular instrument.

(2) Titrate ten samples of the 100 mmol/L standard. Calculate the mean and standard deviation of the values. The mean value should be within 0.5 mmol/L of the value calculated from the amount weighed. If this value is exceeded, recalibrate. The standard deviation should not exceed 1 mmol/L.

(3) Titrate five samples of each of the approximately 75-, 90-, 110-, and 125-mmol/L standards in the same manner as in step (2) above. The range of values for the 75- and 90-mmol/L solutions should not exceed 2 mmol/L and for the 110- and 125-mmol/L standard should not exceed 4 mmol/L. If these ranges are exceeded, recalibrate and repeat (2) above. The mean values for all the standards should be within 0.5 mmol/L of the calculated value. On the data sheet provided, record the standard solution concentrations in mmol/L to 2 decimal places and the experimentally obtained values to at least one decimal place.

(4) A typical calibration curve is shown in figure 2.

b. Calibration for Time Readout Instruments Which Measure Samples Prediluted with Supporting Electrolyte:

(1) Titrate reagent blanks in triplicate and calculate the average time of titration ($\overline{B}$).

(2) Titrate ten samples of the approximately 100 mmol/L working standard. Calculate the gross mean standard

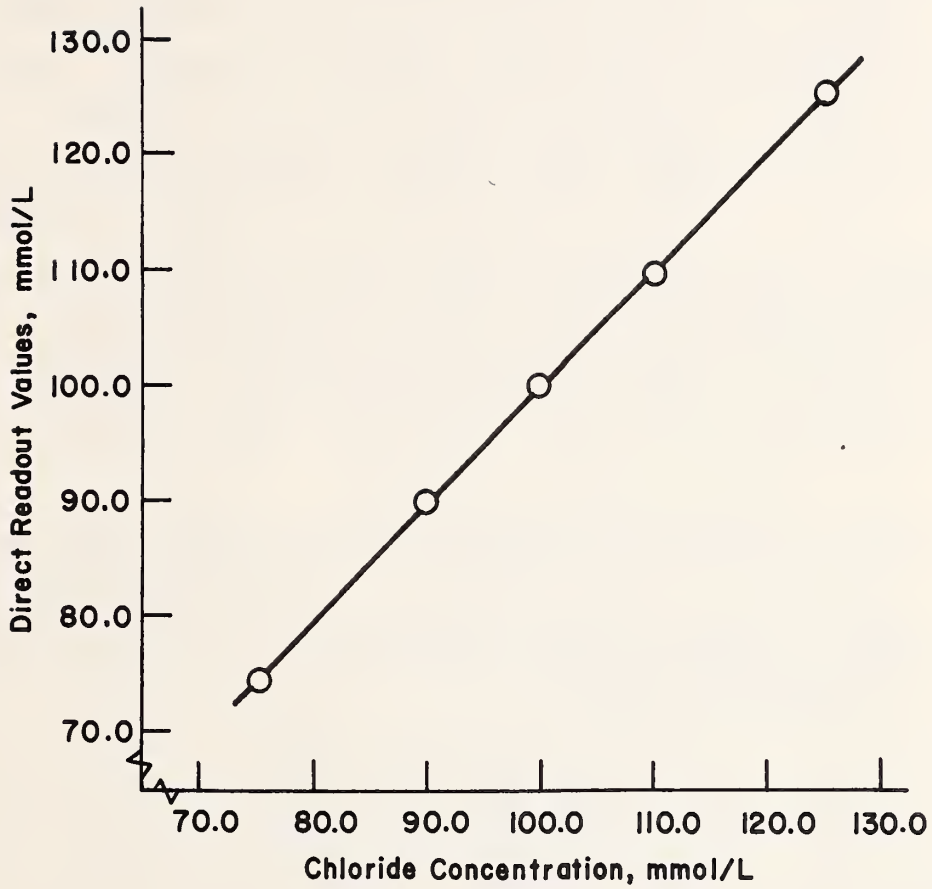

Figure 2. Typical calibration curve for the determination of serum chloride by a coulometric-amperometric technique.

time ($\overline{GS}_{100}$) and standard deviation of the time of titration. The standard deviation should be within 0.4 second.

(3) Calculate the net mean standard time ($\overline{NS}$) by:

$$\overline{NS}_{100} = \overline{GS}_{100} - \overline{B} \qquad (1)$$

(4) Titrate five samples of the approximately 75-, 90-, 110-, and 125-mmol/L standards. Calculate the gross mean standard times. For $\overline{GS}_{75}$, $\overline{GS}_{90}$, $\overline{GS}_{110}$, and $\overline{GS}_{125}$, the values for the standard deviations should not exceed 0.5 seconds. If this limit is exceeded, trouble-shoot the system. Calculate $\overline{NS}_{75}$, $\overline{NS}_{90}$, $\overline{NS}_{110}$, and $\overline{NS}_{125}$ as in (3) above and record the data on the sheet provided.

(5) Plot on rectilinear graph paper the $\overline{NS}$ values as ordinate versus the corresponding calculated standard concentrations expressed in mmol/L of chloride. The plot should be linear passing through the origin ±1 second.

c. The standard deviation of fit for both types of readouts can be calculated from the deviations, d_i, of the N points from the least squares fitted calibration line:

$$s_{fit} = \sqrt{\sum_{i=1}^{N} (d_i^2)/(N-2)}. \qquad (2)$$

If on visual inspection, one point of the plot exhibits a large residual from a smooth curve drawn through the remaining points, remeasure that standard solution. If the remeasured value for the solution continues to exhibit the large deviation, prepare that standard solution again, remeasure it, and compare the values obtained.

d. Sample Measurements

(1) Transfer the working sample to the sample titration cell by the appropriate pipetting technique. Titrate the working sample solution and select the two working standard solutions whose direct readout values or titration times most closely bracket that of the sample.

(2) Titrate the lower working standard, the working sample, and the higher working standard in that order and record each reading in the set.

(3) Repeat step d(2) until 3 'sets' of data are obtained.

(4) Repeat steps d(1), (2), and (3) for all of the samples.

(5) If one chloride value (working standard or working sample) in the measurement format is wrong due to a known error in technique, it may be disregarded and a repeat titration for that particular solution should be run immediately. However, if one chloride value appears to be in error and cannot be excluded due to known faulty technique, report that set of values. A repeat of the measurement format should be run for that particular working sample and both formats are to be reported with appropriate explanation.

e. Data Recording and Calculations

(1) On the data sheet, record the concentrations of the standard solutions in mmol/L of chloride to two decimal figures and the corresponding instrument readings to as many decimal places as possible.

(2) Calculate the concentration $\hat{C}$ of chloride present in the sample in mmol/L by mathematical interpolation as follows:

$$\hat{C} = C_1 + \frac{(C_2-C_1)(Y-X_1)}{(X_2-X_1)} \qquad (3)$$

where

$\hat{C}$ is the sample concentration of chloride in mmol/L,

C_1 is the low standard concentration of chloride in mmol/L,

C_2 is the high standard concentration of chloride in mmol/L,

Y is the chloride concentration in mmol/L or the titration time for the working sample minus the blank,

X_1 is the chloride concentration in mmol/L or the titration time for the low working standard minus the blank, and

X_2 is the chloride concentration in mmol/L or the titration time for the high working standard minus the blank.

(3) Record the $\hat{C}$ calculated values to a minimum of two decimal places in the column provided on the data sheet.

(4) Average the results for the three aliquots of the serum analyzed to obtain the 'single measurement' value. (See Section IV.)

(5) Average the four single measurement values to obtain a chloride value for the serum pool analyzed.

IV. RESULTS AND STATISTICAL ANALYSIS

The main objective of the statistical analyses of the round robin data is to derive measures of precision and accuracy for the micro- and macropipetting versions of the reference method. Precision is characterized by the variability of the protocol measurements within a single laboratory, $\hat{\sigma}_{within}$, and by the total variability of a laboratory's protocol measurements, $\hat{\sigma}_{total}$. This latter uncertainty includes the variability of 'between laboratory' measurements. Accuracy relates to the comparison between reference method and definitive method values and is related to the magnitude of the bias.

Each reported data point (test result) is the end product of three sets of data, the number of sets specified by the protocol. For simplicity of discussion, each reported data point is referred to as a single measurement, meaning that each is the product of a single run-through of the protocol. When "replication" is mentioned, replication of the entire protocol process is meant, and "replication error" thus refers to the variability among the end results of repeated run-throughs of the protocol. Each interlaboratory exercise is discussed separately; the final, detailed statistical analysis is reported for the results from IE-II.

A. Interlaboratory Exercise Results

1. Preliminary Interlaboratory Exercise (Dates Run: April-August 1975).

a. Objectives: To allow the participating laboratories to become familiar with and comment on the protocol and to determine interlaboratory precision.

b. Samples: Three vials, each containing a sample from the same serum pool. Each participating laboratory was to analyze a single portion of each sample within one day.

c. Procedure: The micro- or macropipetting protocol was used.

d. Data: The three data points reported by the individual laboratories are summarized in Table 4. The data are presented graphically in figure 3 as the percent difference from the collective average of the reported values. Essentially all reported values are within three percent of the collective average of 103.9 mmol/L with a relative standard deviation (CV) of less than two percent.

e. Comments and Protocol Deviations: The following laboratory comments or protocol deviations were received:

(1) Lab 3: Schales-Schales titration procedure used rather than C-A procedure.

(2) Lab 4: Instrument designed for 1:40 dilution rather than 1:50 specified in protocol.

(3) Lab 8: The three samples were turbid. (Growth of microorganisms?)

(4) Lab 11: Stock standard solutions prepared in 500 mL quantities. Glassware cleaning done with detergents.

(5) Lab 13: Instrument designed for 1:100 dilution rather than 1:50 specified in the protocol.

f. Direction: On examining these results with the statisticians and the Experts Committee, it was concluded that an interlaboratory exercise should be undertaken using samples with chloride concentration values determined by the definitive method.

Table 4. Serum chloride concentrations reported by the participating laboratories for the Preliminary Interlaboratory Exercise.

	[Cl], mmol/L[a]			
Laboratory	Vial 1	Vial 2	Vial 3	Laboratory Average
1	105.0	104.7	105.3	105.0
2	102.0	100.3	100.7	101.0
3	105.0	103.7	105.0	104.6[b]
4[c]	104.5	104.7	104.9	104.7
5	101.1	103.3	104.5	103.0
6[c]	103.5	103.7	104.6	103.9
7	105.6	105.6	104.2	105.1
8	103.2	104.1	103.5	103.6
9	104.5	104.3	104.2	104.3
10	102.0	104.0	103.7	103.2
11	101.7	102.0	102.7	102.1
12	104.7	105.7	105.0	105.1
13[c]	104.4	104.6	105.3	104.8

[a]Each value represents a single measurement on a sample.

[b]Schales-Schales titration procedure was used. Value was not used to determine collective laboratory average.

[c]Macropipetting procedure.

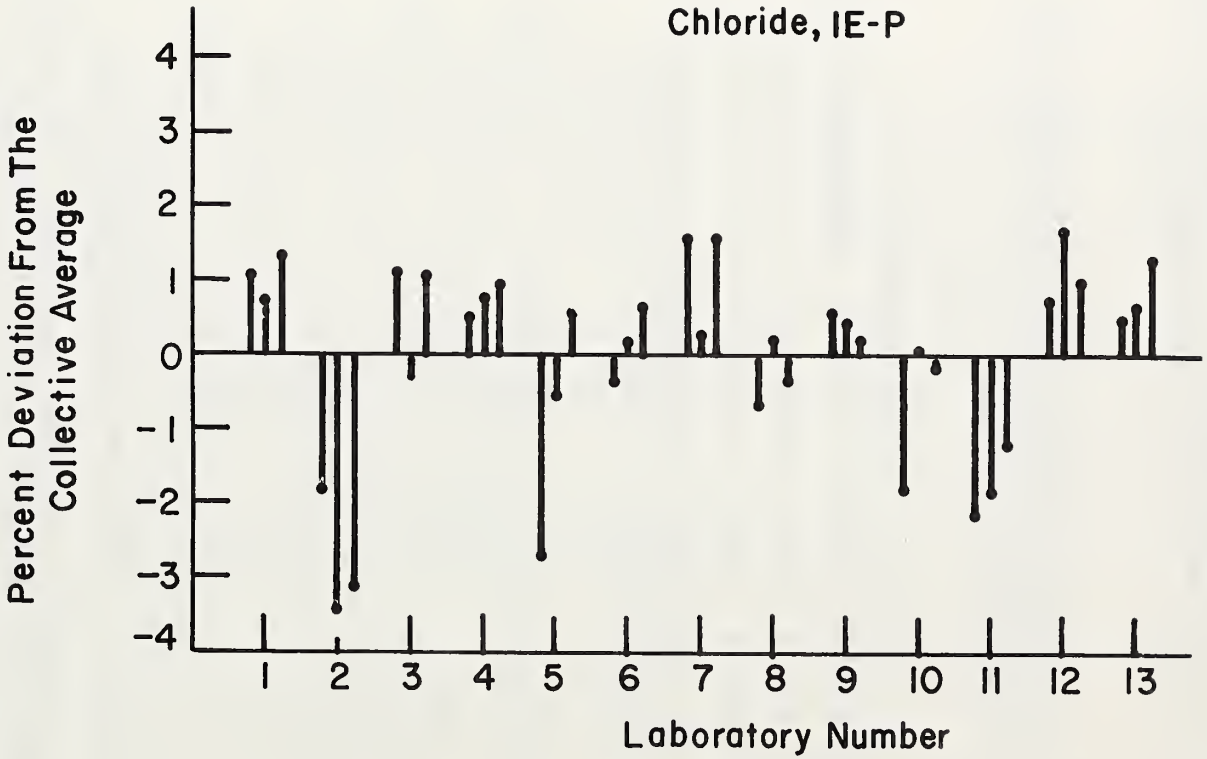

Figure 3. Percent deviation of individual results from the collective average of the measurements obtained in the Preliminary Interlaboratory Exercise.

2. Interlaboratory Exercise I (IE-I) (Dates Run: October-December 1975.)

a. Objectives: To test the micro- and macropipetting procedures on serum samples having a wider range of chloride values and to determine the imprecision and bias of the test results.

b. Samples: IE-I was a test series run on 12 samples – four vials (samples) of each of three different concentrations (Pools 2, 4, and 6). Each laboratory was to analyze two vials of each pool on one day and the remaining pairs of samples on a subsequent day with the requirement that a minimum of one day and a maximum of seven days should elapse between analyses.

c. Protocol: The micro- and macropipetting protocols were used.

d. Comments and Protocol Deviations: The following laboratory comments or protocol deviations were received:

(1) Lab 3: Schales-Schales titration procedure used.

(2) Lab 4: Used 1:40 dilution (instrument required) rather than 1:50 dilution.

e. Data: The single-measurement data reported by the laboratories for both pipetting alternatives are summarized in Table 5. The data are presented graphically in figure 4 as percent deviation of each one-day 'single measurement' average from the definitive method value. In general, the data reported by most laboratories were within three percent of the definitive method values. Most of the laboratories reported results that bracketed

Table 5. Concentrations of serum chloride reported by the participating laboratories for Interlaboratory Exercise I, micro- and macropipetting protocols.

Laboratory	[Cl], mmol/L[a]					
	Pool 2		Pool 4		Pool 6	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	84.71	88.60	102.49	100.69	114.52	110.19
	89.40	79.89	101.53	99.26	120.04	109.84
2	82.67	81.00	100.00	100.33	110.00	109.67
	83.00	82.33	100.33	100.33	110.00	109.33
3[b]	89.67	90.98	108.67	107.33	108.75	117.33
	89.37	89.67	105.86	108.28	116.67	117.12
4[c]	84.35	84.55	101.43	101.62	112.15	113.53
	84.32	84.49	101.62	102.61	112.62	113.25
5[c]	85.44	84.55	100.74	100.56	112.52	112.02
	84.52	83.44	100.52	101.26	113.28	110.97
6[c]	83.91	85.52	102.56	101.86	112.81	114.55
	84.61	85.17	101.24	102.68	113.95	115.32
7	84.60	84.45	101.49	101.82	112.72	113.35
	84.68	84.90	101.63	102.82	114.47	113.63
8	85.66	83.96	102.27	101.61	113.30	113.24
	84.28	84.71	102.43	102.18	112.69	113.03
9	84.27	84.57	102.23	102.20	112.17	112.27
	84.57	84.93	99.80	101.87	112.63	112.03
10	84.83	84.67	102.80	102.17	113.17	113.67
	84.83	84.67	102.00	102.67	112.17	114.17
11[c]	84.73	83.46	101.86	102.03	113.83	113.86
	84.78	83.46	103.62	102.24	116.29	112.78
12	85.37	84.53	102.40	102.10	114.27	112.33
	84.47	84.37	102.40	102.27	114.40	111.43
13	85.00	85.67	102.33	102.67	113.00	114.67
	85.33	85.67	102.33	103.00	113.33	114.67

continued

Table 5 (continued)

Laboratory	[Cl], mmol/L[a]					
	Pool 2		Pool 4		Pool 6	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
14	85.33	85.33	104.77	102.40	114.37	114.80
	85.37	84.73	104.37	102.77	116.43	113.77
15	84.61	84.77	102.29	102.13	113.28	113.19
	84.67	84.70	102.05	102.09	113.36	113.21
15[c]	84.29	85.01	102.16	101.97	113.63	113.32
	84.63	85.19	102.14	101.95	113.41	113.21
Definitive Method Values	84.5		101.8		112.9	

[a]Each value is the single measurement average of three C-A readings made on a single sample.

[b]Schales-Schales titration method, data for comparison only.

[c]Macropipetting protocol.

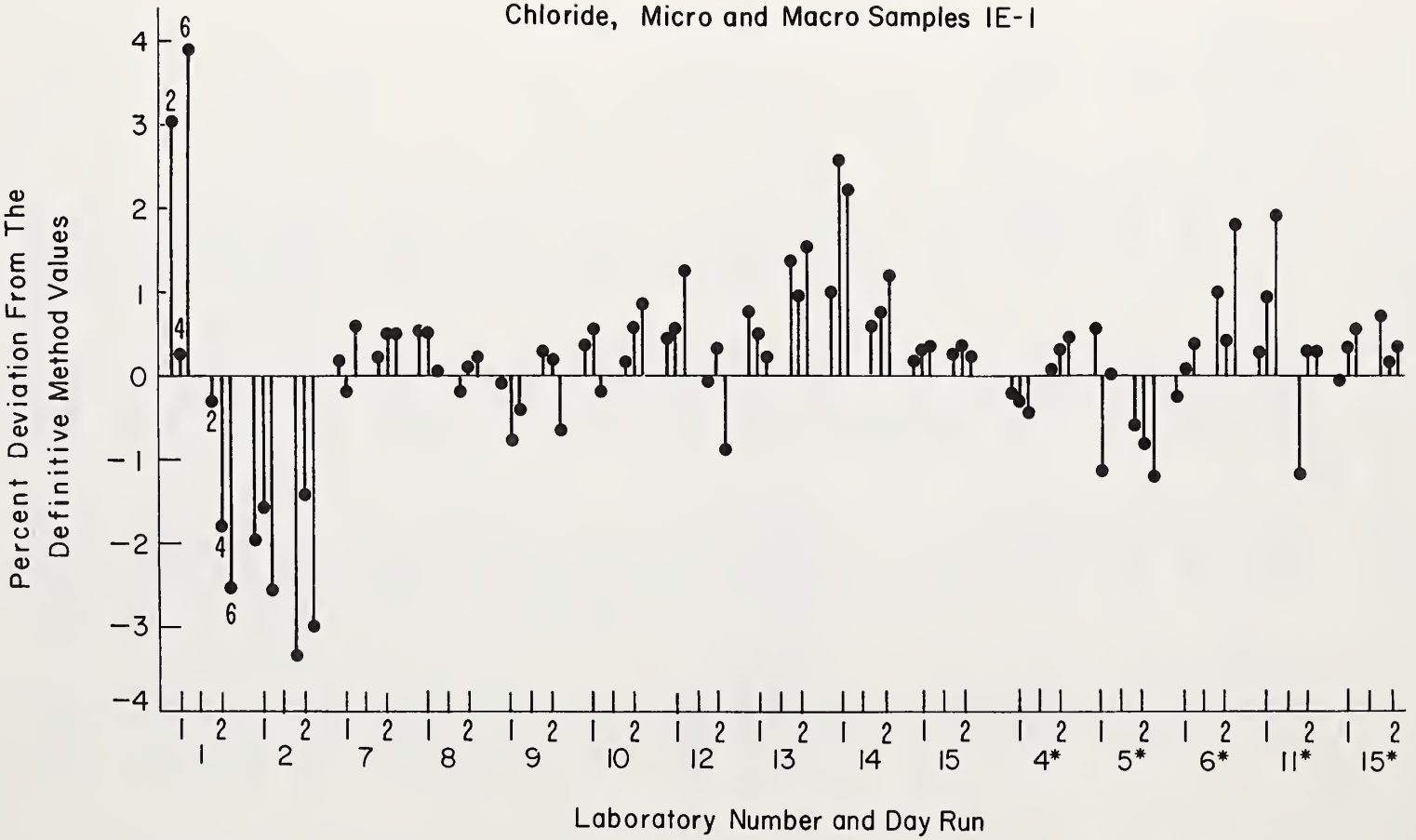

Figure 4. Percent deviations of the Interlaboratory Exercise I measurements using micro- and macropipetting from the definitive method values. The analyzed pools are identified by the numbers 2, 4, and 6 next to the data from laboratory 1. The presentation is similar for the remaining results. The numbers 1 and 2, placed above the laboratory number, represent the first day and subsequent day test results, respectively. The asterisk next to the laboratory number designates those laboratories using the macropipetting procedure.

or were quite close to the definitive method values.

f. Direction: A second Interlaboratory Exercise (IE-II) was to be run using both the micro- and macropipetting protocols. Test samples would cover the full range of chloride concentrations.

3. Interlaboratory Exercise II: (IE-II. Dates Run: February-April 1976.)

a. Objective: To test both the micro- and macropipetting alternatives of the protocol on samples with chloride concentrations over the nominal range of 79.2 to 116.8 mmol/L.

b. Samples: IE-II was a test series run on a total of 20 samples – four vials of each of five different chloride concentrations (Pools 1, 3, 4, 5, and 7). Each laboratory was to analyze two vials of each concentration on the first day and the remaining pairs of samples after the elapse of a minimum of one day and a maximum of seven days.

c. Protocol: The micro- and macropipetting versions of the protocol were used.

d. Data Statistical Analysis: Results from IE-II are given in Tables 6-7 and illustrated in figure 5.

e. Statistical Analysis: The data are presented as two-way tables in which the rows represent the different participating laboratories and the columns represent the different sample pools. The sample pool concentrations ranged from 79.2 to 116.8 millimoles of chloride ion per liter of serum. The results for the micropipetting procedure and for the macropipetting procedure are listed separately, and all single measurements reported

Table 6. Concentration of serum chloride reported by the participating laboratories for Interlaboratory Exercise II, micropipetting protocol.

	- - - - - - - - - [Cl], mmol/L - - - - - - - - -				
Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
1-1	79.53	94.70	102.38	108.57	117.21
	80.05	94.77	102.65	108.47	117.74
1-2	80.32	93.57	103.20	107.91	117.28
	80.14	95.45	102.31	108.16	117.64
2-1	77.00	91.60	99.60	106.62	116.10
	76.75	90.70	101.70	104.90	113.90
2-2	76.36	92.10	99.95	104.52	114.20
	77.94	92.66	99.95	106.10	115.00
4-1	79.37	94.88	101.90	108.00	117.71
	79.95	94.28	101.82	107.64	117.80
4-2	79.49	95.37	102.70	108.26	118.45
	79.67	94.73	101.88	107.65	118.44
7-1	78.76	94.47	101.96	107.01	117.49
	79.20	95.31	100.00	107.20	117.68
7-2	79.45	95.40	101.77	107.59	118.12
	80.36	93.57	102.05	107.29	115.49
8-1	78.05	94.33	102.01	106.92	116.64
	79.10	94.96	101.31	106.68	117.17
8-2	79.43	94.40	102.07	107.21	115.79
	78.60	94.00	101.30	107.26	116.16
9-1	80.20	94.83	101.13	106.53	114.53
	79.50	94.10	100.87	107.23	113.50
9-2	80.43	94.17	100.97	106.30	116.73
	78.53	93.70	100.17	106.07	116.30
10-1	78.35	94.50	100.40	105.20	115.60
	78.53	94.03	99.80	106.90	114.00
10-2	80.10	93.10	99.41	107.29	116.95
	77.80	93.63	102.50	106.14	116.99

continued

Table 6 (continued)

	- - - - - - - - - [Cl], mmol/L - - - - - - - - -				
Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
13-1	80.00	94.67	102.70	107.30	117.20
	79.70	95.00	102.00	107.00	116.50
13-2	79.80	95.00	102.00	106.33	117.20
	79.50	95.00	101.90	107.20	117.00
14-1	81.50	96.60	103.30	109.60	118.90
	80.20	96.50	106.60	109.90	117.30
14-2	80.40	95.10	101.40	107.00	117.30
	79.30	94.30	102.70	107.80	116.50
15-1	79.46	94.59	101.93	107.47	116.92
	79.54	94.76	101.99	107.52	116.89
15-2	79.51	94.81	102.01	107.40	116.99
	79.39	94.68	101.97	107.23	116.86
Definitive Method Values	79.2	94.0	101.8	107.2	116.8

[a]The laboratory designation consists of two parts: the initial digit(s) represents the assigned laboratory number and the last digit represents either the first or second day's results.

Table 7. Concentration of serum chloride reported by the participating laboratories for Interlaboratory Exercise II, macropipetting protocol.

	- - - - - - - - - [Cl], mmol/L - - - - - - - - -				
Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
5-1	79.87	94.74	100.85	106.51	116.45
	78.33	94.14	100.49	106.81	115.27
5-2	79.45	93.44	101.58	105.68	116.70
	78.68	94.17	102.04	107.14	116.64
6-1	78.24	94.73	101.88	106.43	117.08
	78.63	95.01	101.12	106.24	117.00
6-2	78.56	93.48	101.34	106.01	115.95
	78.30	93.61	101.63	106.68	116.52
11-1	79.58	93.53	101.26	107.12	115.22
	79.14	95.09	103.43	107.03	115.11
11-2	80.11	94.48	101.98	108.01	116.74
	79.60	94.87	102.00	107.63	116.84
Definitive Method Values	79.2	94.0	101.8	107.2	116.8

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or second day's results.

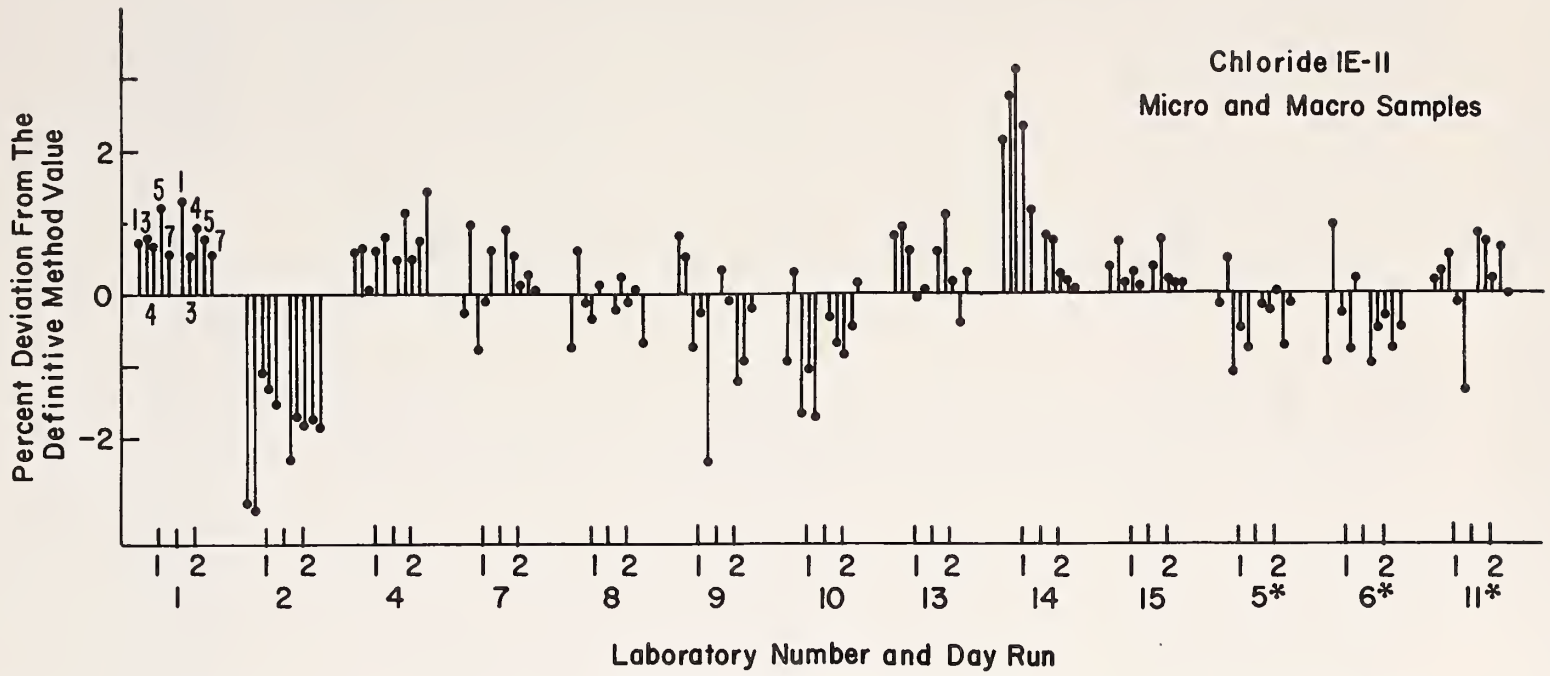

Figure 5. Percent deviations of the Interlaboratory Exercise II measurements using micro- and macropipetting from the definitive method values. The analyzed pools are identified by the numbers 1, 3, 4, 5, and 7 next to the data from laboratory 1. The presentation is similar for the remaining results. The numbers 1 and 2, placed directly above the laboratory number, designate the first day and subsequent day test results, respectively. The asterisk next to the laboratory number designates those laboratories using the macropipetting procedure.

are included in the tables. The definitive method values for the chloride concentrations in the sample pools are listed at the bottom of Tables 6-7.

The following statistical analysis was made. First the data were inspected by calculating the percent deviation of each day's results for each pool from an overall average for that sample pool. These percent deviation values for all laboratories using the two pipetting procedures are listed in Tables 8-9.

Table 8. Percent deviations from averages for chloride in serum from Interlaboratory Exercise II, micropipetting protocol.

Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
1-1	0.64	0.40	0.79	1.25	0.70
1-2	1.20	.16	1.03	.79	.69
2-1	-3.04	-3.40	-1.04	-1.33	-1.42
2-2	-2.69	-2.10	-1.73	-1.75	-1.76
4-1	.48	.24	.15	.59	.94
4-2	.38	.73	.57	.72	1.54
7-1	-.38	.56	-.71	-.07	.80
7-2	.79	.13	.20	.24	.13
8-1	-.89	.30	-.05	-.36	.21
8-2	-.34	-.17	-.02	.05	-.58
9-1	.72	.11	-.69	-.28	-2.26
9-2	.25	-.45	-1.12	-.93	-.12
10-1	-1.06	-.10	-1.58	-1.06	-1.59
10-2	-.42	-1.05	-.74	-.44	.27
13-1	.72	.51	.63	-.03	.17
13-2	.46	.68	.24	-.39	.38
14-1	1.98	2.32	3.19	2.39	1.24
14-2	.72	.36	.34	.20	.21
15-1	.28	.34	.25	.29	.21
15-2	.21	.41	.28	.12	.23
Average used in calculations, mmol/L	79.28	94.36	101.71	107.18	116.65

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or second day's results.

Table 9. Percent deviations from averages for chloride in serum from Interlaboratory Exercise II, macropipetting protocol.

Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
5-1	0.07	0.18	-0.95	-0.11	-0.37
5-2	.03	-.50	.17	-.34	.32
6-1	-.77	.63	-.13	-.41	.64
6-2	-.77	-.77	-.15	-.40	-.05
11-1	.40	.04	.70	.28	-.97
11-2	1.03	.43	.35	.98	.43
Average used in calculations, mmol/L	79.04	94.27	101.63	106.77	116.29

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or second day's results.

A comparison was next made of the ability of each laboratory to replicate its values relative to that of the average replication ability of all laboratories. This was done by comparing the standard deviation for each day's measurements for each pool against the average standard deviation for all laboratories for that pool (see Tables 10-11). If all of the participating laboratories were of the same population in regard to replication error, the standard deviation ratios reported in Tables 10-11 would be larger than 2.44 and 2.14, respectively, only about one percent of the time. In practice, it is not too uncommon to encounter

Table 10. Ratios of standard deviations to average standard deviation for chloride in serum from Inter-laboratory Exercise II, micropipetting protocol.

Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
1-1	0.70	0.12	0.30	0.17	0.74
1-2	.24	3.11	.98	.43	.50
2-1	.34	1.49	2.32	2.95	3.08
2-2	2.14	.93	.00	2.71	1.12
4-1	.78	.99	.09	.62	.13
4-2	.24	1.06	.91	1.05	.01
7-1	.59	1.39	2.16	.33	.27
7-2	1.23	3.03	.31	.51	3.69
8-1	1.42	1.04	.77	.41	.74
8-2	1.12	.66	.85	.09	.52
9-1	.95	1.21	.29	1.20	1.44
9-2	2.57	.78	.88	.39	.60
10-1	.24	.78	.66	2.91	2.24
10-2	3.11	.88	3.41	1.97	.06
13-1	.41	.55	.77	.51	.98
13-2	.41	.00	.11	1.49	.28
14-1	1.76	.17	3.64	.51	2.24
14-2	1.49	1.32	1.43	1.37	1.12
15-1	.11	.28	.07	.09	.04
15-2	.16	.22	.04	.29	.18
Average standard deviation, mmol/L	0.523	0.427	0.641	0.413	0.505

[a]The laboratory designation consists of two parts: the initial digit(s) represents the assigned laboratory number and the last digit represents either the first or second day's results.

Table 11. Ratios of standard deviations to average standard deviation for chloride in serum from Inter-laboratory Exercise II, macropipetting protocol.

Laboratory[a]	Pool 1	Pool 3	Pool 4	Pool 5	Pool 7
5-1	2.36	0.98	0.53	0.58	3.37
5-2	1.18	1.19	.68	2.83	.17
6-1	.60	.46	1.12	.37	.23
6-2	.40	.21	.43	1.30	1.63
11-1	.68	2.54	3.21	.17	.31
11-2	.78	.63	.03	.74	.29
Average standard deviation, mmol/L	0.461	0.435	0.478	0.364	0.247

[a]The laboratory designation consists of two parts: the initial digit(s) represents the assigned laboratory number and the last digit represents either the first or second day's results.

ratios that are somewhat larger, as this is a reflection of some heterogeneity of the laboratory population in regard to replication error. (As long as the standard deviation ratios are not too large, this is normally not used as a reason for rejection of a laboratory. However, laboratories with large standard deviation ratios are advised to reexamine their procedures for possible sources of excessive replication error.)

The calculations on IE-II data were made on the data in Tables 6 and 7 using a weighted least squares fit to the following model [21]:

$$Y_{ijk} = \mu_i + \beta_i (X_j - \tilde{X}) + \lambda_{ij} + \varepsilon_{ijk} \qquad (4)$$

where:

Y_{ijk} = the sample concentration reported by the i^{th} laboratory, for the j^{th} sample, and for the k^{th} replicate measurement,

μ_i = a constant factor associated with the average bias for laboratory i,

β_i = a slope factor for laboratory $\underline{i}$, expressing the relation of bias to concentration,

X_j = the observed average concentration for sample pool j (this average is taken over all laboratories),

$\tilde{X}$ = the weighted average concentration for all samples (this average is taken over all laboratories and over all sample pools),

λ_{ij} = a random sample interference factor (matrix effect) for laboratory i and sample pool j, and

ε_{ijk} = a random replication error.

The above model is quite general, and extensive experience has shown that it is well suited to describe a number of measurement factors in inter-laboratory tests [22].

Weighted analyses of variance were made on the data in the two-way tables using the fits to the above model. (A modified version of the weighting procedure reported in reference 23 was used.) From the analyses it is possible to derive the following estimates for three components of variability, each characterized by its standard deviation:

$\hat{\sigma}_{\varepsilon} = \hat{\sigma}_{\varepsilon(Repl)}$ = the uncertainty observed for replicate measurements in a given laboratory on a given day,

$\hat{\sigma}_{D} = \hat{\sigma}_{Day}$ = the additional uncertainty that is observed when measurements are made on different days within the same laboratory, and

$\hat{\sigma}_{L} = \hat{\sigma}_{Lab}$ = the additional uncertainty that is observed when measurements are made by different laboratories.

From the analyses, it was observed that the ranges of values for the $\hat{\sigma}_{\varepsilon}$, $\hat{\sigma}_{D}$, and $\hat{\sigma}_{L}$ components of standard deviation were small, and that the values did not depend significantly on the chloride ion concentration. Because of this, only average $\hat{\sigma}_{\varepsilon}$, $\hat{\sigma}_{D}$, and $\hat{\sigma}_{L}$ values are reported. These components of standard deviation are given in Table 12.

Table 12. Components of standard deviation in mmol/L for all IE-II chloride ion levels (79-117 mmol/L).

	$\hat{\sigma}_{\varepsilon(Repl)}$	$\hat{\sigma}_{Day}$	$\hat{\sigma}_{Lab}$
Micropipetting Protocol (Pooled results from 10 labs)	.69	.42	.90
Macropipetting Protocol (Pooled results from 3 labs)	.53	.37	.36

Because of the relatively small size of the chloride ion IE tests, the individual components of standard deviation are considered to be only advisory in nature. Nevertheless, they do seem to indicate that the three components ($\hat{\sigma}_{\varepsilon}$, $\hat{\sigma}_{D}$, and $\hat{\sigma}_{L}$) are all of about the same order of magnitude. The final,

statements of uncertainty are made through the recombination of these components.

One such final statement is $\hat{\sigma}_{within}$, the expected uncertainty within a single laboratory from running the complete protocol (2 replicates/day for 2 days). The $\hat{\sigma}_{within}$ results are reported in columns three and seven in the top section of Table 13, and are calculated as follows:

$$\hat{\sigma}_{within} = \sqrt{\frac{\hat{\sigma}_{\varepsilon}^{2}}{4} + \frac{\hat{\sigma}_{D}^{2}}{2}} \quad . \tag{5}$$

These are the expected uncertainties that a single average laboratory could see by repeating the complete protocol a number of times and observing the variability of its results. This $\hat{\sigma}_{within}$ is not the total uncertainty since there is also a "between laboratory" component, $\hat{\sigma}_{Lab}$.

The standard deviation of the total uncertainty expected as a result of a single laboratory running the complete protocol is calculated as follows:

$$\sigma_{Total} = \sqrt{\frac{\hat{\sigma}_{\varepsilon}^{2}}{4} + \frac{\hat{\sigma}_{D}^{2}}{2} + \hat{\sigma}_{L}^{2}} \quad . \tag{6}$$

Columns four and six in the top section of Table 13 list such standard deviations for the micro- and macropipetting protocol data from IE-II. The precision goal for the reference method is listed in column five. Comparison of the tabulated standard deviations and the goal shows that the precision goals have been met.

Table 13. Summary of imprecision and bias results in mmol/L for chloride ion in serum, IE-II.

	- - - - - - - - - - - - - 1σ Precision - - - - - - - - - - - - -						
	Micropipetting Protocol				Macropipetting Protocol		
Cl Level mmol/L	$\hat{\sigma}_{comp}$	$\hat{\sigma}_{within}$	$\hat{\sigma}_{total}$	Goal	$\hat{\sigma}_{total}$	$\hat{\sigma}_{within}$	$\hat{\sigma}_{comp}$
79-117	.32	.46	1.01	1.5	.52	.37	.30

	- - - - - - - - - - - - - Accuracy - - - - - - - - - - - - -		
	Micropipetting Protocol		Macropipetting Protocol
Cl Level mmol/L	Round Robin Composite Bias $(X_{obs}-X_{DM})$	Goal	Round Robin Composite Bias $(X_{obs}-X_{DM})$
79	.1	±2.0	-.2
94	.4	±2.0	.3
102	-.1	±2.0	-.2
107	0	±2.0	-.4
117	-.1	±2.0	-.5

The standard errors of the IE-II composite values are given in columns two and eight of the top section of Table 13. These standard errors are calculated from the components of standard deviation as follows:

$$\hat{\sigma}_{Comp} = \sqrt{\left(\frac{1}{N}\right)\left[\frac{\hat{\sigma}_{\varepsilon}^{2}}{4} + \frac{\hat{\sigma}_{D}^{2}}{2} + \hat{\sigma}_{L}^{2}\right]} \qquad (7)$$

where N represents the 10 or 3 laboratories participating in the micro- or macropipetting procedures, respectively. The bottom section of Table 13 lists the observed biases between the reference method

interlaboratory composite values and the definitive method values. The observed biases are within the goals for the reference method.

The composite IE-II sample averages ± twice the standard error for the micro- and the macropipetting versions, and for the corresponding definitive method values are listed in Table 14.

The accuracy of the IE-II results is within the recommended goal of the reference method.

Table 14. Summary of chloride ion in serum values ±2 standard errors.

IE-II - Composite Values (mmol/L)		Definitive Method Values (mmol/L)
Micro	Macro	
79.3 ± .64	79.0 ± .60	79.2 ± .4[a]
94.4 ± .64	94.3 ± .60	94.0 ± .5
101.7 ± .64	101.6 ± .60	101.8 ± .5
107.2 ± .64	106.8 ± .60	107.2 ± .5
116.7 ± .64	116.3 ± .60	116.8 ± .6

[a]Estimated maximum error of 0.5 percent of the value as reported by NBS Analytical Mass Spectrometry Section. This estimated maximum error includes both imprecision and an estimated upper bound for possible systematic errors. The estimated maximum error is believed to be equal to or greater than the true error for the 95 percent confidence limits.

Auxiliary Statistical Analysis

The protocol requires a check on the automated titrimeter by running a calibration curve each day using freshly prepared standard solutions. The necessity of these curves also provides a check on the correct preparation of the standard solutions. The data reported here on the calibration curve check are advisory in nature since in the actual analytical procedure only the pair of calibrating solutions nearest to the unknown concentration is used. The calibration curve data for the micro- and macropipetting procedures were reported and are given in Tables 15 and 16. Straight line least square fits were made to these data and the resultant standard deviations of fit are given in Table 17. These standard deviations of fit are expressed in units of chloride ion concentration (mmol/L). The calibration curve data show excellent linearity with all curves having standard deviation of fit of 1.23 mmol/L or less, and 12 of the 14 curves having s_{fit} of 0.79 mmol/L or less. This analysis indicates that if in the calibration step it is found that any calibration point deviates from the calibration curve by more than 1.3 mmol/L, then the standard solutions and the instrument should be checked for sources of excessive error before proceeding further into the analysis.

Table 15. Calibration curve data for chloride in serum, Interlaboratory Exercise II, micropipetting protocol.

Lab. No.[a]		Std. 1	Std. 2	Std. 3	Std. 4	Std. 5
1-1	X[b]	75.0	90.0	100.0	110.0	125.0
	Y[c]	75.16	90.02	100.28	110.08	124.74
1-2	X	75.0	90.0	100.0	110.0	125.0
	Y	75.00	90.70	99.95	109.80	125.30
4-1	X	75.02	90.01	100.00	110.00	125.00
	Y	75.31	89.73	100.36	110.38	125.47
4-2	X	75.02	90.01	100.00	110.00	125.00
	Y	74.62	89.86	99.81	109.72	124.82
8-1	X	75.00	90.00	100.00	110.00	125.00
	Y	75.0	89.8	99.8	110.2	124.8
9-1	X	75.01	90.00	100.00	110.03	125.01
	Y	74.4	89.4	99.9	109.4	124.8
13-1	X	75.00	90.00	100.00	110.00	125.00
	Y	75.0	90.0	100.0	110.0	125.0
14-1	X	75.0	90.0	100.0	110.0	125.0
	Y	76.0	90.0	99.0	109.0	124.0
14-2	X	75.0	90.0	100.0	110.0	125.0
	Y	75.0	90.0	100.0	111.0	124.0
15-1	X	75.00	90.00	100.00	110.00	125.00
	Y	47.14	56.14	62.16	68.23	77.23

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or second day's results.

[b]X = standard solution values in mmol/L.

[c]Y = instrument readings.

Table 16. Calibration curve data for chloride in serum, Interlaboratory Exercise II, macropipetting protocol.

Lab. No.[a]		Std. 1	Std. 2	Std. 3	Std. 4	Std. 5
5-1	X[b]	75.01	90.01	100.01	110.00	125.00
	Y[c]	23.2	27.9	31.0	34.2	39.0
5-2	X	75.01	90.01	100.01	110.00	125.00
	Y	23.1	27.8	31.0	34.2	38.8
6-1	X	75.00	89.96	99.93	110.27	124.87
	Y	79.06	94.40	104.85	115.58	130.36
11-1	X	74.99	90.03	100.04	110.08	124.98
	Y	26.62	30.34	33.5	36.72	41.42
11-2	X	74.99	90.03	100.04	110.08	124.98
	Y	25.74	30.9	33.94	38.22	42.84

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or second day's results.

[b]X = standard solution values in mmol/L.

[c]Y = instrument readings.

Table 17. Calibration curve results for chloride in serum as standard deviation of fit (s_{fit}) in mmol/L.

- - - - - Micro - - - - -		- - - - - Macro - - - - -	
Laboratory Number[a]	s_{fit}	Laboratory Number[a]	s_{fit}
1-1	0.18	5-1	0.16
1-2	.41	5-2	.18
4-1	.30	6-1	.13
4-2	.10	11-1	1.23
8-1	.20	11-2	.98
9-1	.28		
13-1	(0)		
14-1	.79		
14-2	.51		
15-1	.04		

[a]The laboratory designation consists of two parts: the initial digit(s) represent the assigned laboratory number and the last digit represents either the first or the second day's results.

V. DISCUSSION

A. Candidate Protocol:

1. Preliminary Tests

Generally, in the development of a reference method where the state of analytical knowledge leaves an uncertainty in the choice of a candidate reference method, it is essential that investigations be undertaken to assure optimized analytical conditions, minimized interferences, and freedom from other sources of bias. Such preparation helps avoid initiating the interlaboratory testing process with inappropriate procedures. However, in the case of chloride, the similarity of test results obtained by use of the C-A method and the highly specific IDMS method, led the Committee to decide to proceed directly to the interlaboratory exercise phase with the C-A method, without further preliminary studies.

2. Specifications

In light of prior experience [8,9,10,14,15,24], the written protocol is explicit as to reagent and glassware specifications, pipetting, and directions for dilution of standard and sample. Thus, Class A or equivalent glassware, reagent grade or equivalent chemicals, 'tested' water, analytical balances with a ±0.1 mg weighing capability, and micropipets with specified accuracy and precision are used. In addition, the reference method provides for the use of analytical techniques that should reduce the combined error due to weighing, pipetting, and dilution to below one percent of the measured values.

3. Coulometric–Amperometric Instrumentation

Coulometric-amperometric instrumentation has been used extensively for the determination of serum chloride in the clinical laboratory. In the Committee's judgment, it was felt that these instruments might provide the necessary

accuracy and precision in a clinical reference method, and that instructions for the use of specific instruments should not be given in the protocol. Instead, it should be left to the investigator to use the manufacturer's instructions for the specific instrument he employs, since different readouts, electrolytes, dispersants, micropipets, and sample volumes might be used. Thus specifications were not presented other than a requirement for stable instrument operating conditions. As in sample preparation and handling, the human element in achieving accuracy and precision is critical. It is essential that operators be thoroughly familiar with their instruments and alert to the onset of instrumental difficulties. Instrument linearity requirements are not included in the protocol since the bracketing method for obtaining valid measurements is used to ensure minimal error attributable to instrumental variation.

Instruments produced by five different manufacturers[11] were used in the interlaboratory exercise. All gave excellent results and are listed in Table 18. On examination of the data reported for calibration curves, excellent linearity was found over the range of chloride concentrations from 75 to 125 mmol/L. More than 92 percent of the calibration curves showed standard deviations of fit of 1.00 mmol/L or less. A standard deviation of fit larger than 1.30 mmol/L would clearly warrant a laboratory's examination of its operation of the procedure and/or preparation of the standard solutions.

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[11] To describe instruments, it was necessary to identify commercial products by the manufacturer's name. In no instance does such identification imply endorsement by the National Bureau of Standards, nor does it imply that the particular product is necessarily the best available for that purpose.

Table 18. Instruments, pipets, and various protocol parameters used by the participating laboratories in Interlaboratory Exercise I and Interlaboratory Exercise II[a].

Lab.	Instrument	Readout Direct	Readout Time	Electrolyte	Dispersant	Micropipet	Protocol Micro	Protocol Macro	Sample Volume (μL)
1	Buchler	X		NR[b]	NR[b]	Clay Adams 4625	X		—
2	Corning 920M	X		Corning Acid Buffer	NR[b]	Dade Micropipettes	X		100
4	Buchler 4-2500	X		Acetic/Nitric	Gelatin Reagent	Micromedic	X	X	100
5	AMINCO Co.	X		NR[b]	Gelatin Reagent	—		X	—
6	Buchler 4-2088		X	Acetic/Nitric	Polyvinyl Alcohol	—		X	—
8	Corning-EEL 920	X		Acetic/Nitric	Gelatin Reagent	—		X	—
9	Radiometer CMT-10	X		Radiometer LS 101	(Premixed)	NR[b]	X		20
10	AMINCO J4-4411	X		Acetic/Nitric	Gelatin Reagent	Automatic	X		40
11	AMINCO 4-4433		X	Acetic/Nitric	Gelatin Reagent	—		X	—
13	Radiometer CMT-10	X		Acetic/Nitric	Polyvinyl Alcohol	SMI Micro/Pettor	X		20
14	Beckman Cl/CO_2	X		.12 $\underline{N}$ H_2SO_4	Polyvinyl Alcohol	Beckman	X		10
15	AMINCO J4-4415		X	Acetic/Nitric	Gelatin Reagent	—		X	—
15	AMINCO J4-4415		X	Acetic/Nitric	Gelatin Reagent	Micromedic	X		100

[a] Laboratory 3 information not included since the Schales-Schales titration procedure was used.
[b] Not reported.

4. Statistical Analysis

The results discussed here are based on the analysis of four replicate samples analyzed as pairs on two separate days. Adherence to this pattern of replicate analysis facilitated analysis of the performance of the reference method.

The imprecision and bias goals of 1.5 and 2.0 mmol/L, respectively set at the 100 mmol/L level, were in fact reached over the total concentration range by the laboratories using either the micro- or macropipetting protocols. We believe there are no differences in the imprecision or bias values obtained by the two pipetting alternatives. In Table 13, the $\hat{\sigma}_{total}$ value for the macropipetting procedure was observed to be approximately one-half that for the micropipetting procedure. Because of the limited three-laboratory sample in the macropipetting procedure, the $\hat{\sigma}_{total}$ has a lower reliability than that for the micropipetting procedure which involved 10 laboratories. Comparison of results from IE-I with those from IE-II suggests that the imprecisions and biases for both pipetting procedures are essentially equal (see, for example, figures 4 and 5). The micropipetting procedure results may be considered indicative of the imprecisions and biases for both pipetting procedures.

Considering the uncertainties in the definitive method values, the results from the micro- and macropipetting procedures show no significant bias from the definitive method values, Table 14.

In summary, for laboratories in the population typical of those participating in this study (i.e., clinical laboratories that have practiced the reference method and are in good quality control) have imprecisions ($\hat{\sigma}_{total}$) within 1.0 mmol/L and biases within 0.5 mmol/L can be expected in the performance of this reference method.

Auxiliary statistical analysis on the calibration curve data showed excellent linearity with all curves having standard deviations of fit of 1.23 mmol/L or less and 12 of the 14 curves having s_{fit} of 0.79 mmol/L or less.

VI. CONCLUSIONS

A candidate reference method, specified by a written protocol for the determination of serum chloride employing commercial coulometric-amperometric instrumentation, was evaluated by comparing the results of analyses run on serum and aqueous samples in a selected group of laboratories that used the method against definitive method values for the same samples. The results for samples having chloride concentrations in the 79 to 117 mmol/L range, showed a total imprecision ($\hat{\sigma}_{total}$) of approximately 1.0 mmol/L and a maximum bias of 0.5 mmol/L. In IE-I and IE-II, similar imprecisions and biases were found whether macropipetting or micropipetting was used in the C-A procedure.

Statistical analysis of the results shows that the C-A method can be carried out with the accuracy and precision expected of a reference method for serum chloride. Hence, the candidate method is considered to be the reference method. This reference method may be used to establish the accuracy of field methods for chloride by comparative testing. It may also be used to determine reference material values for serum chloride. Each of these uses would require an appropriate experimental design to ensure its achievement of accuracy and precision equal to those demonstrated here.

We would like to especially thank the principal investigators and other scientists in the participating laboratories (listed in Appendix A), who, through their efforts, made this work meaningful and possible. We thank Dr. David Bayse and Ms. Sue Lewis, CDC, for providing excellent, homogeneous serum pools used in the interlaboratory testing process.

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[24] Clinical reference methods are also being developed at NBS for lithium and magnesium.

APPENDIX A

Scientists not previously acknowledged who contributed to this study are:

Mrs. Harriet Bailey
Dr. Daniel Grisley
New England Deaconess Hospital

Ms. Leyda Ortega
Mr. G. S. King
American Hospital Supply

Ms. J. Schmitz
Veterans Administration Hospital

Ms. K. Koch
Beckman Instruments

Miss C. Wong
Mrs. Shirley Wertlake
University of California

Mr. Frank Doherty
Pennsylvania Department of Health

Ms. R. Stauffenberg
Food and Drug Administration

U. S. Department of Commerce
Frederick B. Dent
Secretary

National Bureau of Standards
Richard W. Roberts, Director

National Bureau of Standards Certificate of Analysis

Standard Reference Material 919

Sodium Chloride
(Clinical Standard)

This Standard Reference Material is certified for use in the calibration and standardization of procedures employed in the determination of sodium and chloride ions in clinical analyses. The sample consists of highly purified sodium chloride. Chemical assay as well as analyses for specific impurities indicate that the material may be considered essentially pure, except for occluded moisture.

Purity ..... 99.9 ± 0.0 percent

The above value for the purity of the material is based on a sample dried over magnesium perchlorate for 24 hours. At room temperature sodium chloride is hygroscopic above 60 percent relative humidity. The sorbed water can be removed, however, by desiccation over freshly exposed P_2O_5 or $Mg(ClO_4)_2$ for 24 hours. Chloride was determined using the coulometric method of Marinenko and Taylor [J. Res. NBS, 67A, 31 (1963)].

Based on 8 independent measurements of chloride content, the sample is considered homogeneous.

When the material is crushed and dried at 200 °C for 18 hours, the loss of moisture is about 0.08 percent. Coulometric determinations of chloride on the dried material indicate 99.995 ± 0.004 percent purity.

The sodium chloride used for this Standard Reference Material was obtained from the J. T. Baker Chemical Company, of Phillipsburg, New Jersey. Analyses were performed by G. Marinenko, J. R. Baldwin, M. Darr, and T. C. Rains.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. A. Durst.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Washington, D. C. 20234
August 6, 1973

J. Paul Cali, Chief
Office of Standard Reference Materials

(over)

This material was examined for compliance with the specifications for reagent grade sodium chloride as given in Reagent Chemicals, 4th edition, published by the American Chemical Society. The material meets or exceeds the minimum requirements in every respect.

Sodium was assayed using a gravimetric procedure in which the sodium chloride was converted to sodium sulfate. Approximately 250 mg of sodium chloride (dried at 500 °C for 4 hours in a platinum crucible) was dissolved in ultrapure sulfuric acid solution (1:1) and evaporated to dryness. Ammonium carbonate was added and the crucible slowly heated to 600 °C, then 900 °. This treatment was repeated until the weight of sodium sulfate remained constant. Based on 6 determinations, the sodium assay is 39.3_2 weight percent or 99.9_6 percent of the amount computed for perfectly pure, stoichiometric NaCl.

A semiquantitative survey for trace elements by emission spectroscopy indicated less than 10 μg/g calcium, copper, iron, and magnesium. A value of less than 3 μg/g magnesium was obtained by atomic absorption spectrometry. Flame emission spectrometry indicated the presence of the following elements: potassium, 11 μg/g; calcium and cesium, less than 2 μg/g; and rubidium and lithium less than 0.5 μg/g.

This Standard Reference Material is intended for "in vitro" diagnostic use only.

This material is intended for use as a standard for the determination of sodium and chloride ions in clinical chemistry.

Sodium is most frequently determined by flame emission photometry. The operative details of this methodology vary from instrument to instrument and are discussed at length in their respective operating manuals. A standard solution of 100 mmol of sodium chloride per liter (suitable for both sodium and chloride determinations) may be prepared by placing 5.85 g of SRM 919 (dried at 110 °C) in a 1-liter volumetric flask and adding 3 ml of concentrated nitric acid (ACS Reagent Grade) and 100 ml of deionized water. After the NaCl is dissolved, dilute to the mark with deionized water. The concentration required for analysis may be prepared by accurate dilutions with distilled water.

This Standard Reference Material should be stored in the well-closed original bottle under normal laboratory conditions. It is recommended that weighing and other manipulations not be made when the relative humidity exceeds 60 percent.

Solutions of SRM 919 are stable indefinitely when stored in a well-stoppered, all-glass container. All such solutions should be clear and display no turbidity.

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This Standard Reference Material has been measured and certified at the laboratories of the National Bureau of Standards, Gaithersburg, Maryland. All inquiries should be addressed to:

Office of Standard Reference Materials
Room B311, Chemistry Building
National Bureau of Standards
Washington, D. C. 20234

The date of issuance and certification of this Standard Reference Material was August 6, 1973.

APPENDIX C

DEFINITIVE METHOD
Thermal Ionization Isotope Dilution Mass Spectrometry

Isotope dilution mass spectrometry (IDMS), because of its innate accuracy, has been used extensively at NBS to accurately determine the concentration of elements in Standard Reference Materials and other reference materials. An accurate measurement may be defined as one that is both precise and free of unevaluated systematic errors. The concentration determined by this procedure represents the "true value" within the stated uncertainty limits.

At NBS the most accurate methods available to measure a given chemical property are known as definitive methods. A definitive method can be defined as one which has a valid and well described theoretical foundation, has been experimentally evaluated so that reported results have negligible systematic errors, and has high levels of precision. Thermal ionization isotope dilution is regarded at NBS as a definitive method.

In isotope dilution mass spectrometry the quantity of an element present is estimated from the change produced in its isotope composition by the addition of a known quantity of an isotope of the element which alters the analyte's isotopic composition. This alteration is measured by mass spectrometry and the initial amount of the element present in the sample is calculated.

The method, as we use it, consists essentially of the following steps:

1) The addition to a weighed sample of a known amount of a separated isotope of the element to be determined. We prefer to make this addition as a weighed portion of a solution of known concentration.

2) Dissolution of the sample by appropriate means, and thorough mixing of the resulting solution to ensure equilibration of the isotopes from the spike and the sample. This may necessitate chemical treatment to convert the separated isotope and natural element to the same oxidation state.

3) Chemical extraction of the isotopically altered element in a form suitable for mass spectrographic analysis. A major advantage of IDMS is the fact that since we are only concerned with the ratio of isotopes, recoveries need not be quantitative after equilibration.

4) Measurement of the altered isotopic ratio by thermal ionization mass spectrometry. The instruments used are solid sample, single stage instruments, designed and built at NBS. Essential detailed descriptions of these instruments have been published [1,2].

5) Calculation of the amount of element present in the sample from the isotope ratio measurement. The calculation for an element with isotopes a and b is shown by equation (1).

$$\mu g/g = \frac{W_{sp} C[A_{sp} - RB_{sp}]}{BR - A} \cdot \frac{M}{W_s} \qquad (1)$$

where

R = measured isotopic ratio
W_{sp} = weight spike solution, g
A_{sp} = mole fraction a in spike
B_{sp} = mole fraction b in spike
A = mole fraction a in natural element
B = mole fraction b in natural element
M = atomic weight natural element
W_s = weight of sample, g

(This calculated concentration must be corrected for blank.)

An analysis of the possible errors in IDMS shows that errors could result from the following sources:

a) Error in the calibration of the concentration of the spike isotope. The accuracy of a determination depends on the "spike calibration". The spike solution is calibrated against at least two different solutions of the pure natural element by what has been called reverse isotope dilution. Whenever possible, SRMs are used as the natural material. The error from the spike calibration will be the same as for the element determination and is due to the imprecision of the ratio measurement.

b) Chemical errors. These could be caused by: 1) Incomplete dissolution of the sample. This is a problem common to all wet analytical methods, and the analyst must devise a system that ensures complete sample solution or decomposition. 2) Loss of the element from sample or spike due to volatility or adsorption during dissolution. This can usually be detected by spiking some samples before dissolution and some samples after dissolution. 3) Incomplete mixing or equilibration of spike and natural isotopes. This can be caused by differences of oxidation state or by the occurence of the natural element in a complex or chelated form. This source of error can be eliminated by proper chemical treatment, for example, oxidation or reduction and wet-ashing. 4) Isotopic fractionation in the chemical treatment if the separation is not quantitative. This is usually not a problem but may occur with some techniques, such as in the separation of lithium by ion exchange chromatography. This can be detected by isotopic analysis of small fractions of the natural material that have been put through the partial separation.

In regard to the total chemical error, no undetected errors should arise from this source in a well designed analysis.

c) Contamination or blank. Contamination or blank may arise from reagents, apparatus, or dust from the laboratory environment. It can be minimized by carrying out the chemical operations in a carefully controlled atmosphere and by using special high purity reagents. The total blank may be estimated by carrying a number of "blanks" through all the steps of the analysis. The average blank value can be treated as a systematic error and used as a correction. The uncertainty of this correction is equal to the randomness of its measurement. For concentrations where the blank represents a significant fraction of the element determined, the blank may become the largest source of error.

d) Interferences. Interferences mainly occur between elements with isobars, that is different elements that have isotopes of equal mass. This problem may be avoided by basing the analysis on those isotopes of the element that are not isobaric, or by chemically separating the interfering element. Fortunately, most of the elements with isobars are in different groups of the periodic table, so separations are not difficult. No concealed systematic error should arise from this source.

For example, ^{40}K is isobaric with ^{40}Ca, but K can be easily separated from Ca by ion exchange chromatography. To be sure that the ^{40}K level is insignificant, the analyst can monitor ^{39}K which is four orders of magnitude more abundant than ^{40}K in natural potassium.

e) Instrumental errors. Instrumental errors may be caused by mass discrimination or fractionation, but are usually cancelled since the same percentage error would be present in the ratio measurement for the spike calibration. With some elements, impurities in a sample can cause a different fractionation pattern from pure material. These effects are usually small (less than 0.1%) and can usually be corrected by repurifying the sample.

This analysis of the possible sources of systematic error shows that these errors can be eliminated or corrected as in the case of the blank. We are left then with the random errors. Random errors are present in the isotope ratio measurements for both the determination and the spike calibration and the blank correction. If the blank correction is insignificant, then the total error in a careful determination reduces to the combined random error for the spike calibration and the determination. This error is reflected in the precision of the isotopic measurements which for many elements is of the order of 0.05 to 0.25 percent. Therefore, absolute accuracies of 0.1 to 0.5 percent are possible even for very low concentrations in complex materials.

When the blank correction is significant then the uncertainty from this source must be added to the uncertainties from the ratio measurements.

Determination of Chloride

Introduction

Some years ago, the absolute $^{35}Cl/^{37}Cl$ ratio was measured at NBS during a redetermination of the atomic weight of chlorine [3]. The absolute $^{35}Cl/^{37}Cl$ ratio was determined to be 3.127 ± 0.008. A study of chlorine minerals had shown this ratio to be invarient in natural samples within the measurement uncertainty.

With the exception of sample size, the same mass spectrographic method in which chloride was analyzed using an ammonical solution of silver chloride, seemed to be applicable to the determination of chloride in blood serum by isotope dilution. However, preliminary experiments with serum revealed that silver nitrate solution precipitated not only silver chloride, but also some other material, probably protein, so attempts were made to first remove the protein.

The Folin-Wu method [4] in which protein is precipitated by sodium tungstate from an acidified serum solution, produced a clear "protein-free" filtrate. Unfortunately, the blank for chloride was too high due principally to the chloride content of the sodium tungstate. A modification of this method using ammonium molybdate, which also precipitates protein, and nitric acid instead of sulfuric acid reduced the chloride blank to about 1 μg Cl or less than 0.1 percent of the chloride contained in 1 g of serum.

During actual IDMS determination of Cl in serum, it was found that further purification from organic matter was necessary. Some samples became dark on drying on a filament and gave a different fractionation pattern from the reference during mass spectrometric analysis. When these samples were further purified by allowing ammonia gas to escape and AgCl to crystallize slowly from ammonical solution, the problem disappeared.

Procedure

Four randomly selected vials (5 for lot 1) from each of the seven pools of bovine blood serum were used for the determination of chloride ion concentrations. The frozen samples were allowed to thaw and equilibrate to room temperature. One-gram sub-samples were taken from each of the vials and a duplicate sample was taken from one of the vials from each lot. Each sub-sample was then treated as follows:

The 1-g sub-sample was spiked with ^{37}Cl (sufficient to give a 35/37 ratio of approximately one), 8 mL of 0.1 mol/L HNO_3 was added, and the solution was thoroughly mixed and heated for two hours to equilibrate natural chloride with the spike chloride. Protein was then precipitated with ammonium molybdate solution and removed by filtration.

The chloride in the "protein-free" filtrate was precipitated as silver chloride which was allowed to settle from the solution. The supernatant solution was removed by decantation and the AgCl was dissolved with a minimum of ammonium hydroxide solution. AgCl was reprecipitated by the addition of nitric acid, separated by centrifugation and redissolved in concentrated ammonium hydroxide. Further purification was accomplished by allowing ammonia gas to escape and AgCl to slowly crystallize. The supernatant liquid was withdrawn and AgCl was again recrystallized from ammonium hydroxide solution. Finally, the recrystallized AgCl was dissolved in concentrated ammonium hydroxide to give a chloride concentration of 3 mg/mL.

Two different mass spectrometers were used for chlorine isotopic ratio measurements. One instrument was dedicated to blanks and separated isotope samples and the other to spiked samples and the natural isotopic standard. The mass spectrometric technique is described in NBS Technical Note 277 [5]. Major modifications in the procedure were the use of degassed filaments and reduction of the sample size per analyses to approximately 60 μg.

Results

The results of these determinations are shown in Table 1. The blank correction based on six blank determinations was 0.7 μg or less than 0.03 percent of the amount of chloride present in any sample. The results are reported both on a weight and a volume basis using an average density of 1.0312 g/mL at 23 °C for all lots of serum. The indicated uncertainty is an estimated limit of error, and is larger than the 95 percent confidence limits for a single determination ($t\sigma$) calculated from the concentration data. The additional allowance is made for possible error contributions

from spike calibrations and background signals. The estimated accuracy is 0.5 percent and includes allowance for all known sources of systematic error.

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Table 1. Chloride in CDC Serum.

Pool	Sub-Sample	Chloride Concentration	
		mmol/kg	mmol/L
P1	1-1	76.95	79.35
	2-1	76.91	79.31
	3-1	76.63	79.02
	4-1	76.92	79.32
	5-1	76.85	79.25
	5-2	76.92	79.32
	Average	76.9 ± 0.4	79.2 ± 0.4[a]
P2	1-1	81.83	84.38
	2-1	82.06	84.62
	3-1	81.90	84.46
	4-1	81.94	84.50
	4-2	81.95	84.40
	Average	81.9 ± 0.4	84.5 ± 0.4[a]
P3	1-1	91.18	94.02
	2-1	91.12	93.96
	3-1	91.24	94.09
	4-1	91.14	93.98
	4-2	91.22	94.06
	Average	91.2 ± 0.5	94.0 ± 0.5[a]
P4	1-1	98.62	101.69
	2-1	98.76	101.85
	3-1	98.68	101.76
	4-1	98.70	101.78
	4-2	98.66	101.74
	Average	98.7 ± 0.5	101.8 ± 0.5[a]
P5	1-1	103.77	107.00
	2-1	104.00	107.25
	3-1	103.95	107.20
	4-1	103.86	107.10
	4-2	103.98	107.22
	Average	103.9 ± 0.5	107.2 ± 0.5[a]

continued

Table 1 (continued)

Pool	Sub-Sample	Chloride Concentration			
		mmol/kg		mmol/L	
P6	1-1	109.45		112.86	
	2-1	109.51		112.93	
	3-1	109.56		112.98	
	4-1	109.50		112.92	
	4-2	109.53		112.95	
	Average	109.5	0.5	112.9	0.6[a]
P7	1-1	113.15		116.68	
	2-1	113.15		116.68	
	3-1	113.34		116.87	
	4-1	113.38		116.92	
	4-2	113.14		116.67	
	Average	113.2	0.6	116.8	0.6[a]

[a]Estimated maximum error of 0.5 percent of the value as reported by NBS Analytical Mass Spectrometry Section. This estimated maximum error includes both imprecision and an estimated upper bound for possible systematic errors. The estimated maximum error is believed to be equal to or greater than the true error for the 95 percent confidence limits.

APPENDIX D

Note 1:

A temperature range of ±2 °C is designated as the operating temperature. In this temperature range the maximum difference in aqueous solution volumes due to thermal expansion of the liquid is 0.102 percent and the difference in volume due to the volumetric glassware is very small since the coefficient of expansion for borosilicate glass is 0.00001 per °C. (J. Lembeck, "Calibration of Small Volumetric Laboratory Glassware", NBSIR Report 74-461, 1974, Institute for Basic Standards, National Bureau of Standards, Washington, D. C. 20234). We judge these errors to be acceptable for this reference method. Larger temperature variations may necessitate appropriate correction.

Note 2:

Glassware Required:

a) Micropipetting alternative:

Volumetric Flasks: Five 250-mL.

Pipets: Micropipets as required for instrument with the accuracy requirement specified in Section IIIC-2.

b. Macropipetting alternative:

Volumetric Flasks: Ten 250-mL plus as many additional 250-mL volumetric flasks as there are samples to be analyzed.

Pipets: One 5-mL; five 4-mL plus as many additional 4-mL pipets as there are samples to be analyzed.

Note 3:

Cleaning of Glassware:

a) Clean the glassware in the following manner:

(1) soak glassware for 60 min in 0.77 mol/L HNO_3.

(2) Rinse six times with a volume of water equal to at least 10 percent of the container volume.

(3) Use immediately or air dry (inverted in a dust-free environment) for later use.

Note 4:

The atomic weights used in this report are those reported in : Pure and Applied Chemistry, 47, 75 (1976).

Note 5:

If the wash solution does not drain cleanly from the pipet, wash with 0.77 mol/L HNO_3, H_2O, MeOH, 70:30 v/v $CHCl_3$:MeOH, MeOH, and H_2O in that order. Then repeat the water wash and check that the pipet does drain properly.

Note 6:

The three following pages are examples of the data sheets returned from each laboratory after each round robin test.

ELECTROLYTES IN SERUM — CLINICAL REFERENCE METHOD

CHLORIDE ION

LABORATORY 5 ANALYST XY INTERLABORATORY EXERCISE II

DATE SAMPLES RECEIVED 2-18-76 DATES ANALYZED (1) 2-24 (2) 2-26

INSTRUMENT MANUFACTURER American Instrument Co. MODEL Aminco-Cotlove Chloride Titrator

METHOD: MICRO ____ MACRO X DIRECT READING ____ TIME X

VOLUME TRANSFERRED TO TITRATION CELL 4.0 (ML)

TYPE MICROPIPET USED ____

ELECTROLYTE USED 6.4 mL conc. Nitric Acid + 100 mL Glacial Acetic QS to 1 liter

DISPERSANT USED Gelatin Reagent (6.2 g to 1 liter of hot water)

		Weight ratio
Composed of:	Knox Gelatin #1	60
	Thymol Blue	1
	Thymol	1

COMMENTS: Due to time involved in performing the macro procedure on 10 samples, the dilutions of the serum samples were made on the day before the titrations were performed. They were allowed to set overnight at room temperature and were shaken vigorously to ensure thorough mixing before the analyses. This change was discussed with and approved by NBS personnel.

DATA SHEET: STANDARD CURVE

LAB 5 DAY 2/24/76

PROTOCOL USED; MANUAL X SEMI-AUTOMATED _____

STANDARD	CALCULATED ION CONCENTRATION, MMOL/L	DIRECT READ-OUT OR TIME
1	75.01	23.2
2	90.01	27.9
3	100.01	31.0
4	110.00	34.2
5	125.00	39.0
6		

DILUENT BLANK 1.7

DATA REPORTING SHEET FOR VALID MEASUREMENTS

PROTOCOL USED: MACRO X MICRO _____

LAB 5 ION Cl^- INTERLABORATORY EXERCISE II

DATE ANALYZED 2/24 OPERATOR XY

SAMPLE # 536603 — TIME OR DIRECT READOUT

STANDARD CONCENTRATIONS MMOL/L (CALCULATED)	VALID SET	LO STD (C_1)	SAMPLE (Y)	HI STD (C_2)	$\hat{C}$
LO 110.00 (C_1)	1.	35.6	37.9	40.5	117.04
HI 125.00 (C_2)	2.	35.8	37.9	40.6	116.56
	3.	35.8	37.6	40.5	115.74

SAMPLE # 496322 — TIME OR DIRECT READOUT

STANDARD CONCENTRATIONS MMOL/L (CALCULATED)	VALID SET	LO STD (C_1)	SAMPLE (Y)	HI STD (C_2)	$\hat{C}$
LO 75.01 (C_1)	1.	24.8	26.0	29.4	78.92
HI 90.01 (CH_2)	2.	24.9	25.9	29.4	78.34
	3.	24.8	26.1	29.3	79.34

SAMPLE # 453700 — TIME OR DIRECT READOUT

STANDARD CONCENTRATIONS MMOL/L (CALCULATED)	VALID SET	LO STD (C_1)	SAMPLE (Y)	HI STD (C_2)	$\hat{C}$
LO 100.01 (C_1)	1.	32.7	34.9	35.9	106.88
HI 110.00 (C_1)	2.	32.6	34.8	36.1	106.29
	3.	32.7	34.8	36.0	106.37

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☐ Document describes a computer program; SF-185, FIPS Software Summary, is attached.

16. ABSTRACT *(A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.)*

Guided by a committee of experts in clinical chemistry, a reference method was established for the determination of serum chloride based on a coulometric titration-amperometric end-point determination (C-A). Its accuracy was evaluated by comparing the values obtained by use of the method in 14 laboratories against the results obtained by a definitive analytical method based on isotope dilution-mass spectrometry (IDMS). Seven serum pools with chloride concentrations in the range 79.2 to 116.8 mmol/L were analyzed. Micro- and macropipetting alternatives were tested using sample sizes of 0.010-0.100 and 5.0 mL, respectively.

The laboratories used several different C-A instruments. The results showed that the standard error for a single laboratory's performance of the procedure was approximately 1.0 mmol/L with a maximum bias of 0.5 mmol/L over the range of concentrations studied. These values are within the accuracy and precision goals that had been set by the committee. The results from the micro- and macropipetting techniques were similar. The calibration curve data showed excellent linearity over the total concentration range, with 12 of 14 curves having standard deviation of fit of less than 0.80 mmol/L.

With appropriate experimental design, the reference method may be used to establish the accuracy of field methods as well as to determine reference chloride values for pooled sera.

17. KEY WORDS *(six to twelve entries; alphabetical order; capitalize only the first letter of the first key word unless a proper name; separated by semicolons)*

Amperometry; chloride analysis; clinical analysis; clinical chemistry; coulometry; definitive method; electrolytes; reference method; serum chloride analysis.

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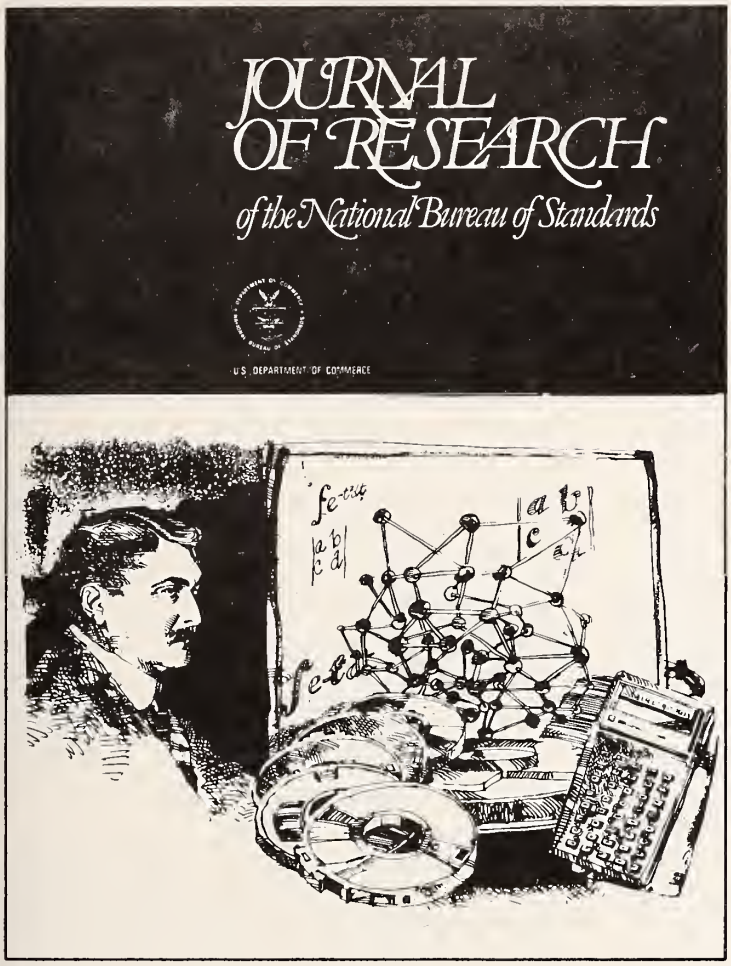

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