TECHNICAL REPORT
4-69
業 績 報 告 書

ULTRAMICRO-COLORIMETRY FOR DETERMINATION OF SERUM CHLORIDE USING SILVER IODATE

ヨウ素酸銀を用いた血清クロライド超微量比色定量法

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ABCC

ATOMIC BOMB CASUALTY COMMISSION

国立予防衛生研究所－原爆傷害調査委員会

JAPANESE NATIONAL INSTITUTE OF HEALTH OF THE MINISTRY OF HEALTH AND WELFARE

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HIROSHIMA AND NAGASAKI, JAPAN

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INTRODUCTION

Mohr's silver nitrate-titration[1] using potassium chromate as the indicator is one of the oldest methods for chloride determination. Subsequently, various other methods were developed including titrations using thiocyanic-Fe[2] (rhodanic Fe) or diphenylcarbasone,[3] as indicators and colorimetry[4] using chloranilic acid mercury. With the recent development of polarography,[5] chloridimetry, and automation, rapid processing, greater specificity and reproducibility are possible

At ABCC, serum Cl is determined routinely by a chloridimeter, which requires 0.1-0.2 ml of serum. Occasionally, however, serum Cl is requested simultaneously with other electrolytes in adults or children from whom it is difficult to obtain a sufficient amount of blood for the determinations.

Therefore, review was made of the reagents and reaction conditions of the method of Hoffman et al,[6] a modification of Sendroy's[7-11] chloride titration that uses silver iodate in a colorimetric method, and a method was developed that required only 0.02 ml of serum.

Values obtained by this method showed very good correspondence with those obtained by the chloridimeter and the macro-method of Hoffman et al.[6] This method also can be used for determinations of Cl in urine and spinal fluid.

THEORY

Protein is removed from the sample by precipitation with phosphoric tungstic acid. Silver iodate is added which reacts with chloride in the sample to produce sodium iodate and insoluble silver chloride:

$$AgIO_3 + NaCl \rightarrow NaIO_3 + AgCl\downarrow \quad \text{..... (Equation 1)}$$

はじめに

最も古い塩化物定量はクロム酸カリウムを指示薬とするMohrの硝酸銀滴定法[1]が挙げられよう．その後，多くの研究者らにより，チオシアン酸鉄[2]（ロダン鉄）やジフェニールカルバゾン[3]などを指示薬にした滴定法，クロラニル酸水銀を用いる比色法[4]などの種々の定量法が開発された．さらに最近ではポーラログラフ法，[5] chloride meterや自動化の出現により，特異性と再現性が向上され迅速定量が可能になった．

ABCCでは，chloride meterを使用し，ルチン検査として血清クロライド値を定量しているが，その使用血清量が0.1－0.2 mlを必要とするため，他の電解質群と同時測定を行なう場合に，小児等の採血困難な患者では，血清量が不足のために時折測定が困難なことに遭遇する．

そこで，Sendroy[7-11]のヨウ素酸銀を用いる塩化ヨード滴定法を比色法に改良したHoffmanら[6]の測定法について試薬，反応条件等を吟味し，この反応術式を応用して使用血清量を0.02mlで測定する定量法を開発した．

本法の実測値はchloride meterおよびHoffmanら[6]のmacro-methodで得る値と非常によく相関しており，また尿中クロライド，髄液クロライドも測定が可能である．

原　理

試料の蛋白をリンタングステン酸ナトリウムで除き，これにヨウ素酸銀粉末を加えると試料中の塩化物がヨウ素酸銀と反応して不溶性の塩化銀を生じヨウ素酸を遊離する．

The precipitated proteins and silver iodide are removed by filtration (or centrifugation) and the remaining solution, containing sodium iodate, is acidified with phosphoric acid and sodium iodide is added. For each equivalent of chloride in the original sample, 6 equivalents of free iodine are liberated:

この遊離した蛋白とヨウ素酸は濾過(または遠心分離)してその上清をとり，リン酸で酸性にして，ヨウ化ナトリウムを加えるとヨウ素酸に相応するヨウ素が生ずる．

$$NaIO_3 + 5NaI + 6H_3PO_4 \rightarrow 3I_2 + 3H_2O + 6NaH_2PO_4 \quad \cdots\cdots\cdots\cdots (2)$$

The amount of free iodine (yellow) is measured spectrophotometrically, thus giving indirectly the amount of chloride present.

したがって，ヨウ素の色(黄色)を近紫外域で比色し，間接的に塩化物の量を測定する．

REAGENTS

試　薬

Phosphoric tungstic acid solution Sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) 6 g is put into a 1000 ml flask, to which 0.15 M phosphoric acid is added to make a solution of 1000 ml.

リンタングステン酸溶液：1000 ml のフラスコにタングステン酸ナトリウム($Na_2WO_4 \cdot 2H_2O$) 6 g 入れ，0.15M リン酸を加えて1000 ml とする．

0.15 M phosphoric acid solution 85% phosphoric acid (H_3PO_4) 10 ml is dissolved with H_2O to make a solution of 1000 ml.

0.15Mリン酸液：85%リン酸(H_3PO_4) 10 ml を H_2O で1000 ml に溶かして作る．

Silver iodate ($AgIO_3$ Fisher Co. "Chloride free" or Wako SG) is used directly.

ヨウ素酸銀　($AgIO_3$ Fisher 社" Chloride free "または Wako SG)をそのまま使用．

0.4% sodium iodide 0.4 g of sodium iodide (NaI) is measured and 0.1 N-NaOH 0.5 ml is added. This is dissolved with H_2O to make a solution of 100 ml (may be used for 2 to 3 weeks).

0.4%ヨウ化ナトリウム：ヨウ化ナトリウム(NaI) 0.4g を測り，0.1 N－NaOH を0.5 ml 加え，H_2O で100 ml に溶解する(2－3 週間使用可能)．

120 mEq/l NaCl standard solution NaCl (special grade dried reagent) is redried for 3-5 hours at 100-110 C, immediately put into a desiccator and sealed. After it has cooled, 7.014 g of this is measured and dissolved with H_2O to make a solution of 1000 ml (may be used for a long period).

120 mEq ／ℓ NaCl 標準液：NaCl (special grade dried reagent)を100－110 C で，3－5時間再乾燥し，ただちにデシケータ中に入れ密封し，冷却したものを 7.014 g 測り H_2O に溶して1000 ml とする(長期間有効)．

Standard working solution 120 mEq/l NaCl solution is diluted with H_2O to make working standards of 30, 60, 90 and 120 mEq/l. These solutions may be stored in sealed glass containers for at least 1 month. When preparing the various concentrations, the solutions can be made easily and accurately by use of Sasaki's[12] standard solution dilution method.

標準使用液：120 mEq ／ℓ NaCl 液を H_2O で希釈して0，30，60，90および120 mEq ／ℓ になるよう希釈し，各濃度液をそれぞれの容器に密封し，保存する(1ヵ月以上有効)．各濃度液を作製する時は，佐々木[12]の基準液希釈法を用いると簡単かつ正確に作れる．

Low Cl content reagents are used, and all solutions are made using previously distilled water, filtered through an ion exchange resin.

注意：すべての試薬類は Cl 含有量の少ないものを使用すべきであり，また試薬調製に用いる H_2O はイオン交換樹脂を通して得たものを使用する．

PROCEDURES

A blank and four standards are prepared by dispensing into five tubes 0.02 ml (20 λ) solutions containing respectively 0, 30, 60, 90, and 120 mEq/l NaCl using a Sanz pipette. Unknowns containing 0.02 ml of serum are similarly prepared. To each tube, 0.5 ml of phosphoric tungstic acid are added and the contents thoroughly mixed. About 20 mg of silver iodate (exact weight unnecessary; the amount scooped onto the tip of a standard Japanese ear currette is sufficient) are added to each tube, thoroughly mixed, allowed to stand for about 5 minutes, and centrifuged at 3000 rpm for 5 minutes.

With a Sanz pipette, 0.02 ml of the supernates from each tube are transferred to other corresponding tubes and 3 ml of 0.4% NaI are added to each. After standing for 10 minutes, spectrophotometry is performed using a 420 mμ filter. The optical densities of the 30, 60, 90 and 120 mEq/l chloride standards are obtained by reading against the blank and a standard calibration curve is constructed from which the chloride content of the unknown samples is determined in the usual way.

RESULTS

A comparison of chloride values in 75 serum samples, determined by the above method, the macro-method of Hoffman et al[6] and chloridimetry is shown in Figure 1. The coefficients of correlation for the ultramicro-method and chloridimetry is 0.991 and that for chloridimetry and the macro-method is 0.986.

Comparisons between the micro-method and chloridimetry, using 20 urine samples were performed and the correspondence was also satisfactory as shown in Table 1.

REVIEW

Absorption Spectra and Standard Calibration Curve

A Hitachi-Perkin-Elmer Type 139 spectrophotometer was used to determine the absorption spectra from 350 mμ to 590 mμ using a blank, and 50 and 100 mEq/l NaCl at a slit width of 0.5 (Figure 2). A maximum absorption peak could not be found within this range. For determinations in the ultraviolet areas, high-class colorimetric apparatus and reagents of high purity are required, making it susceptible to contamination, and therefore, it was decided that for routine tests spectrophotometric readings would be taken in the visible spectrum, specifically at 420 mμ.

操　作

試験管ＡＢＣＤＥおよびＦを用意し，これに標準使用液0，30，60，90および120 mEq／ℓ液および試料（血清）をおのおの0.02ml（20λ）ずつ Sanz pipette で採量し，リンタングステン酸液を0.5 ml ずつ加えよく混合する．ついで約20mgのヨウ素酸銀（正確である必要はなく，耳かき一杯でよい）をおのおのの試験管に加え，よく混合して約５分間放置後，3000 rpm ５分間遠心する．

遠心したそれぞれの上清を，別の試験管に Sanz pipette を用いて0.02ml ずつ移しとり，おのおのに0.4%ヨウ化ナトリウム液を３ml あて注ぎ入れ，10分間放置した後に Filter 420 mμ で比色する．Blank を基準として，30，60，90および120 mEq ／ℓ 液の吸光度を求めて検量線を描き，これを用いて未知試料中のクロライド濃度を通常の方法で決定する．

結　果

血清75例について，本法と従来の Hoffman らの macro-method[6] および chloride meter で得た実測値を比較した（図１）．本法と chloride meter および macro-method との比較では相関係数はそれぞれ 0.991 および 0.986 であった．

20検体の尿についても本法と chloride meter を比較し，表１のごとく満足すべき結果を得た．

吟　味

吸収曲線および検量線

Hitachi-Perkin-Elmer 139型 spectrophotometer を用いて slit 幅 0.5 で blank と50および100 mEq ／ℓ NaCl について，吸収曲線を 350 mμ から 590 mμ まで測定した（図２）．最大吸収の峰はこの範囲には認めなかった．紫外域での測定は高級比色用器械や高純度の試薬が要求され，かつ，汚染が生じやすい．したがって，近紫外域である420 mμ で測定したほうがルチン検査としては有利と考え原法に準ずることにした．

TABLE 1 COMPARISON OF CHLORIDE VALUES IN URINE DETERMINED BY ULTRAMICRO METHOD AND CHLORIDIMETRY

表1　本法と Chloride meter による尿中 Cl の実測値の比較（mEq ／ℓ）

Sample 標本	Chloride in Urine 尿中 Cl 値		Difference 差
	Ultramicro-Method 本法	Chloride Meter	
1	182	180	+2
2	27	28	−1
3	178	174	+4
4	41	41	0
5	133	135	−2
6	120	123	−3
7	118	119	−1
8	172	172	0
9	73	77	−4
10	107	108	−1
11	169	170	−1
12	276	278	−2
13	249	248	+1
14	205	206	−1
15	93	91	+2
16	207	208	−1
17	266	269	−3
18	267	267	0
19	236	239	−3
20	237	238	−1

Figure 3 shows standard calibration curves obtained using Gilford, Coleman Junior II, Coleman 6a and Coleman 6c colorimeters. The curves obtained with Gilford, and Coleman Junior II colorimeters were linear in accordance with Lambert-Beer's law, but the curves obtained with Coleman 6a and 6c colorimeters were slightly arc-shaped.

The deviation from linearity of the standard calibration curve for some of the colorimeters emphasizes the importance of using at least four or five standard solutions of different concentrations to obtain a satisfactory curve. Dependence on a calibration curve derived from a standard solution of only a single concentration can lead to large errors in clinical chemical determinations. Further, standards and unknown samples should be processed in the same manner throughout the entire procedure.

Reagents

Silver iodate: Concentration and commercial variation The effect of increasing concentrations of silver iodate in the procedure (Equation 1) was investigated by adding, to a series of standard test solutions containing 50 mEq/l NaCl, 20 mg increments of silver iodate in a range between 20-120 mg. Commercially available iodate from three different manufacturers, A, B, and C were tested. Judging from the final color reaction (Figure 4), the iodate from companies A and C precipitated a fixed amount of chloride irrespective of the amount of iodate added, but that of company B showed an increase in chloride proportional to

図3は Gilford, Coleman Junior Ⅱ type, 同 6a type および同 6c type の比色計で，それぞれの検量線を描いたものである．Gilford, Coleman Junior Ⅱ type では Lambert Beer の法則に従い直線的であったが，Coleman 6a type および 6c type ではいずれもわずかに弧を描く曲線を得た．

比色計によって検量線の直線性が変動したことは，満足な検量線を得るためには，少なくとも数段階に希釈した4－5本の標準液を用いることがたいせつであることを強く示唆する．ある1濃度のみの標準液を使用して得た検量線によれば，臨床化学定量値に大きな誤差を招くことがある．そこで，数本の標準液を用いて sample と同様に操作して検量線を描くべきである．

試　薬

ヨウ素酸銀：その濃度と市販品　50 mEq ／ℓ NaCl を含有する一連の標準液にヨウ素酸銀を20－120 mgを添加して，前記の操作（反応式1）においてヨウ素酸銀の濃度を増加した場合の影響を調べた．A，B，C 3社の市販ヨウ素酸銀を試験したが，発色の強さからみれば（図4），A社およびC社のヨウ素酸銀では，その添加量に関係なく一定量の塩化物の定量値を得たが，B社のヨウ素酸銀は添加量に比例して塩化物が増加した．これはC社の試薬には塩化

FIGURE 1 COMPARISON OF SERUM CHLORIDE VALUES DETERMINED BY ULTRAMICRO METHOD, CHLORIDIMETRY, AND MACRO METHOD

図1　本法および Macro-method の chloride meter との比較

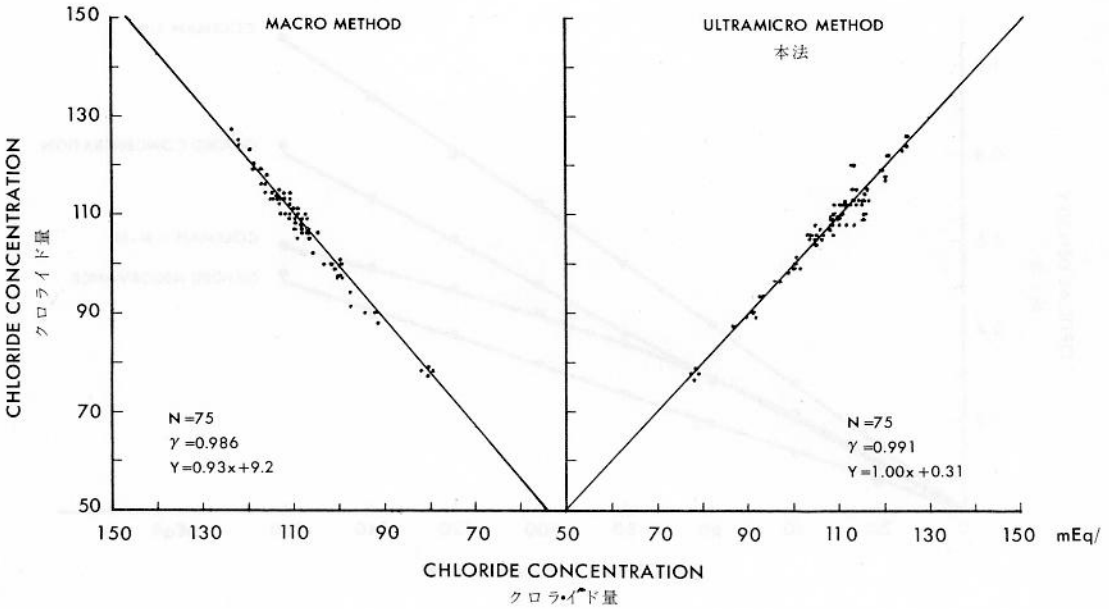


FIGURE 2 ABSORPTION SPECTRA OF CHLORIDE SOLUTIONS

図2　クロライド試験液の吸収曲線

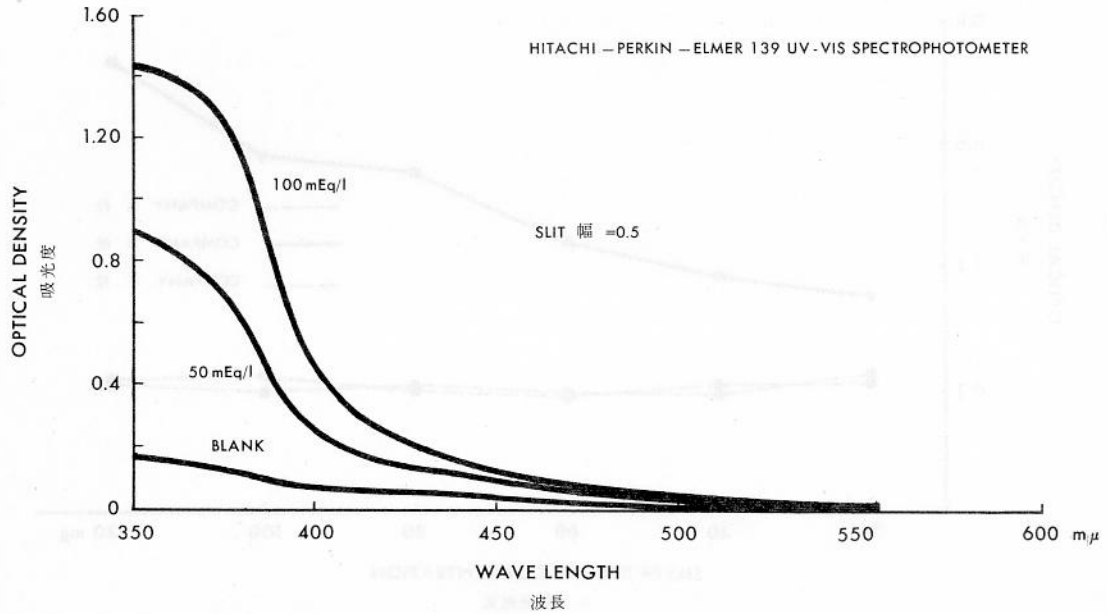


FIGURE 3 STANDARD CALIBRATION CURVES FOR DIFFERENT COLORIMETERS

図3　各種比色計による検量線

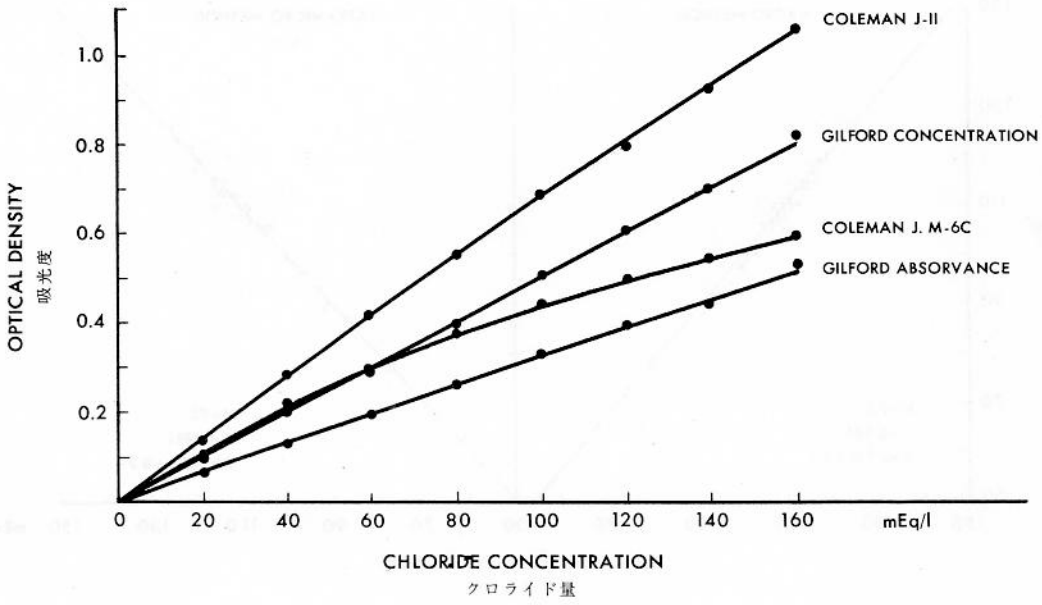
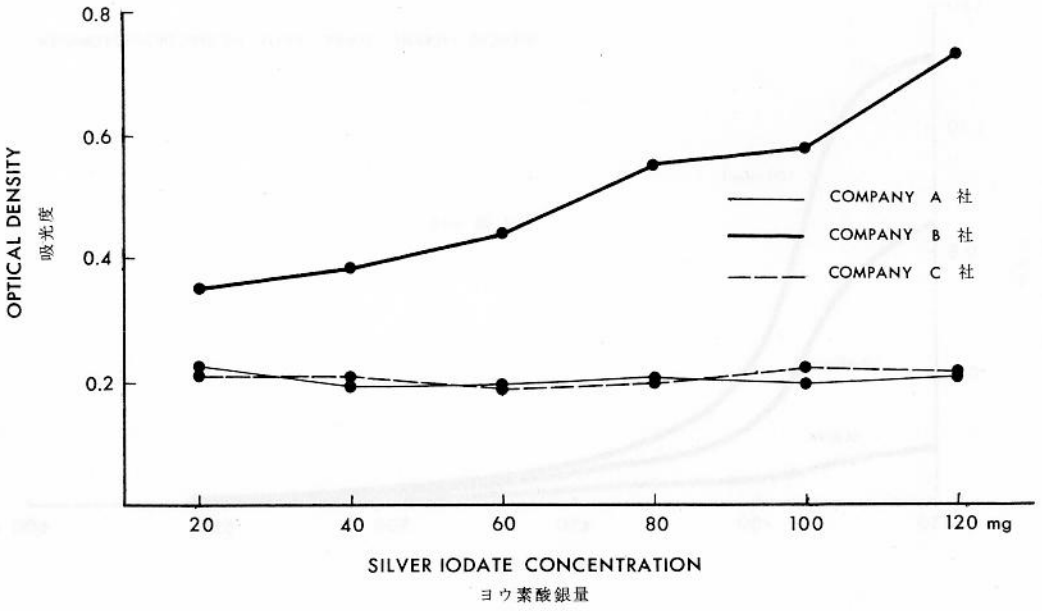


FIGURE 4 EFFECT OF CONCENTRATION OF SILVER IODATE ON COLOR REACTION

図4　発色に及ぼすヨウ素酸銀の量と品質の差



the amount of iodate added, indicating considerable contamination with chloride rendering the reagent unfit for use in the method without further purification.

Comparison of the variation between different lots of silver iodate made by companies A and C showed no major differences. The products of these two companies could be used directly without further purification.

Sodium iodide: Concentration and volume The effect of different concentrations of sodium iodide (Equation 2) on the color reaction was investigated. Standard test samples containing 100 mEq/l NaCl were processed in the usual fashion (see Procedure). To the final 0.02 supernates, 3 ml of sodium iodide were added, in the following concentrations: 0.4%, 0.6%, 0.8% and 1.0%. Over this range of reagent concentration, there were no differences in the optical densities of the test solutions, and the 0.4% concentration was chosen for the procedure.

Review was made of the color reaction when 2,3,4 and 5 ml of 0.4% sodium iodide were added. The color intensity of the reaction decreased with increasing volume of reagent, which is obviously a dilution effect. Adequate color intensity was attained with 2 ml of 0.4% sodium iodide solution. Thus, the volume of sodium iodide may be adjusted according to the size of the colorimeter cuvette. In this laboratory 3 ml is a convenient volume to use.

Color reaction time and fading time

As shown in Figure 5, at 25C, maximum color intensity developed immediately after addition of sodium iodide and remained stable for at least 90 minutes.

Effect of temperature

An incubator with an adjustable temperature control was used to test the effect of temperature at 10,20,30,35,40 and 50C. Within this range, there was no significant effect on the Cl determinations (Figure 6).

Effect of other substances in blood

Protein (human albumin 10g/100ml, hemoglobin (14g/100ml), bilirubin (20 mg/100 ml), glucose (100 mg/100 ml), urea N (100 mg/100 ml), vitamin C (100mg/100ml) and cholesterol (200 mg/100 ml) in amounts of 0.02 ml respectively were added to standard test solutions containing 20,40,60,80 and 100 mEq/l NaCl, and Cl value was determined. No effects due to these substances could be detected.

物の不純物が相当あって，再精製しない限り使用にたえないことを示す．

また，各社のヨウ素酸銀について，各 lot による測定値の変動を比較したが大差はなく，A社，C社の製品は再精製の操作は必要でなく，そのまま使用できる．

ヨウ化ナトリウム：その濃度と使用量 発色に対するヨウ化ナトリウム濃度の影響を検討した（反応式2）．100 mEq／ℓ NaCl を含有する標準 sample を前記の方法（操作の項を参照）で操作し，得られた0.02上清に，0.4%，0.6%，0.8%および1.0%に調製した各ヨウ化ナトリウム液を 3 ml ずつ添加した．この試薬の濃度区間では吸光度には差がなかった．そこで，本検査には 0.4%濃度液を用いた．

0.4%ヨウ化ナトリウム液を 2，3，4 および 5 ml と加えて発色させ，その使用量を吟味した．添加液量に応じて発色の色調が低下したが，これは液量により希釈されるために生ずる現象であり，0.4%ヨウ化ナトリウム液であれば 2 ml でもじゅうぶん発色することを認めた．したがって，比色計のキュベットの大きさによってはヨウ化ナトリウムの量を加減してもよく，当検査室では扱いやすい液量を考慮して 3 ml を使用した．

発色時間および退色

図5のごとく，25Cにおいてヨウ化ナトリウムを添加した直後から発色し，少なくとも90分間は安定していた．

温度による影響

温度調節の可能な恒温槽を使用し，10，20，30，35，40および50Cにおいて温度による影響を調べたが，この範囲の温度ではいずれもクロライド測定値には差を認めなかった（図6）．

他の血中含有物質の影響

Protein（human albumin 10 g／100 ml），Hemoglobin（14 g／100 ml），Bilirubin（20mg／100 ml），Glucose（100 mg／100 ml），Urea N（100 mg／100 ml），Vitamin C（100 mg／100 ml）および Cholesterol（200 mg／100 ml）についてそれぞれ0.02 ml ずつ20，40，60，80および100 mEq／ℓ NaCl 標準液に添加してクロライド値を測定したが，いずれも影響を認めなかった．

Precision and accuracy

Repeatability (Replicate determinations on the same sample, by the same technician in any single run. Measure of precision of individual.) Twenty replicate determinations of the same serum were performed. Figure 7 shows the results: Mean = 108.8 mEq; SD = 0.61; CV (Coefficient of variation = SD/mean × 100) 0.56% indicating excellent repeatability.

Reproducibility (Repeated determinations on the same sample on different days, not necessarily by the same technician. Measure of precision of method.) Determinations were performed over a period of 20 days using the same sample. Figure 8 shows the results. Each point in the upper panel is the average of duplicate determinations for each day. Each point on the lower panel shows the difference in mEq/l between the duplicates for that day. The overall mean for the 20 day period =108.5 mEq; SD = 0.80; CV = 0.73%. Reproducibility for this method is also excellent, though variation is slightly greater than noted under "Repeatability."

精度と正確性

反復性 （同一検査技術員が同一検体について行なう重複測定で，個人の精度を検定する.）同一血清につき20回重複測定を行なった結果，図7が示すとおり平均値＝108.8 mEq ； SD＝0.61； CV（変動係数＝$\frac{\text{標準偏差}}{\text{平均値}}$×100）＝0.56%とすぐれた反復性が示された.

再現性 （同一検査技術員とは限らず同一検体について異なった日時に行なった反復測定で，方法の精度を検定する.）同一血清を用いて20日間測定して，図8に示す結果が得られた．上の図の各点は，1日に行なった重複測定の平均値を示す．下の図の各点は，同じ日に行なった重複測定の誤差を mEq ／ℓ 単位に示したものである．20日間の総合平均は，108.5 mEq ； SD＝0.80； CV＝0.73%であった．本検査の再現性もすぐれているが，反復性の場合よりもばらつきが多少大きい.

TABLE 2 COMPARISON OF CALCULATED AND DETERMINED CHLORIDE CONTENT OF VARIOUS TEST SOLUTION

表2 各種試験液におけるクロライド量の計算値と実測値との比較

Sample 標本		Determined 実測値	Cl Known	Determined 実測値	Calculated 計算値	%
1	10+C*	10.2	30.2	40.2	40.4	99.5
2	20+C	20.0	30.2	49.8	50.2	99.2
3	40+C	39.9	30.2	70.0	70.1	99.8
4	50+C	50.1	30.2	80.2	80.3	99.8
5	80+C	78.1	30.2	105.3	108.3	97.2
6	100+C	96.8	30.2	122.1	127.0	96.1

*C = Ion-free Chemvarion

Recovery experiments Table 2 shows the results obtained by adding known amounts of chloride to test solutions and comparing the calculated results to those obtained by actual determination of the chloride content. Column 2 shows the amount of chloride added to an ion free carrier.† Column 3 shows the chloride value determined colorimetrically for each of these samples. Column 4 gives the chloride content of a commercial preparation ‡ which, when combined with an appropriate volume of the test

回収実験 表2に，試験液に既知量のクロライドを添加して得た成績を挙げ，あわせてクロライド実測値とその計算値を比較した．左から第2欄は無イオン担体†に添加したクロライド量を示した．比色法による各 sample のクロライド値を第3欄に示した．第4欄に示した市販品‡のクロライド量と第3欄に挙げた値を合計すれば，第6

† Chemvarion — Clinton Laboratories, Los Angeles, California, USA.
‡ Versatol — Warner-Chilcott Laboratories, Morris Plains, New Jersey, USA.

FIGURE 5 COLOR DEVELOPMENT AND STABILITY

図5　発色の安定性

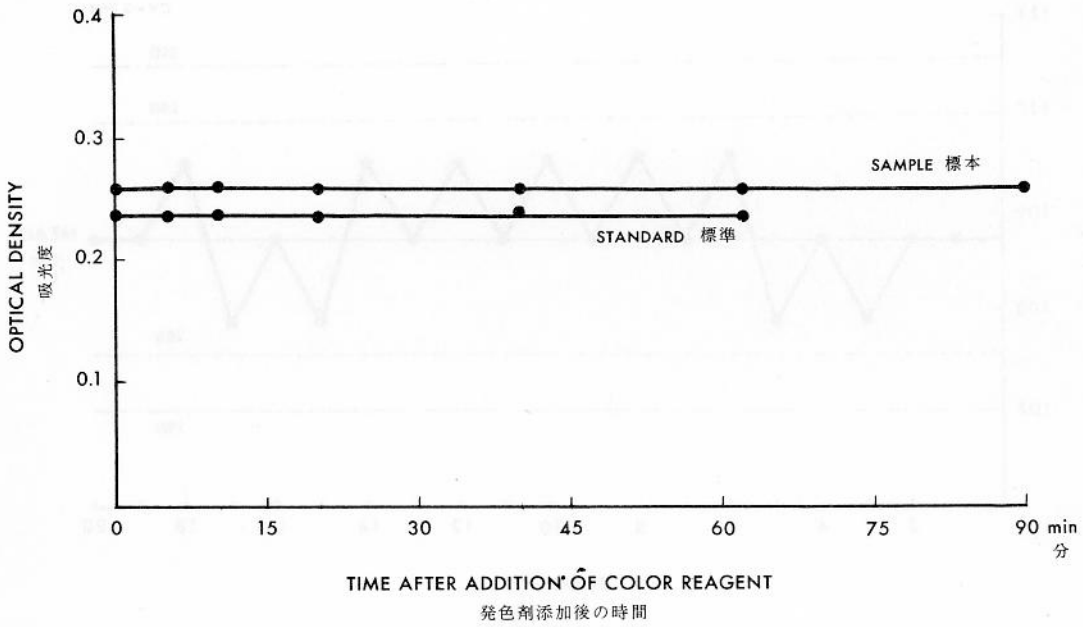


FIGURE 6 EFFECT OF TEMPERATURE ON CHLORIDE DETERMINATION

図6　クロライド測定値に及ぼす温度の影響

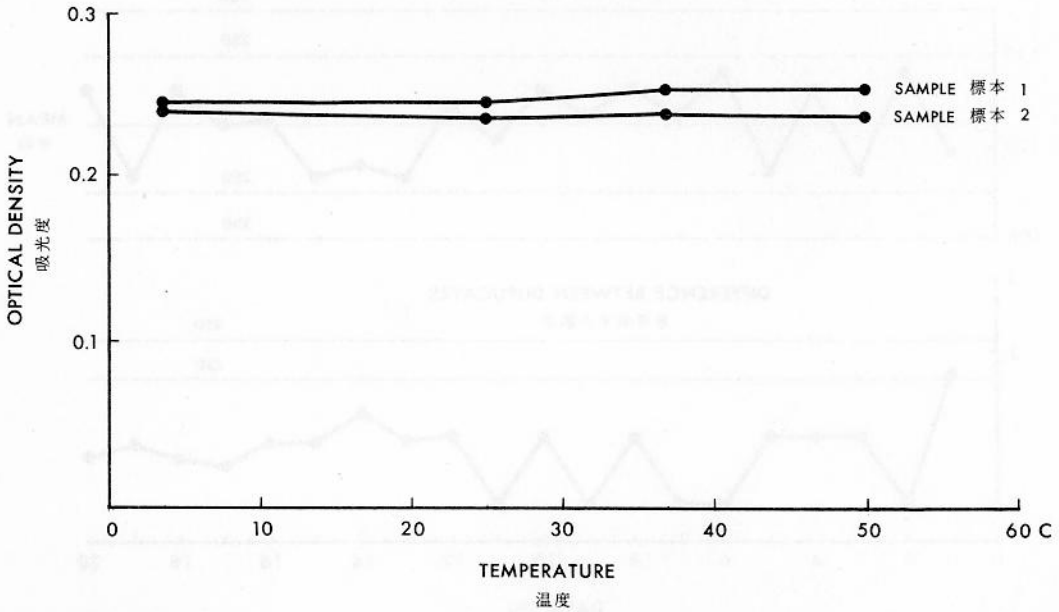


FIGURE 7 REPEATABILITY OF CHLORIDE DETERMINATIONS

図7　クロライド値測定の反復性

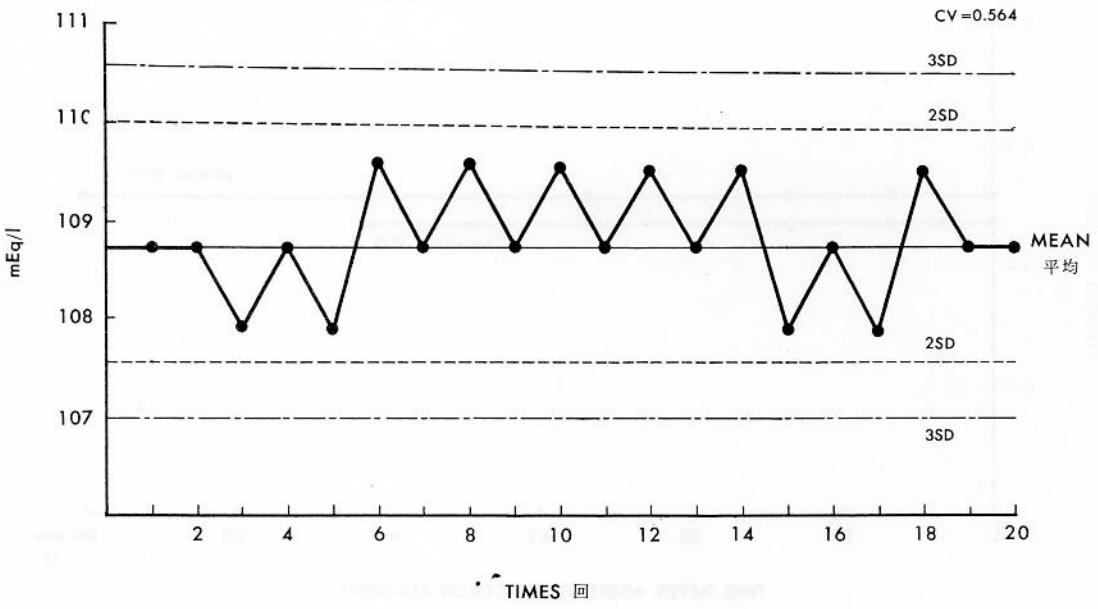
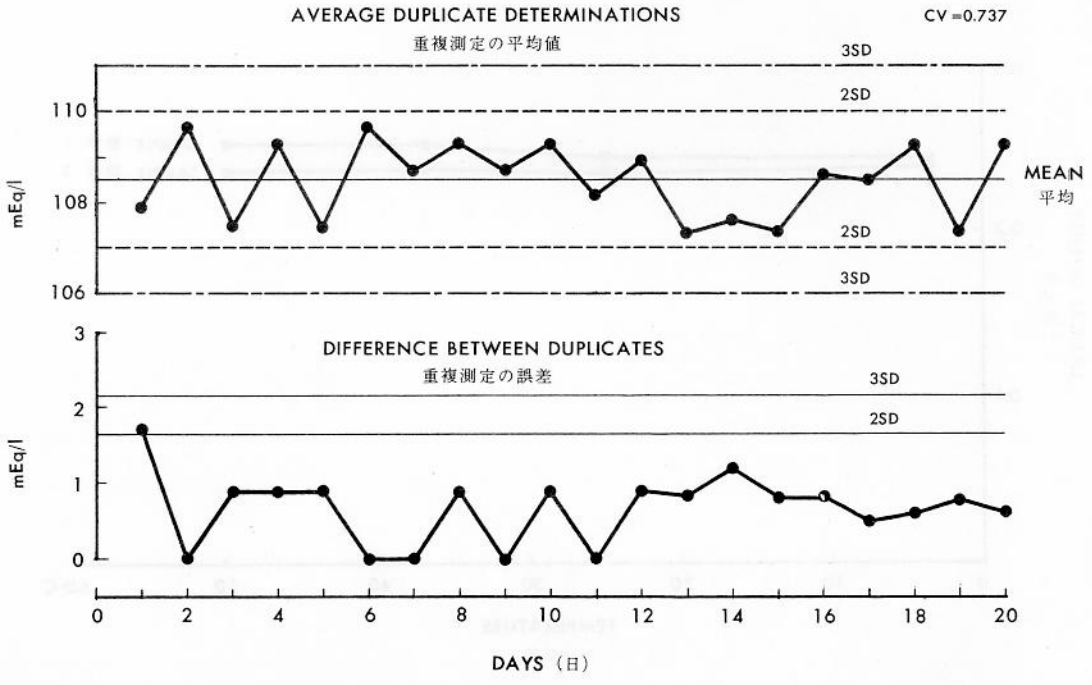


FIGURE 8 REPRODUCIBILITY OF CHLORIDE DETERMINATIONS

図8　クロライド値測定の再現性



solution from column 3, gives a calculated total chloride content shown in column 6. The chloride contents of these artificial mixtures were determined and the results are shown in column 5. Finally, the percent 'recovery' (observed/expected or determined/calculated) is shown in column 7. The percent recovery was over 99% for all but the two higher concentrations of chloride.

欄にある合計クロライド理論値が得られる．これら混合液のクロライド値を測定して，その成績を第5欄に示した．最後に第7欄に回収率（観察値／期待値，または測定値／理論値）を記載した．二つの高濃度クロライド液を除いては，回収率はすべて99%以上であった．

SUMMARY

An ultramicro-colorimetric method was developed based on Hoffman's modification of Sendroy's iodine chloride titration method using silver iodate. This method was shown to be comparable to other methods for determination of serum chloride and can be performed with as little as 0.02 ml of serum.

This method correlates well with chloridimetry (coefficient of correlation 0.991). Repeatability of 20 duplicate determinations was excellent (coefficient of variation = 0.56%); reproducibility was also excellent (coefficient of variation = 0.73%).

要　約

Sendroy のヨウ素酸銀を用いる塩化ヨード滴定法を比色法に改良した Hoffman の測定法に準じて，超微量比色定量法を開発した．本法は他の血清クロライド定量法と同等な精度を有し，わずか0.02ml の血清で測定可能である．

本法は，chloride meter 法に非常によく相関し（相関係数は 0.991），20回重複測定の反復性も（変動係数=0.56%），再現性（変動係数=0.73%）もともにすぐれている．

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