Iodometric and Colorimetric Methods for the Estimation of Calcium in Serum based on the Use of an Improved Permanganate Solution

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In a previous paper (Loureiro & Janz, 1942) we compared the iodometric and oxalic acid titres of permanganate solutions under various conditions, and verified that the values obtained in titrating small samples of oxalic acid in the usual way in hot dilute acid solution may differ appreciably from those yielded by the permanganate-iodide-thiosulphate reaction. This merely confirms a well-known fact on which there is a considerable literature (see McBride, 1912; and Fowler & Bright, 1935), namely, that when excess permanganate is used to oxidize oxalic acid and the excess permanganate is determined iodometrically, the reaction is not stoichiometric. We further ascertained that a closer agreement with theory is obtained when the reaction is carried out in strong acid solution and in the presence of added manganese sulphate. A solution containing 35% H_2SO_4 and 5% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ reacts with oxalic acid at room temperature and with KI in exactly equivalent proportions. This makes it possible to replace the usual titration to the 'pink end-point' by the more exact and sensitive iodometric estimation of unreduced permanganate through the iodide-thiosulphate reaction, or colorimetrically by means of a photoelectric colorimeter.

This greater sensitivity may eventually permit a reduction of the size of the sample required for a satisfactory estimation of calcium in serum to 1 or 0.5 ml. This is not possible with titration to the 'pink end-point', which is already at the limit of sensitivity for the amount of oxalic acid bound by 0.2 mg. Ca. The adaptation of the new method for small samples of serum requires a careful study of the errors involved in the precipitation and washing of the calcium oxalate. This subject will be discussed in a further study.

The present paper is limited to a discussion of the principles of an iodometric and a colorimetric method, which are both more reliable than the usual titration, and to showing that their results agree closely with those obtained by the Clark & Collip (1921) method, when applied to 2 ml. samples of serum.

METHODS

SOLUTIONS

Stock diluent. Dissolve with moderate heat 50 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ in water in a litre flask, cool and add 350 ml. H_2SO_4 . When cool dilute to 1 l.

Standard permanganate solution, approx. N/10. Dissolve 3.5 g. KMnO_4 in 1 l. of stock diluent. The reagent has a characteristic raspberry colour, different from that of the usual aqueous solution. Its loss of titre on storage is very small when it is kept in the ice-box, but is slightly higher at room temperature. This process is due to loss of molecular O_2 and precipitation of MnO_2 ; but, unlike the precipitate formed in the usual aqueous solution, this is black and granular, and sticks to the bottom and walls of the bottle. Since the supernatant fluid remains perfectly clear, no matter how old the solution is, filtration is unnecessary.

From the N/10 solution working dilutions are made in stock diluent. Water should never be added, since it causes precipitation of MnO_2 . Convenient dilutions are approx. N/100 for the iodometric titration and approx. N/500 for the colorimetric estimation.

Standard oxalate solution, N/100. Dissolve 0.335 g. $\text{Na}_2\text{C}_2\text{O}_4$ in 35% H_2SO_4 in 1 l. This solution is required only for the quantitative study of the method and for the estimation of the colorimetric constant.

Standard thiosulphate solution, N/100, exactly adjusted against standard iodate. Addition of 0.2 g. Na_2CO_3 /l. keeps the titre unchanged for many months.

Potassium iodide, 5%.

Soluble starch, 1% in saturated NaCl solution.

Ammonium oxalate, 4%.

Dilute ammonia, 3 vol. of conc. NH_3 solution (sp. gr. 0.88) diluted to 100 vol. with water.

Nitric acid, 50% by vol.

PROCEDURE

Iodometric method

Titration of the blank. Pipette into a beaker 2 ml. of KMnO_4 solution, add about 50 ml. of water and 3-5 ml. KI solution. Titrate at once with $\text{Na}_2\text{S}_2\text{O}_3$.

Titration of the oxalate standard and of unknown solutions. 2 ml. of KMnO_4 and 1 ml. of oxalate standard are mixed, kept 30 min. at room temperature and titrated as stated for the blank. The difference between the vol. of N/100 $\text{Na}_2\text{S}_2\text{O}_3$ used for the blank and the oxalate sample must be exactly 1 ml., equivalent to 0.2 mg. Ca.

Titration of the Clark & Collip calcium oxalate precipitates from serum. The centrifuged, washed precipitate is dissolved in a small drop of 50% HNO_3 , and 2 ml. N/100 KMnO_4 are added. After 30 min., distilled water is added and the contents washed into a beaker. The titration is then performed as for the oxalate solution. 1 ml. N/100 $\text{Na}_2\text{S}_2\text{O}_3$ = 10 mg. Ca/100 ml. serum.

Colorimetric method

The colour density of the N/500 KMnO_4 solution (L) is estimated by means of an Evelyn (1936) photoelectric colorimeter, with a green light filter with maximum transmission at 520 m μ . The Ca-oxalate precipitate from serum

is dissolved in a drop of 50% HNO_3 , and exactly 10 ml. of $\text{N}/500 \text{ KMnO}_4$ are added. After 30 min. the colour density is read (l). The serum Ca is given directly by the equation

$$\text{mg. Ca}/100 \text{ ml.} = K (L - l),$$

where K is a constant depending upon the construction of the apparatus, the colour of the filter and the size of the serum sample.

RESULTS

Iodometric method

Equivalence between permanganate and iodometric titre and its oxidizing strength against oxalic acid. The test was performed ten successive times on $\text{N}/100$ solutions, allowing 2 ml. KMnO_4 to react with 1 ml. of oxalate standard. The expected difference in the $\text{Na}_2\text{S}_2\text{O}_3$ consumptions is 1 ml. Table 1 shows that the oxalic acid and KMnO_4 are strictly equivalent in terms of iodometric titre.

Table 1. Volumes of thiosulphate required in the titration of control and reduced permanganate solutions

Solution titrated	0.01 N- $\text{Na}_2\text{S}_2\text{O}_3$ required ($\mu\text{l.}$)
2 ml. 0.01 N- KMnO_4	1958.0 $\pm$ 0.6
2 ml. 0.01 N- KMnO_4 which had been reduced by 1 ml. 0.01 N-oxalate	959.5 $\pm$ 1.4
Difference	998.5 $\pm$ 0.6

Estimation of calcium in serum and in calcium sulphate solutions. Ca-oxalate precipitates were prepared from 2 ml. of cow sera and from 2 ml. of solutions containing 25 mg. $\text{CaCO}_3/100 \text{ ml.}$ dilute H_2SO_4 . Eight precipitations were made from each serum and half were titrated according to the method of Clark & Collip, the other half by our iodometric technique. In the first group of titrations an average blank value of 0.015 ml. KMnO_4 was subtracted from each reading. In the second group no correction was made because of the much greater sensitivity of the blue starch-iodine endpoint. Table 2 shows that the methods give results in good agreement, the results with the iodometric

procedure usually exhibiting a smaller scatter about the mean than the oxalate-permanganate titration.

Fig. 1. Relation of colour density to volume of permanganate solution. Evelyn photoelectric colorimeter, 520 m μ green light filter. Ordinates: colorimeter readings, L , negative logarithmic scale. Abscissae: $\bullet\bullet\bullet$ ml. of $\text{N}/100 \text{ KMnO}_4$ diluted to 10 ml. with standard diluent; $\times \times \times$ 2 ml. $\text{N}/100 \text{ KMnO}_4$ partially reduced by $(2-n)$ ml. of $\text{N}/100$ oxalate; $\circ\circ\circ$ KMnO_4 solutions, 2 years old, exact titre established by titration, and plotted in terms of ml. $\text{N}/100$.

Colorimetric method

Application of Beer's law. This was verified by measuring the colour density of a series of dilutions of 0.01 N- KMnO_4 (0.2 ml. KMnO_4 diluted to 10 ml. with stock diluent). Stock diluent alone was used as blank. In Fig. 1 the values of the colour density

Table 2. Comparison of the results of estimation of calcium in CaSO_4 solutions and in serum by KMnO_4 titration, and iodometric titration of excess KMnO_4

Method	Calcium content (mg./100 ml.) of					
	CaSO_4 solution			Serum		
	1	2	3	4	5	6
KMnO_4 titration	10.15	10.41	10.54	8.93	8.20	9.19
	10.42	10.17	10.41	8.83	8.43	9.60
	11.33	10.48	—	8.77	8.12	9.19
	10.22	10.56	—	8.88	8.28	9.24
Average	10.25 $\pm$ 0.14	10.40 $\pm$ 0.19	10.47	8.85 $\pm$ 0.08	8.25 $\pm$ 0.14	9.30 $\pm$ 0.23
Iodometric titration	10.43	10.61	10.19	8.81	8.31	9.70
	10.28	10.46	10.04	9.01	8.26	9.75
	10.33	10.51	—	8.71	8.36	9.70
	10.28	10.66	—	8.81	8.31	9.70
Average	10.33 $\pm$ 0.08	10.56 $\pm$ 0.10	10.11	8.84 $\pm$ 0.14	8.31 $\pm$ 0.05	9.72 $\pm$ 0.03

are plotted against the KMnO_4 concentrations. Since they fall in a straight line, the solution conforms to Beer's law.

Stoichiometric relationship between permanganate and oxalate and colour density. A second series was prepared from 2 ml. of $\text{N}/100$ KMnO_4 and decreasing volumes of $\text{N}/100$ sodium oxalate diluted to 10 ml. with stock diluent. The readings were recorded after 30 min. (Fig. 1). A straight line is again obtained.

This not only confirms the results of the iodometric titration, but also shows that all available oxidant is in the state of MnO_4^- ions, the Mn of which is reduced from Mn^{VII} to the lowest oxidation level, Mn^{II} . Were intermediate oxidation products such as MnO_2 present, the relationship would deviate from the straight line.

Colour density of old and new permanganate solutions. Measurements were made of four different solutions, two recently prepared and two old ones. One of these, kept at room temperature for nearly 2 years, had lost seven-tenths of its original strength. The solutions were diluted to strengths between $\text{N}/500$ and $\text{N}/1000$ and the exact strengths determined by titration. In Fig. 1 the colour values are plotted against the titre. All fall within narrow limits on the straight line previously established. The constancy of the titre-colour density relation was regarded as proved when the same constant was obtained in parallel experiments and with various standard reductants.

Establishment of colorimetric constant for Evelyn colorimeter by measurement of colour of standard permanganate solutions partially reduced by standard reductants. Potassium iodide and thiosulphate, oxalic acid, ferrous ammonium sulphate and arsenite were used, but As_2O_3 was discarded because it requires heating and in these conditions the end-point is not sharp. 5 ml. of an exact $\text{N}/500$ solution of the standard reductant in 35% H_2SO_4 were mixed with 5 ml. of $\text{N}/250$ KMnO_4 . The colour density of this partially reduced sample was measured in an Evelyn photoelectric colorimeter, as likewise that of $\text{N}/500$ KMnO_4 (5 ml. $\text{N}/250$ KMnO_4 and 5 ml. standard diluent). The difference between the colour density of $\text{N}/500$ KMnO_4 (L) and that of the partially reduced sample (l) expresses the loss of colour due to the action of 10 ml. of a $\text{N}/1000$ solution of standard reductant. The relation between the titre and the colour of the solution measured in a photoelectric colorimeter is given by the equation

$$K = \frac{10^{-3}}{L-l},$$

and K is a constant for the colorimeter used. This is the method of choice for ascertaining the constant of any colorimeter, using $\text{Na}_2\text{C}_2\text{O}_4$ as the reference standard. Table 3 presents the constants deter-

mined for the Evelyn colorimeter used in our work. The results with the three standards agree within the limits of their probable errors. Since, however,

Table 3. Colorimetric constant for Evelyn colorimeter of the new permanganate solution estimated with different standard reductants

Standard reductant	No. of exps.	$K \times 10^3$
Iodide thiosulphate	13	4.679 ± 0.067
Oxalate	11	4.623 ± 0.019
Ferrous ammonium sulphate	12	4.653 ± 0.042

The colour density at $520\text{m}\mu$ was measured for $\text{N}/500$ KMnO_4 (L) and for $\text{N}/500$ KMnO_4 partially reduced by $\text{N}/1000$ standard reductant (l), and the relation between normality and colour density (a constant for the instrument) calculated from the equation $K = 10^{-3}/(L-l)$.

the iodometric value tends to be slightly high on account of the end-point of the titration, and since the end-point with the ferrous salt is not so stable as with the oxalate standard, the latter probably yields the most reliable value of K . With the colorimeter used the titre of a permanganate solution may be expressed by the equation

$$\text{Normality} = 4.63 \times L_{520} \times 10^{-3}.$$

Application to the estimation of calcium in serum. The equation yielding the calcium content of serum, when 2 ml. samples are used, is

$$\text{Ca (mg./100 ml.)} = 46.3 (L_{520} - l_{520}).$$

The results of a comparison of the iodometric and colorimetric methods in three different cow sera are recorded in Table 4. The agreement is good, and again the main difference is in the scatter of duplicates, which is slightly higher in the colorimetric series.

Table 4. Comparison of duplicate results of iodometric and colorimetric estimations of calcium in serum

Method	Calcium content (mg./100 ml.) of serum		
	1	2	3
Iodometric titration	9.60	9.35	9.30
	9.50	9.40	9.45
	9.55	9.45	9.40
	9.60	9.20	9.45
Average	9.56 ± 0.05	9.35 ± 0.12	9.40 ± 0.08
Colorimetric estimation	9.53	9.19	9.31
	9.70	9.77	9.44
	9.53	9.27	9.35
	9.70	9.41	9.44
Average	9.61 ± 0.11	9.41 ± 0.30	9.38 ± 0.07

DISCUSSION

At a first glance, it may appear that the results of the techniques here described do not improve on those obtained by the Clark & Collip method for the estimation of calcium in serum, since their mean values are in good agreement. A discussion of the

experimental errors will show, however, that an improvement has been secured.

The most unsatisfactory part of Clark & Collip's technique is the final KMnO_4 titration. The inconvenience of keeping the solution at 80° , the significant variations resulting from differences in speed of titration, the uncertainty of the end-point, the risk of over-titration, etc., cause the result to be somewhat uncertain. The large number of slightly different techniques that have been advocated for the standardization of aqueous KMnO_4 solutions is a sign that none of these variations has entirely solved the difficulties of titrations involving this reagent. It is hoped that the drastic change in the conditions of the reaction introduced by the use of the new KMnO_4 solution will settle satisfactorily this long-standing difficulty.

As shown in Table 1 the new solution is capable of oxidizing oxalic acid at room temperature and its oxidizing strength is exactly the same whether it is measured against oxalic acid or by an iodometric method. This is the important feature of the method, and the *conditio sine qua non* for an exact iodometric or colorimetric estimation of unreduced permanganate.

Table 1 shows, further, that in the titration of a standard oxalate solution the scatter of duplicates is very small. The sensitivity of the iodine-starch reaction is such that the 'end-point error' with $N/100$ solutions (0.1 – 0.3%) is well below the combined volumetric error of the method. This means that a further reduction in the titre of the thio-sulphate standard solution and in the size of the samples to be titrated is permissible without prejudice to the accuracy.

In Table 2 the scatter of duplicates expresses the combined errors of the precipitation and of the titration. The probable error for the iodometric estimation of calcium in serum ($\pm 0.8\%$) is two or three times that of titration. With the Clark & Collip method the combined error is still larger ($\pm 1.5\%$); and, since the errors of titration to the 'pink end-point' exceed those involved in the isolation of the precipitate from 2 ml. of serum, there is no possibility of utilizing this type of titration for smaller amounts of serum without proportionately increasing the final error of the method.

The convenience of the colorimetric estimation is particularly apparent in mass investigations, not only on account of the absence of titration, but also because the stability of the end-point permits

the readings to be checked whenever necessary. Another advantage of the colorimetric method is the elimination of a titrimetric standardization of the solution. Hoffman (1940) has already suggested the standardization of aqueous KMnO_4 solutions with a photoelectric colorimeter. With the new solution, an exact parallelism between the colour density and the titres against oxalic acid, ferrous ammonium sulphate, and potassium iodide has been demonstrated. Therefore, when once a photoelectric colorimeter has been calibrated by the method described, the titres of unknown solutions will be estimated directly from their colour density. Table 3 shows that the error of this estimation is about 1% when all three standards are considered together, much less when oxalic acid, the most reliable of the three, is considered alone.

In the estimations of calcium in serum (Table 4), the probable error of the colorimetric method ($\pm 1.6\%$) is slightly higher than that of the iodometric ($\pm 0.8\%$). This is not surprising, even with a good colorimeter, and it does not mean that the error would increase in proportion to the reduction in the absolute colour density and to the differences between blank and experiment, if the method were to be adapted to smaller samples.

SUMMARY

1. A modified permanganate solution which reacts stoichiometrically at room temperature with oxalic acid and with potassium iodide is described.

2. The use of this solution permits the uncertain titration to the 'pink end-point' to be replaced by an exact iodometric estimation of the unreduced permanganate.

3. Application of the method to 2 ml. samples of serum is described, and the agreement with the results of the Clark & Collip method verified.

4. The colour of this permanganate solution follows Beer's law, and its colour density expresses exactly its oxidizing strength against oxalic acid, ferrous ammonium sulphate and potassium iodide.

5. The colorimetric estimation of calcium in serum is described, and found to be in agreement with the results of the iodometric titration.

6. Examination of the experimental errors shows that, while in the Clark & Collip method the errors of titration exceed those involved in the isolation of the calcium oxalate precipitate from 2 ml. serum, the reverse is the case with the new method. This makes possible its adaptation to smaller amounts of serum.

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