CHAPTER 15

PREPARATION OF REAGENTS

Since the level of iron in serum is so low, contamination is the most important cause of inaccuracy. Therefore, a great care should be taken to avoid any possible iron contamination during the preparation of reagent which are to be used for the purpose of serun iron estimations. It may also be necessary to discard certain batches of reagents if they produce a strong colour in the Blank.

REAGENTS TO BE PREPARED

(1) 1 N Oxalic acid.

(2) 1 N Sodium hydroxide.

(3) Alkaline acetate solution.

(4) Trichloracetic acid (60% W/V).

(5) 1 N Hydrochloric acid.

(6) 0.01 N Hydrochloric acid.

(7) Ascorbic acid solution.

(8) Dipyridyl reagent.

(9) Iron-free water.

IMPORTANT CONSIDERATIONS

(1) All the reagents must be of "Anala-R" Grade (A.R.Grade) (free from iron-contamination).

(2) The various ingredients should be weighed accurately on an analytical balance.

(3) All the reagents and solutions should be made with iron-free water (triple glass-distilled water).

(4) The reagents and solutions should be stored in previously prepared "iron-free" glass-stoppered reagent bottles.

(5) All the bottles should be labelled properly, corked tightly and preserved carefully.

(6) A labelled pipette should be kept SEPARATE for each reagent and must be used only for that particular reagent/solution.

PREPARATION OF SOLUTIONS

(1) 1N Oxalic acid solution :

Normal oxalic acid solution is used for preparing normal sodium hydroxide. This is because Oxalic acid is a very stable substance and can be weighed very accurately. Hence, the normal solution can be prepared easily, accurately and is stable (i.e. remains for a long time as such) so that it can be used for standardising normal sodium hydroxide

solution at any time.

Formula : $(COOH)_2 .2H_2O$ (Crystalline Oxalic acid)

The equivalent weight of Oxalic acid is found as follows :

$$2NaOH + \begin{matrix} COOH \\ | \\ COOH \end{matrix} = \begin{matrix} COONa \\ | \\ COONa \end{matrix} + 2H_2O$$

90

(Anhydrous Oxalic acid) (Sodium Oxalate)

Hence, the equivalent weight of anhydrous oxalic acid is 90/2 = 45. The equivalent weight of crystalline Oxalic acid is $\frac{90 + 36}{2} = \frac{126}{2} = 63$.

Therefore, 63 gm. of crystalline oxalic acid when dissolved in water to give 1 litre of final solution will give Normal solution of oxalic acid.

Method :

A watch glass was weighed accurately on an analytical balance. Then 6.3 gm. of crystalline oxalic acid was weighed accurately in this watch glass. The oxalic acid used was of C.P. grade (chemically pure grade) or of A.R. grade (Analytical reagent grade).

The oxalic acid so weighed was transferred to a 100 ml. flask through a funnel. The washings of the watch glass, funnel and sides of the flask were added. Some more distilled water was also added and the oxalic acid was dissolved

by shaking. The volume was made to 100 ml. by adding more distilled water, and the solution was stirred well. The normal solution was thus ready.

(2) 1N Sodium Hydroxide Solution :

Sodium hydroxide was supplied in form of small pellets. It is deliquescent substance and absorbs moisture and carbon dioxide from air so rapidly that it becomes liquid and forms sodium carbonate. It always contains some carbonate. Hence, this substance cannot be weighed accurately. (If one tries to weigh it accurately, during the time required, it absorbs considerable moisture and carbon dioxide from air).

The equivalent weight of sodium hydroxide is found from the following equation:

$$\underset{36.46}{HCl} + \underset{40}{NaOH} = NaCl + H_2O$$

Hence, equivalent weight of NaOH is 40. At first the solution is always to be prepared more concentrated than what is required so that afterwards the required concentration can be accurately adjusted by necessary dilution.

Method :

The watch glass was weighed. Then, approximately (not accurately) 20.5 gm. of sodium hydroxide pellets were weighed. (NaOH is handled with forceps). 50 ml. of triple glass-distilled water was taken in a beaker

which was then kept in a cold water basin. (The beaker was kept in cold water because much heat is evolved - it being an exothermic reaction when NaOH is added to water). The weighed NaOH was transferred to the beaker quickly. It was allowed to cool. During this period it dissolved by itself. (The beaker was not shaken lest NaOH may absorb CO_2 from air). The contents of the beaker were transferred to a 500 ml. volumetric flask. The volume was made to 500 ml. by adding triple glass-distilled water. This approximately 1N NaOH solution was standardised against 1N Oxalic acid solution.'

STANDARDISATION AGAINST 1N OXALIC ACID SOLUTION

$$2\ NaOH + \begin{matrix} COOH \\ | \\ COOH \end{matrix} = \begin{matrix} COONa \\ | \\ COONa \end{matrix} + 2H_2O$$

2 x 40 — 2 x 45 (Anhydrous oxalic acid)

Hence 40 gm. of NaOH is equivalent to 45 gm. of anhydrous oxalic acid, or to 63 gm. of crystalline oxalic acid.

$\therefore$ 1000 ml. of 1N NaOH $\equiv$ 1000 ml. of 1N oxalic acid.

$\therefore$ 20 ml. of 1N NaOH $\equiv$ 20 ml. of 1N oxalic acid.

Procedure :

20 ml. of 1N oxalic acid was measured accurately, and transferred to flask. 1-2 drops of phenolphthalein as indicator were added. (Phenolphthalein gives colourless appearance in acidic medium and pink colour in basic medium). Approximate 1N NaoH was taken in burette.

Development of persistant pinkish colour marked the end point. The amount of 1 N NaOH solution required was noted. (This should be less than 20 ml. because instead of 20 gm. 20.5 gm. of NaOH was added to 500 ml. This approximately 1 N NaOH solution is always to be prepared more concentrated than normal solution so that it can be easily adjusted to normal by dilution. If it is more dilute than normal solution, NaOH will have to be added to it and then to be titrated again).

Results of First Titration :

TABLE NO. 17

STANDARDISATION AGAINST 1 N OXALIC ACID SOLUTION

Obs. No.	Amount of 1 N oxalic acid solution in flask (in ml.)	Approx. 1 N NaOH solution in Burette				Indicator
		Initial reading (in ml)	Final reading (in ml.)	Difference (in ml)	Mean reading (in ml)	
1.	20.0	0.0	19.4	19.4	19.4	Phenolphthalein.
2.	20.0	2.0	21.4	19.4		

The end point was marked by development of persistent pinkish colour.

The end point was reached at 19.4 ml. of approximate 1 N NaOH solution.

∴ Every 19.4 ml. of approximate 1 N NaOH solution ≡ 20 ml. of (exact) 1 N NaOH solution.

∴ 19.4 ml. of approximate 1 N solution should be diluted to 20 ml. to give exactly 1 N solution.

∴ 19.4 ml. of approximate 1 N solution should be diluted to 200 ml. This dilution was carried out accordingly. Then, it was retitrated against 1 N oxalic acid solution for confirmation.

Results of Second Titration :

TABLE NO. 18

STANDARDISATION AGAINST 1 N OXALIC ACID SOLUTION

Obs. No.	Amount of 1 N oxalic acid soln. in flask (in ml.)	Approximate 1 N NaOH solution in Burette				Indicator
		Initial reading (in ml.)	Final reading (ml)	Difference (in ml)	Mean reading (ml)	
1.	20.0	0.0	20.0	20.0	20.0	Phenolphthalein.
2.	20.0	5.0	25.0	20.0		

The end point was marked by development of persistent pinkish colour.

Thus, the diluted NaOH solution was retitrated similarly against 20 ml. of 1N oxalic acid solution to confirm that exactly 20 ml. of 1N NaOH solution neutralised 20 ml. of 1N oxalic acid solution.

The standardisation of 1N NaOH solution is also possible by using oxalic acid crystals. But the use of oxalic acid solution is more convenient and accurate because the solution once prepared can be used for any number of titration, without any chances of variation in the strength of oxalic acid solution.

(3) Alkaline Acetate Solution :

Alkaline acetate solution is prepared by saturating 1N Sodium hydroxide solution (vide supra) with solid sodium acetate (25 gm. of solid sodium acetate per 100 ml. of 1N sodium hydroxide solution). Deposits are formed at the bottom of the bottle. Portions of solution are removed for use without disturbing the deposits.

(4) Trichloracetic Acid Solution (60% W/V) :

300 gm. of trichloracetic acid was weighed out accurately and transferred to a 500 ml. volumetric flask. The washings were added to the flask. The volume was made to 500 ml. by adding triple - glass - distilled water. The contents after stirring well were transferred to the reagent bottle.

(5) 1 N Hydrochloric Acid solution :

The commercial conc. Hydrochloric acid has 1.19 sp. gr. and contains about 36% Hcl. by weight (i.e.36 gm. of Hcl. in 100 gm. of the conc.acid.) 100 ml. of conc. acid will weigh 100 x 1.19 = 119 gm.

But 100 gm. of conc. acid solution contains 36 gm.of acid. .'. 119 gm. of conc. acid solution will contain

$$\frac{119 \times 36}{100} = 46.74 \text{ gm. of acid.}$$

i.e. 100 ml. of conc. acid solution contains 46.74 gm.of acid.

.'. Approx. 79 ml. of conc. acid solution contains 36.46 gm. of acid.

.'. to prepare a slightly stronger solution than normal, 90 ml. of conc. HCl were measured in a graduated measuring cylinder. (This was done in a hood because fumes are disagreeable) and was added to 1 litre volumetric flask containing 800 ml. of cold triple-glass-distilled water. (A strong acid or alkali is to be added to water,and not water to the acid or alkali. The reaction of acid or alkali with water is exothermic and much heat is evolved. Hence, to keep the temperature as low as possible, acid or alkali is added to water which is first taken in the container.) It was diluted to the mark with triple-glass-distilled water, and was mixed well. This approximate 1 N solution of HCl (slightly stronger than normal)was

standardised against pure anhydrous sodium carbonate (C.P.) by using Methyl Orange as an indicator (yellow in basic medium and organge in acidic medium), or it may be standardised against 1 N NaOH solution by using Phenolphthalein as an indicator (colourless in acidic medium and pink in basic medium).

Standardisation against 1 N NaOH Solution :

$$HCl + NaOH = NaCl + H_2O$$

36.46 40

$\therefore$ 1000 ml. of 1N NaOH $\equiv$ 1000 ml. of 1 N HCl.

$\therefore$ 20 ml. of 1 N NaOH $\equiv$ 20 ml. of 1 N HCl.

HCl was taken in flask and phenolphthalein was used as an indicator.

Results of First Titration :

TABLE NO. 19

STANDARDISATION AGAINST 1 N NaOH SOLUTION

Obs. No.	Amount of approx. 1N HCl. in flask.(ml)	1 N NaOH solution in Burrette				Indicator
		Initial reading (in ml)	Final reading (in ml)	Difference (in ml)	Mean (in ml)	
1.	20.0	0.0	21.3	21.3	21.3	Phenolphthalein.
2.	20.0	3.0	24.3	21.3		

The end point was marked by development of persistent pinkish colour.

$\therefore$ Every 20.0 ml. of approx. 1 N HCl $\equiv$ 21.3 ml. of (exact) 1 N HCl.

$\therefore$ Every 20.0 ml. of approximate 1 N HCl should be diluted to 21.3 ml. to give 1 N HCl.

Accordingly, the dilution was carried out and the normality was confirmed by retitrating it against 1 N NaOH solution.

Results of the Second Titration :

TABLE NO. 20

STANDARDISATION AGAINST 1 N NaOH SOLUTION

Obs. No.	Amount of (exact) 1 N HCl. in flask. (ml.)	1 N NaOH Solution in Burette				Indicator
		Initial reading (ml.)	Final reading (ml.)	Difference (ml.)	Mean (ml.)	
1.	20.0	2.0	22.0	20.0	20.0	Phenol-phthalein
2.	20.0	0.0	20.0	20.0		

The end point was marked by development of persistent pinkish colour.

Thus, 1 N HCl solution was ready for the use .

(6) 0.01 N Hydrochloric acid solution :

This was prepared by 1 in 100 dilution of 1 N hydrochloric acid with triple-glass-distilled water.

(7) Ascorbic acid Solution :

0.25 gm. of ascorbic acid was weighed out accurately on an analytical balance and was transferred carefully to a small test tube. It was dissolved in 3 ml. of 1 N Hydrochloric acid solution. The tube was shaken well till the ascorbic acid dissolved completely. The solution was prepared FRESHLY Each Day.

(8) Dipyridyl Solution :

0.2 gm. of ascorbic acid and 0.02 gm. of 2 : 2' Dipyridyl (Anala - R Grade) were weighed out accurately on Mettler Balance (Photograph No.89) as follows :

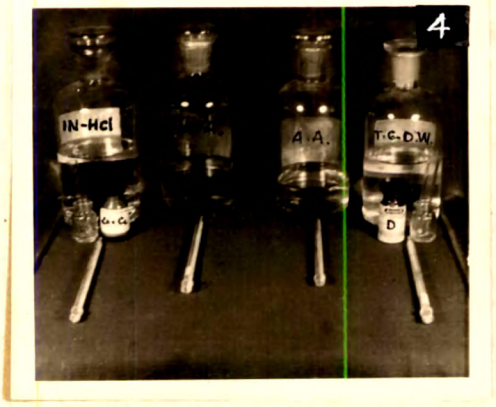
Weight	
0.153	gm. paper slip
+	
0.200	gm. ascorbic acid
0.353	Total
+ 0.020	gm. Dipyridyl.
0.373	Total weight

Then it was transferred to a small "Iron-free" test tube, containing 4 ml. of triple-glass-distilled water. It was mixed well till a clear solution was obtained. The solution was made FRESHLY each day.

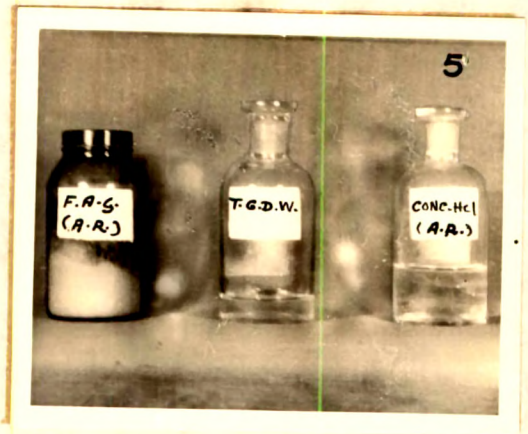
(9) Iron-free Water :

The 'iron-free' water is one of the most important and commonest requirements for Serum Iron Estimations. The

triple-glass-distilled water was used for the purpose. It was prepared by distillation of water in a metal apparatus, then re-distillation in an all-glass apparatus and again re-redistillation in an all-glass apparatus. The bottles of triple-glass-distilled water should be corked nicely, labelled properly and preserved carefully.



Main Reagents required for Serum Iron Estimation with a SEPARATE labelled pipette for each reagent.



Requirements for preparing
Stock Iron Standard

PROCEDURE

(1) Stock Iron Standard :

An "iron-free" watch glass was weighed accurately on an analytical balance. Then exactly 21.59 gm. of Ferric ammonium sulphate (A.R.) was weighed out accurately. The contents of the watch glass were carefully transferred to a 500 ml. volumetric flask through an "iron-free" funnel. The washings of the watch glass, funnel and sides of the flask were added. 50 ml. of concentrated hydrochloric acid (A.R.) and some amount of triple-glass-distilled water were added to the flask. Ferric ammonium sulphate was dissolved by shaking till a transparent clear solution was obtained. More triple-glass-distilled water was added to make the volume to 500 ml. It was transferred

to a iron-free reagent bottle. The stock iron standard solution was thus ready, containing 5 mg. (5000 mcg.) Fe per 100 ml.

The stock iron standard solution was preserved in a Brown coloured screw capped bottle in refrigerator. The brown colour of the bottle prevents the action of light on the contents of the bottle when the latter is exposed to light. The bottle should be kept in refrigerator immediately after the use. The refrigerator favourably supplies low temperature and darkness.

Preservation of stock standard (S.S)

(2) Dilute Iron Standard :

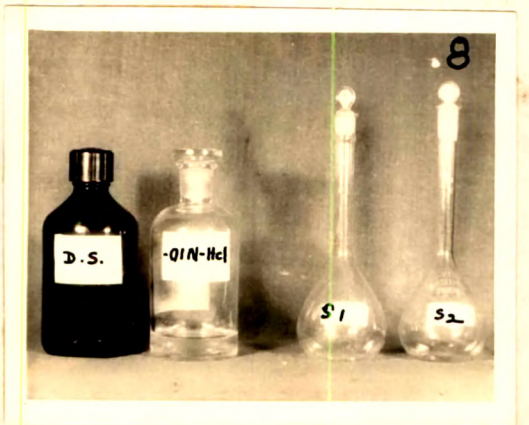
The dilute iron standard was prepared by 1 in 10 dilution of stock standard solution with 0.01 N hydrochloric acid. Hence exactly 1 ml.of stock iron standard was pipetted out in a test tube or 50 ml. beaker containing 9 ml. of 0.01 N HCl. The

contents were mixed well. The dilute iron standard solution thus prepared contains 500 mcg. Fe per 100 ml.

Preparation of Dilute standard (D.S.)

(3) Working Iron Standard :

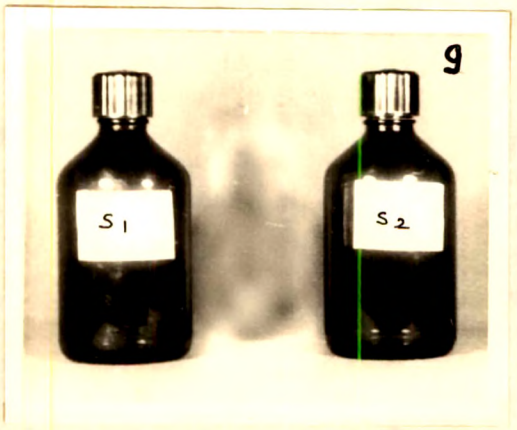
The working iron standards were prepared by diluting exactly 0.2 ml. and 0.4 ml. respectively of dilute iron standard to 100 ml. with 0.01 N hydrochloric acid.



Preparation of working standard (W.S.)

The two working iron standards (S_1 and S_2) thus prepared contains 100 and 200 mcg. Fe per 100 ml. respectively.

The working iron standards (S_1 and S_2) are to be prepared from stock iron standard freshly each time. Alternatively, if the working iron standards(S_1 and S_2) prepared above are preserved carefully in brown coloured screw capped bottles in refrigerator, the same may be readily used as and when required.

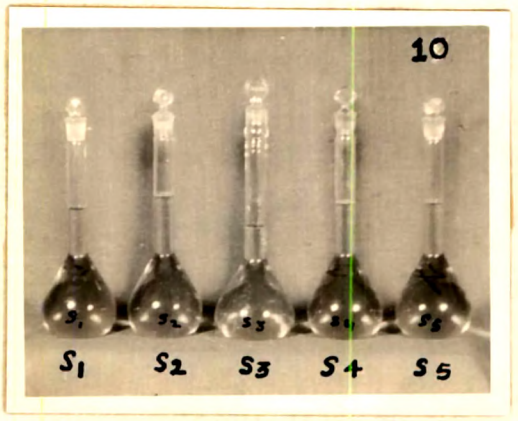


Preservation of S_1 and S_2

The use of ready made working standards saves much time and labour to be spent at each set of experiment. Simultaneous use of fresh working standards and stored working standards during the course of present study has given practically the same results.

to each flask respectively and the volume in each flask was made to 100 ml. by adding 0.01 N HCl.

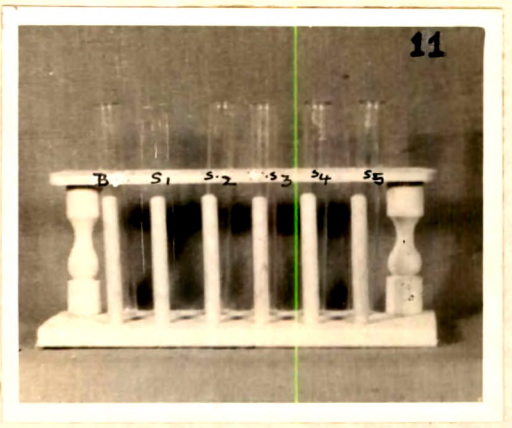
		S_1	S_2	S_3	S_4	S_5
(1)	Amount of dilute iron standard (containing 500 mcg. %) in ml.	0.1	0.2	0.3	0.4	0.5
(2)	Final volume to be made(by adding 0.01 N HCl)in ml.	100	100	100	100	100
(3)	Iron concentration in mcg. per 100 ml.	50	100	150	200	250



Progressive Dilution of
Dilute Iron Standard

After the dilution, the contents of each flask were mixed thoroughly by shaking. It was not possible for the pipette to enter the narrow mouth of flask. Therefore,five iron-free test tubes were taken and labelled as S_1, S_2, S_3, S_4 and S_5. The five different working iron standards prepared above were transferred to these test tubes respectively.

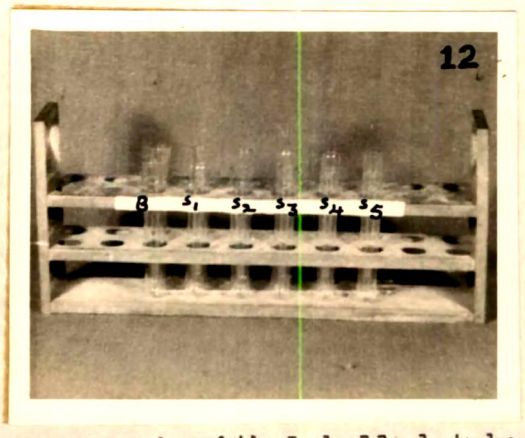
A test tube containing triple-glass-distilled water (Blank) was also included.



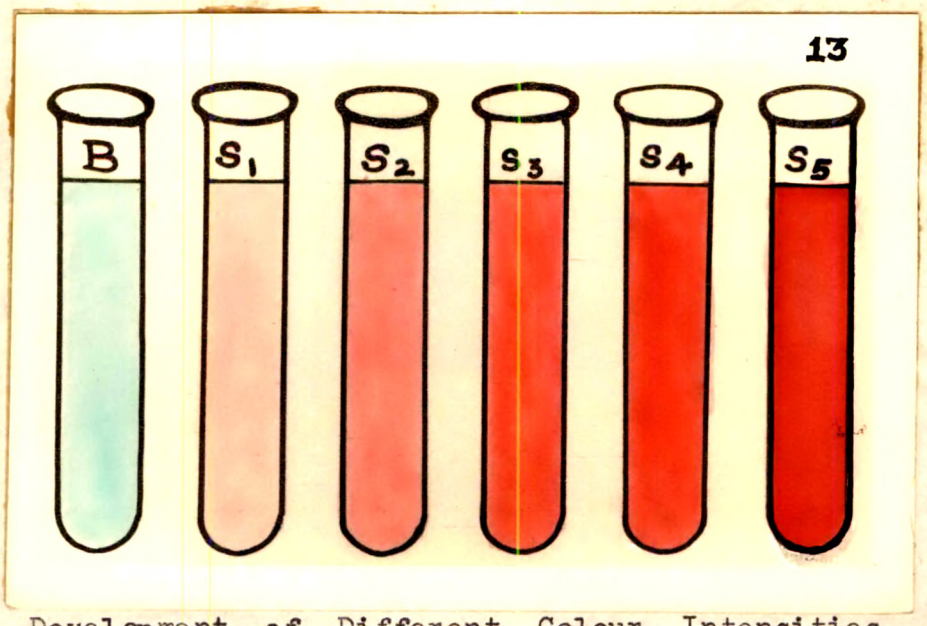
Blank and five different working iron standards in test tubes

PROCEDURE

Twelve small test tubes (3" x 1/2") were arranged on a copper rack in two rows of six each. Each row of six test tubes was labelled as B, S_1, S_2, S_3, S_4 and S_5.



Copper rack with labelled tubes



Development of Different Colour Intensities

(12) Measure the optical density in Beckman Spectrophotometer at 520 m u, using the Blank solution(distilled water Blank) to set the instrument to zero.

RESULTS

Five such experiments were run and the results of each experiment are given in separate tables along with the Iron Standard Curve (chapter 26).