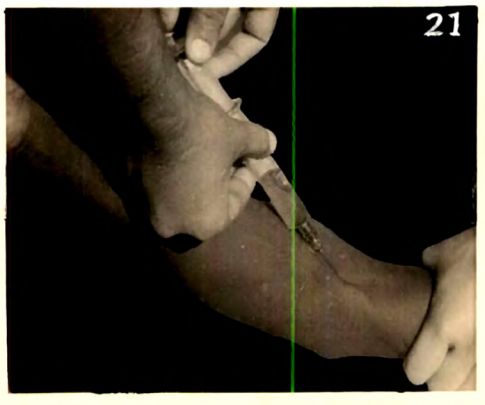
CHAPTER 19

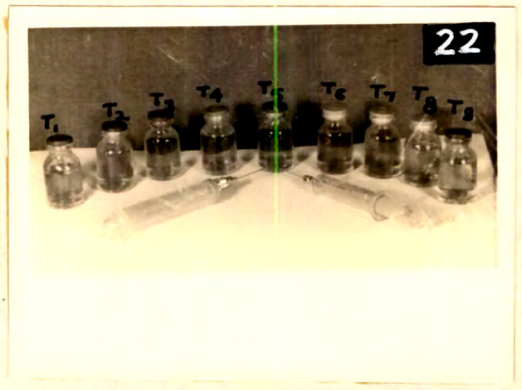
SERUM IRON ESTIMATION

On the day of starting the Iron Therapy, Fasting blood sample (10 c.c.) was collected in plain bulb. Then a dose of 3 tablets of ferrous sulphate or ferrous fumarate was given to the patient stat after lunch only. The second and third sample of blood was collected 4 hours and 8 hours respectively after the administration of drug.



Collection of blood sample

In this way three blood samples were collected from each patient. The patients were taken up for Serum Iron Estimation in batches of three. Thus, the total number of blood samples collected at each set of experiment was nine. A separate all-glass iron-free syringe was utilised for collecting each of the nine blood samples. Similarly, nine blood samples were collected from the same three patients on 21st day of treatment.



Nine blood samples collected at each set of experiment

PRINCIPLE

The iron is liberated and maintained in the reduced state with ascorbic acid. Ferrous iron when allowed to react with 2:2' Dipyridyl (Anala-R) produces pink colour. The optical density of the colour so produced is measured in Beckman Spectrophotometer.

DIPYRIDYL REAGENT

Formula : $(C_5H_4N)_2$ = 156.19

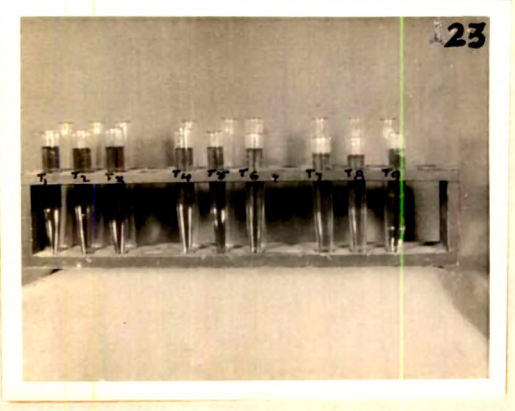
2:2' Dipyridyl is also known as α-α' Dipyridyl. This iron-reagent used for the purpose must be of "Anala-R" grade. Interestingly, the SENSITIVITY of 2:2' Dipyridyl (Anala-R) to IRON (Fe) is 1:10,000,000 MINIMUM.

PROCEDURE

The serum iron was estimated by the method[78] described by Marrack (1956), which was modified and simplified during the present study. The following are the actual steps of the procedure employed :

(1) Detach the blood clots carefully from the wall of the plain bulbs with a thin glass rod(a separate glass rod for each sample).

(2) Pour out the upper clear portions of sera from plain bulbs into the centrifuge tubes of the front row.



18 centrifuge tubes in two rows

(3) Centrifuge the sera at 3000 r.p.m. for 15 minutes to obtain completely clear samples.

Centrifugalisation of Sera

(4) Pipette out exactly 4 ml. of each test sample of serum into the corresponding centrifuge tubes of the back row, using a separate pipette for each sample.

(5) Add 0.2 ml. of freshly prepared ascorbic acid solution to each tube.

(6) Mix well by stirring with a thin glass rod (a separate glass rod for each sample).

(7) Allow to stand for 5 minutes at room temperature.

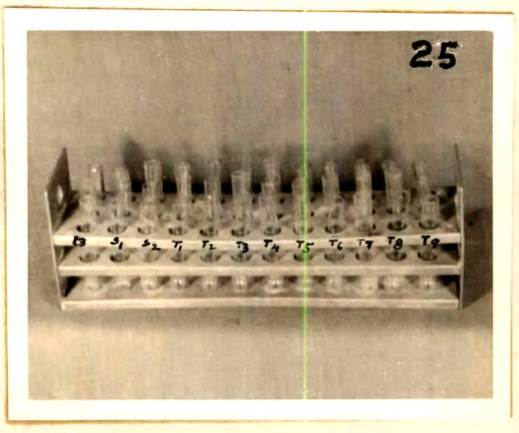
(8) Add 0.4 ml.of Trichloracetic acid solution to each tube.

(9) Mix the contents of each tube thoroughly by Vigorous shaking for 1 minute. (The vigorous shaking is to be carried out immediately after the addition of trichloracetic acid solution in a particular tube).

(10) Allow to stand for 10 minutes at room temperature.

(11) Centrifuge at 3000 r.p.m. for 15 minutes.

(12) Pour out the supernatant fluids from the centrifuge tubes into the corresponding small test tubes of the front row (out of the three rows) arranged on a copper rack.



Copper rack holding 36 small test tubes arranged in 3 rows of 12 each

(13) Centrifuge at 3000 r.p.m. for 5 minutes.

(14) Pipette out exactly 2 ml. of clear aliquote of the protein-free supernatant fluid accurately into the

corresponding tubes of the middle row, using a clean separate pipette for each. Besides this, pipette out 2 ml. of triple-glass-distilled water in the first tube (labelled as B) and 2 ml. of each standard 1 and standard 2 in the 2nd and 3rd tube respectively (labelled as S_1 and S_2) as follows :

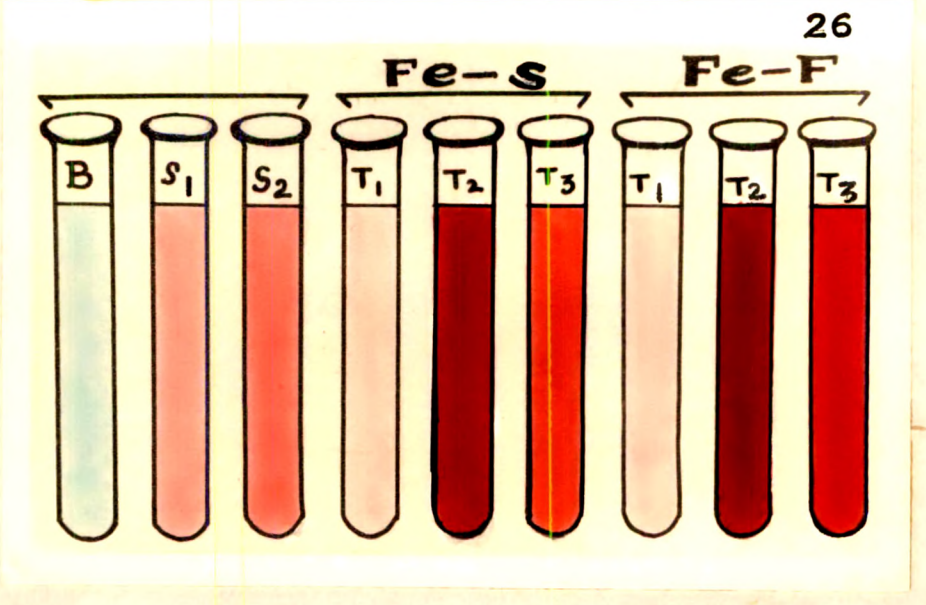
	Middle row of tubes (all values in ml.)			
	B	S_1	S_2	T_1 to T_9
a. Triple-glass-distilled water	2.0	-	-	-
b. Standard 1 (containing 100 mcg. per 100 ml.)	-	2.0	-	-
c. Standard 2 (containing 200 mcg. per 100 ml.)	-	-	2.0	-
d. Respective supernatant fluid	-	-	-	2.0

(15) Pipette out 0.4 ml. of Alkaline acetate solution followed by 0.3 ml. of freshly prepared 2:2' dipyridyl solution in-to each of the tubes of the back row as follows :

	Back row of tubes (All values in ml.)			
	B	S_1	S_2	T_1 to T_9
a. Alkaline acetate solution	0.4	0.4	0.4	0.4
b. Dipyridyl solution (freshly prepared)	0.3	0.3	0.3	0.3

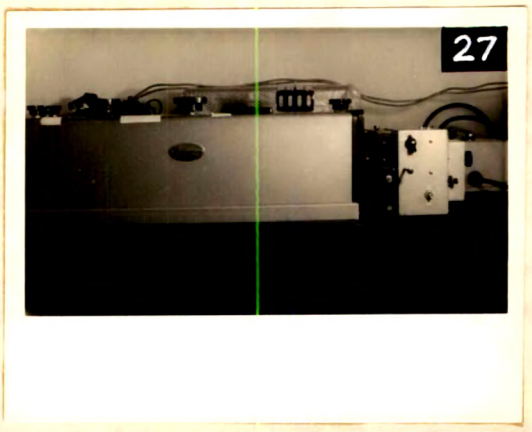
(16) Add the contents of the tubes of back row to those of the corresponding tubes of the middle row (i.e. add the mixture of Alkaline Acetate Solution and Dipyridyl solution to the supernatant fluid).

(17) Mix well and allow to stand for 10 minutes. Different intensities of pink colour are developed.

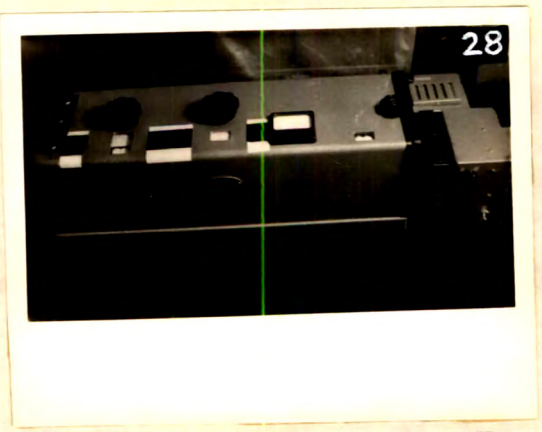


Development of Different Colour Intensities

(18) Measure the optical density within next 20 minutes, in Beckman Spectrophotometer at 520 mu, with Quartz type of cells having 2 ml. capacity, using the Blank solution (distilled water blank) to set the instrument to zero. (If the readings are to be taken at a wave length more than 400 mu,i.e. within the Visible range, Quartz cuvettes are used whereas if the readings are to be taken at a wave length less than 400 mu, i.e. within the Ultraviolet range, Silica Cuvettes are used).



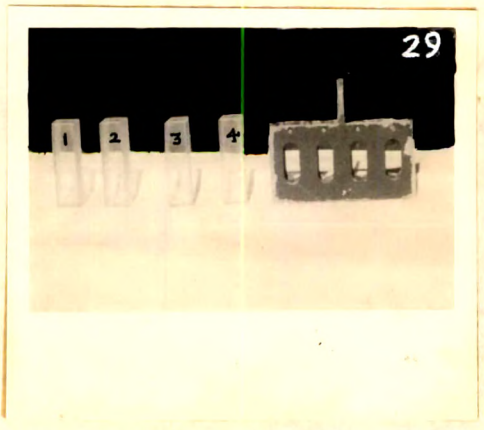
Beckman Spectrophotometer - Front view



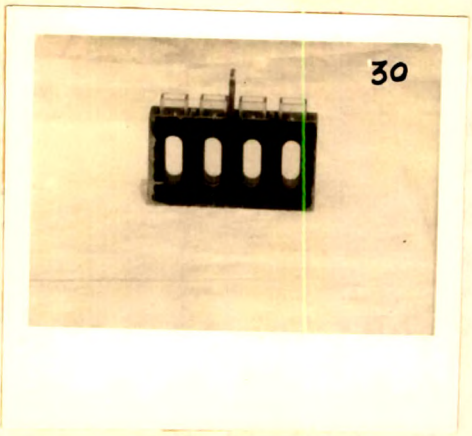
Beckman Spectrophotometer - Top View

In one cuvette Blank Solution was taken and in rest of the three cuvettes next three samples were taken.

The instrument was adjusted to zero against the Blank. Then the first set of readings of the three samples were taken. The instrument was once again adjusted to zero against the same blank and then the second set of readings were taken. After this the cuvette containing the blank solution was kept in situ, but rest of the three cuvettes were emptied (by transferring the contents to the respective test tubes), washed with triple-glass-distilled water after rinsing with 1 N HCl and dried. Next three samples were then transferred to these three cuvettes and the cycle was repeated. Similarly, the readings of all the rest of the samples were taken.

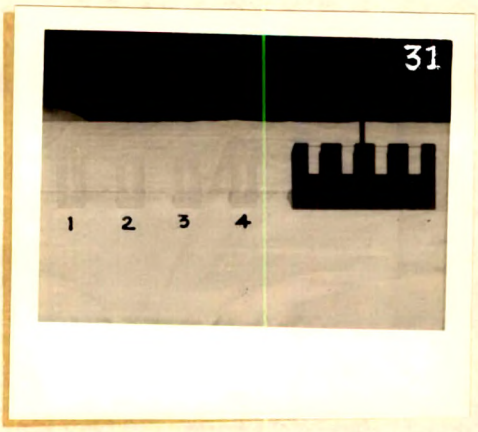


Four Cuvettes (2 ml.capacity) and the Cuvette holder

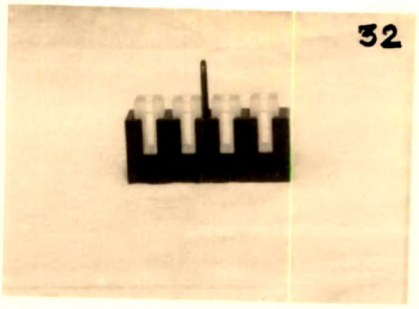


Cuvette holder holding the same cuvettes in situ

Small cuvettes (1 ml. capacity), if available, have an advantage that they require less amount of serum for the iron estimation.



Four small cuvettes (1 ml. capacity) and the cuvette holder for the same.



Cuvette holder holding same Cuvettes in situ

PROCEDURE FOR ESTABLISHING THE STABILITY OF COLOUR

For studying the stability of the colour developed for the spectrophotometric reading in serum iron estimation, the readings of serum iron values were taken immediately (after 10 minutes, but within 30 minutes of the development of the colour) and also 24 hours after the development of colour, and then the immediate readings were compared with the readings after 24 hours.

For taking the readings after 24 hours, a special procedure was adopted. The following are the actual steps of the procedure employed.

(19) Plug all the test tubes tightly with non-absorbable cotton soon after taking the 'immediate readings'.

(20) Immediately put the rack holding all the test tubes in refrigerator for 24 hours (Confirm that the labelling of the tubes is carried out with wet-proof adhesive tape and water-proof ink).

(21) After 24 hours, take out the rack from refrigerator. Centrifuge all the samples (B, S_1, S_2 and T_1 to T_9)x at 3000 r.p.m. for 5 minutes only.

(22) Gently but rapidly transfer the contents of the tubes to the cuvettes.

(23) Take the readings in Beckman Spectrophotometer at 520 mu, with the cell having 2 ml. capacity, using the blank solution (distilled water blank) to set the instrument to zero.(First and second set of readings were taken as before).

(24) Compare the immediate readings with the readings obtained after 24 hours.

MODIFICATIONS

The method for serum iron estimation (Marrack 1956) is much simplified and modified during the course of the

present study, by introducing new steps as also by modifying few original steps. Accordingly, step No.4, 9 and 15 are modified whereas step No.12, 13 and 14 are newly introduced. The procedure for taking the readings after 24 hours i.e. step No.19, 20, 21, 22, 23 and 24 is also newly introduced. Besides this, the procedure for standardisation is much modified and is described step-wise with all the necessary details. Moreover, theprecedure for rendering the glassware iron-free is also modified by using 1 N HCl instead of 2.5 N or 6 N HCl and by replacing the boiling procedure, as mentioned in the original method, by prolonged immersion of glassware in 1 N HCl.

Step No. 4 :

"Pipette out exactly 4 ml. of each test sample of serum into the corresponding centrifuge tubes of the back row, using a separate pipette for each sample."

In the original method 2 ml. of serum was taken and the final volume for spectrophotometric reading was 1.3 ml. In the present study, the volume of the serum, reagents etc. was doubled to obtain the final volume 2.6 ml. for spectrophotometric reading because the capacity of the cuvettes used was 2 ml. The 2 ml. capacity cuvettes are much cheaper and easily available. This type of change, therefore, made it possible to carry out the serum iron estimation studies with the available cuvettes.

1 ml. capacity cuvettes (Photograph No.31 and 32) which require 2 ml. of serum to begin with, are very costly and are rarely available. (These cuvettes were obtained with great endeavour and that too when the study was almost nearing completion.

Step No. 9 :

"Mix the contents of each tube thoroughly by Vigorous Shaking for 1 minute. (The Vigorous shaking is to be carried out immediately after the addition of trichloracetic acid solution in a particular tube)."

This modification of vigorous shaking and that too immediately after the addition of trichloracetic acid is a vital step for obtaining clear and completely protein-free supernatant fluid. The supernatant fluid must be clear, as even the slightest opalesence will greatly increase the error of the spectrophotometric reading.

Stirring with a glass rod as mentioned in the original method failed to mix the serum thoroughly with trichloracetic acid, subsequently resulting in incomplete precipitation of protein and opalesent or at times even cloudy supernatant fluid.

Step No.12 :

"Pour out the supernatant fluids from the centrifuge tubes into the corresponding small test-tubes of the front row."

This newly introduced step is meant for separating out the supernatant fluid from the solid mass of protein-precipitates. This separation in turn facilitates the purpose of Step No.13.

Step No. 13 :

"Centrifuge at 3000 r.p.m. for 5 minutes."

This newly introduced step of recentrifuging is a vital step for obtaining crystal-clear supernatant fluid free from any floating particles of the protein precipitates.

Step No. 14 :

"Pipette out exactly 2 ml. of clear aliquote of the protein-free supernatant fluid accurately into the corresponding tubes of the middle row, using a clean separate pipette for each. Besides this,pipette out 2 ml. of triple-glass distilled water in the first tube (labelled as B) and 2 ml. of each standard 1 and standard 2 in the 2nd and 3rd tube respectively (labelled as S_1 and S_2)."

This newly introduced vital step of measuring all supernatant fluids at a time in a SEPARATE set of test tubes (and the reagents in another set of test tubes) is of greatest practical importance because of the following reasons :

(i) Thorough mixing of the Supernatant Fluid & the reagent :

Stirring with glass rod or shaking of the tube was not an adequate and quick procedure for mixing of the supernatant fluid and the reagent thoroughly. Therefore, thorough mixing was carried out by pouring the contents from one test tube to another a number of times. This method of properly mixing the two solutions is indispensable, and there is much to recommend it.

(ii) Taking the Spectrophotometric readings within the specified time-limit :

The sharp time-limit of taking the spectrophotometric readings (10 minutes after the development of the colour but not longer than 30 minutes after the development of colour i.e. a total period of only 20 minutes) is a great handicap because the number of operations required for preparing the final solutions as also for taking the spectrophotometric readings are so numerous that the amount of time consumed for carrying them out will fall short to meet the above mentioned time-limit requirements. The operations required are as follows :

(a) Measuring 2 ml. of 12 samples viz. B, S_1, S_2, and T_1 to T_9.

(b) Thorough mixing of each of these 12 samples with the reagents.

(c) Transferring first four samples viz. B, S_1, S_2 and T_1 to the respective four previously prepared iron-free cuvettes.

(d) Adjusting the instrument (Backman Spectrophotometer) to zero for first set of readings.

(e) Taking the first set of readings of S_1, S_2 and T_1.

(f) Readjusting the instrument to zero for the second set of readings.

(g) Taking the second set of readings of the same samples.

(h) Transferring the three samples viz. S_1, S_2 and T_1 to the test tubes.

(i) Washing three cuvettes with 1 N HCl (to prevent iron contamination) and rinsing the same with triple-glass-distilled water (to remove the acid).

(j) Drying the three cuvettes (to prevent dilution).

... ...

(k) Repeatation of last eight operations (c to j) for next three samples viz. T_2, T_3 and T_4.

(l) Repeatation of the same eight operations (c to j) for next three samples viz. T_5, T_6 and T_7.

(m) Repeatation of same eight operations (c to j) for next two samples viz. T_8 and T_9.

Total Number of Operations :

No.of operations involved in (a) to (j)	...	10
No.of operations involved in (k)	...	8
No.of operations involved in (l)	...	8
No.of operations involved in (m)	....	8
TOTAL	...	34

Thus, totally 34 operations are involved. This would make it impossible to take the readings accurately within the specified time-limit.

Much time is consumed for performance of all the above operations when three patients i.e. nine test samples are studied at a time. (This makes a total of 12 samples on inclusion of the blank and two standards). Even if only one patient i.e. 3 test samples are studied at a time, still it will not be possible to finish the estimation within the specified time-limit because then also the inclusion of Blank, Standard-1 and Standard-2 is inevitable. Simultaneously, it is uneconomical also to study only one patient i.e.three test samples at a time because the duration of time, the amount of work and the quantity of materials (1 N HCl, triple glass-distilled water and reagents) required for the preparation of glassware and also for the colour development in Blank, S_1 and S_2 each time will be comparatively much more

than those required for the study of three patients i.e. nine test samples at a time. This is all due to the fact that Blank, S_1 and S_2 are to be repeated at each set of experiment even if one patient is studied at a time, whereas they will be common when more than one patients are studied at a time. Lastly, the total duration of time required for the study of a particular number of cases will be far greater when only one patient is studied at a time, because at the most only two sets of experiments can be conducted per week.

Step No.15 :

"Pipette out 0.4 ml. of Alkaline acetate solution followed by 0.3 ml. of freshly prepared 2:2' dipyridyl solution into each of the tubes of the back row."

This step is modified slightly by taking the measured amount of dipyridyl solution instead of counting the number of drops because the size of the drops is likely to vary depending upon the bore of the droper and the angle at which the droper is held. This measurement supplies accuracy in a particular set of estimation and maintains uniformity all throughout the study.

STABILITY OF THE COLOUR :

In order to study the stability of the colour, developed for the spectrophotometric readings, the whole

procedure for taking the readings after 24 hours (i.e. Step No. 19 to 24) is also newly introduced during the course of this study.

The problem of detecting the duration of stability of the colour for more than the specified time-limit mentioned in all the methods for serum iron estimation used so far, arose because of the shortness of the time interval. The present study has brought it out clearly that by using the modification of the method adopted for serum iron estimation in this study, the developed colour is stable for spectrophotometric reading as long as upto 24 hours instead of the specified time of only 1/2 hour.

Step No. 19 :

"Plug all the test tubes tightly with non-absorbable cotton soon after taking the immediate readings."

The ascorbic acid maintains the serum iron in reduced state and the ferrous iron when treated with 2:2' Dipyridyl produces pink colour.

This ascorbic acid if exposed to atmospheric air is oxidised. Hence to prevent such oxidation, the test tubes were tightly plugged with cotton. Non-absorbable cotton was used for this purpose to prevent the absorption of the contents of the tube (volume decreases) and also to prevent the absorption of moisture within the refrigerator

(cotton plug becomes loose).

Step No. 20 :

"Immediately put the rack holding all the tubes in refrigerator for 24 hours.(Confirm that the labelling of the tubes is carried out with wet-proof adhesive tape and water-proof ink)."

The colour developed for spectrophotometric reading was found to be photo-sensitive as well as thermo-sensitive. Hence, it was decided to preserve the tubes in refrigerator with minimum exposure to atmospheric temperature and light while taking the 'immediate readings' and the 'readings after 24 hours.'

Step No.21 :

"After 24 hours take out the rack from refrigerator. Centrifuge all the samples (B, S_1, S_2 and from T_1 to T9) at 3000 r.p.m. for 5 minutes only."

By this, the few particles which are quite often seen in the coloured samples and particularly so in the serum samples are made to settle down so that they may not give rise to fluctuation, in the spectrophotometric readings. Secondly, during the period of and act of centrifugalisation, the low temperature of the contents of the tubes is rapidly raised to room temperature. If this care is not exercised, the moisture on the outer surfaces of the cuvettes is bound

to give wrong spectrophotometric readings.

PROCEDURE FOR STANDARDISATION :

The procedure for standardisation is as such not given in the original method (Marrack 1956). During the present study, the whole procedure is laid down in an easily practicable way and is described step-wise with all the details. Moreover, the Iron Standard Curve is also prepared and presented.

PREPARATION OF GLASSWARE :

The procedure for the preparation of glassware is much modified and simplified. The whole procedure is described step-wise in an easily practicable manner. The following modifications have been made :

(a) Use of 1 N HCl (instead of 2.5 N HCl) :
Marrack in his original method has recommended the use of Dilute HCl for rendering the glassware iron-free. According to British Pharmacopia 'Dilute HCl' is approximately 2.5 N HCl (concentrated HCl is 36 per cent and is about 10 N whereas dilute HCl is 10 per cent). The use of 2.5 N HCl was expensive, as every time fresh HCl has to be used. Hence, during the study of 'Trial Cases' the use of 1 N HCl was also given a trial. Interestingly,

1 N HCl was proved to be quite efficient for rendering the glassware iron-free.

(b) Prolonged Immersion in Acid (instead of Boiling) :

Boiling the glassware in dilute HCl(2.5 N HCl) as recommended in the original method (Marrack 1956)has been omitted and successfully replaced by prolonged immersion of the glassware in 1 N HCl at room temperature. This important modification has rendered the procedure very simple and economical. Omission of boiling solved many practical difficulties of obtaining the big glass container required for boiling, the rich source of heat required for boiling, continuous supervision etc.

(c) Use of Gloves:

The use of gloves which was introduced during the present study is indispensable and there is much to recommend it. Severe burning of the skin of hands due to prolonged contact with the Hydrochloric acid during the preparation of glassware was the greatest practical handicap. Thus, the use of gloves completely solved the problem.

RESULTS

1. Serum Iron Values in Normal Subjects :

The Serum Iron values in normal adult males and in normal adult females are separately presented in a tabular form along with the statistical mean, standard deviation and range in chapter 27. The serum iron distribution curves in normal adult males and in normal adult females are separately represented photographically.

2. Serum Iron Values in Anaemic Subjects :

The serum iron values in male adult patients and in female adult patients are separately presented in a tabular form along with the statistical mean, standard deviation and range in chapter 27. The serum iron distribution curves in male adult patients and in female adult patients are separately represented photographically.

3. Serum Iron Response :

The serum iron values before and after the administration of respective iron preparations are also given in chapter 27.

4. Stability of the Colour :

On comparing the serum iron values obtained from the 'Immediate Readings' with those obtained from the "Readings after 24 hours", it was found that both the values fall

within ± 5 per cent difference. This difference of ± 5 per cent is within the experimental error and can even be due to the electrical fluctuations.

It is, therefore, clear that when the samples are preserved as described in the present method, the developed colour is stable for not less than 24 hours for spectrophotometric readings.