Improved automated technique for the estimation of iron in ferrioxamine

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SYNOPSIS A modified automated technique for the estimation of iron in the ferrioxamine complex is described. The use of sodium dithionite as the reducing agent gave greater recovery from ferrioxamine-containing solutions when compared with a standard manual method and automated method currently used. In addition errors due to urinary pigments are eliminated by dialysis.

Available methods for the determination of serum iron appeared inadequate for the measurement of urinary iron, particularly when chelated with desferrioxamine. A simple technique using a Technicon AutoAnalyzer is described and the results compared with two other established methods.

METHOD

PRINCIPLE The iron is released from ferrioxamine and reduced to the ferrous form by sodium dithionite. It is then separated from urinary pigments by dialysis and reacted with tripyridyl-triazine. The resulting complex, buffered with ammonium acetate, produces a colour which is measured at 600 m μ .

REDUCING REAGENT Sodium dithionite (1.5 g.) is dissolved in 100 ml. of iron-free water and the pH is adjusted rapidly to 7.0 with 2N-NaOH. No analytical grade of this reagent is available and general purpose reagent grade is adequate.

BUFFER SOLUTION Ammonium acetate AR (10 g.) is dissolved in 100 ml. of iron-free water and 1 drop of 20% Tween 20 is added.

COLOUR REAGENT 2, 4, 6-Tripyridyl-triazine AR (10 mg.) is dissolved in 100 ml. of 0.05 N-HCl, and 1 drop of 20% Tween 20 is added.

Iron-free water is prepared by passing distilled water through a mixed-bed exchange resin.

IRON STANDARD A stock solution (5 mg. of iron per ml.) is prepared by dissolving 4.32 g. of ferric ammonium sulphate in 100 ml. of N-HCl. Working standards containing 50, 100, 200, 300, 400, and 500 μ g. iron per 100 ml. are prepared from the stock solution by dilution with iron-free water.

Ferrioxamine (Ciba) containing 9.130 mg. of iron per 100 mg. was used.

Specimens of urine obtained from normal healthy adults found to contain no iron by prior estimations were used.

AUTOANALYZER ASSEMBLY Using the standard components of an AutoAnalyzer system¹ the apparatus is set up as described by Young and Hicks (1965). The assembly is set for analysis at a rate of 60 per hour with alternate cups containing iron-free water for an adequate wash. Iron-free water is first run through all lines for 15 minutes. The reagents are then run, with the sample line in iron-free water, for 15 to 30 minutes. A continuous line on the recorder indicates that the system is iron free and the baseline is determined while the reagents are running. Standards up to 500 μ g. iron per 100 ml. are run with each batch of urines.

RESULTS

REPRODUCIBILITY Duplicate estimations of urinary iron were made on 18 specimens to which amounts of ferrioxamine varying from 50 to 500 μ g.% had been added. The values obtained for each pair of estimations varied by less than $\pm 1.2\%$.

RECOVERY Recovery experiments were performed by estimating urinary iron after known amounts of ferrioxamine had been added to normal urine and to distilled water. In addition, experiments were performed by adding known amounts of ferric ammonium sulphate to normal urine. For comparison, these experiments were performed on the same samples by the automated method described by Young and Hicks (1965) and by the manual method of Bothwell and Mallett (1955).

Experiment 1 (method of Young and Hicks) Figure 1 shows the recovery of iron from the three test solutions. The recovery from ferric ammonium sulphate added to urine was 100% at all concentra-

FIG. 1. Recoveries of iron from the test solutions by the automated technique of Young and Hicks (1965). The figures in the parentheses indicate the number of estimations. ○—○ represents recoveries from ferric ammonium sulphate added to urine. This corresponds with the theoretical line of 100% recovery. ●—● represents recoveries from ferrioxamine in urine. ▲—▲ represents recoveries from ferrioxamine in water.

FIG. 2. Recovery of iron from the test solutions by the manual method of Bothwell and Mallett (1955). Each point on the graph is the mean of two estimations. Upper broken line indicates the theoretical line of 100% recovery. ●—● recoveries from ferric ammonium sulphate added to urine. ×—× recoveries from ferrioxamine added to urine. ▲—▲ recoveries from ferrioxamine added to water.

tions. The recovery from ferrioxamine added to urine was 50% at a concentration of 50 $\mu\text{g.}\%$, and it gradually declined to 45%. The recovery from ferrioxamine in water showed a similar decline from 50% to 20% as the concentration of iron as ferrioxamine increased from 50 $\mu\text{g.}\%$ to 500 $\mu\text{g.}\%$.

Experiment 2 (method of Bothwell and Mallett) Figure 2 shows the recovery of iron from the three test solutions. The recovery from ferric ammonium sulphate in urine averaged 90% of all concentrations and was somewhat lower than that obtained by the method of Young and Hicks. The recovery from ferrioxamine in urine varied from 30% to 80% as the concentration increased from 50 $\mu\text{g.}\%$ to 500 $\mu\text{g.}\%$ of iron.

Experiment 3 (automated technique using sodium dithionite) Figure 3 shows the results of recovery from the three test solutions. The recovery from ferric ammonium sulphate in normal urine was

FIG. 3. Recoveries of iron from the test solutions by the present method. Upper broken line represents the theoretical line of 100% recovery. ○—○ represents recoveries from ferric ammonium sulphate added to urine. ▲—▲ represents recoveries from ferrioxamine in urine. ●—● represents recoveries from ferrioxamine in water.

100% at all concentrations. The recoveries from ferrioxamine in urine and water were identical and ranged from 100% to 85% as the concentration increased from 50 $\mu\text{g.}\%$ to 500 $\mu\text{g.}\%$ of iron.

Figure 4 shows a comparison of the three methods in the recovery of iron from solutions containing ferrioxamine. Recovery was greatest when sodium dithionite was used as the reducing agent. The recovery by the automated technique of Young and

FIG. 4. Comparison of recoveries of iron-ferrioxamine containing solutions. Figures in parentheses indicate the number of estimations. Upper broken line indicates the theoretical line of 100% recovery. ●—● represents the recoveries by the present method. ○---○ represents the recoveries by the manual method of Bothwell and Mallett. ●-.-● represents the recoveries by the automated method of Young and Hicks.

Hicks (1965) was lowest, ranging from 40% to 50% of the added amount. The manual method of Bothwell and Mallett gave an intermediate recovery. As shown in Fig. 4 no iron can be recovered when the concentration of ferrioxamine is equivalent to less than 40 $\mu\text{g.}\%$ of iron.

DISCUSSION

To estimate iron when bound in an organic complex it is necessary to release and reduce it before reaction with tripyridyl-triazine. In the method of Young and Hicks (1965) this is achieved by the use of 0.1N-HCl-ascorbic acid. This is adequate for estimation of protein-bound iron in the serum but is unsuitable in the presence of E.D.T.A. In the present study it has been shown that HCl-ascorbic acid is an unsatisfactory reducing agent for the desferrioxamine-iron complex. The recovery of iron from the solutions containing ferrioxamine was 40–50% at all

concentrations of iron (Fig. 4). The use of 2N-HCl as a reducing agent in the manual method of Bothwell and Mallett (1955) gave a higher recovery, but the results of experiments from all the test solutions showed a wider scatter around the mean line (Fig. 2). No iron could be recovered from ferrioxamine at concentrations of less than 40 $\mu\text{g.}\%$ of iron (Fig. 4).

When sodium dithionite was used to release iron from the ferrioxamine complex there was complete recovery at a low iron concentration (50 $\mu\text{g.}\%$), but this declined to 85% when the concentration was increased to 500 $\mu\text{g.}\%$ of iron as ferrioxamine is shown by the 100% recovery from added ferric ammonium sulphate at all concentrations (Fig. 3).

The method of Bothwell and Mallett (1955) has the additional disadvantage of interference produced by the urinary pigments. This is confirmed by 100% recovery of added ferric ammonium sulphate by the present technique as well as by the method of Young and Hicks (1965), but a lower recovery from the same solution by the method of Bothwell and Mallett (1955) (Figs. 1, 2, and 3). This difficulty was not overcome by substituting diluted urine for the reagent blank. The use of dialysers in the automated techniques effectively removed the interfering urinary pigments.

Sodium dithionite tends to deposit sulphur in the mixing coils and it was found necessary to limit the concentration to 1.5 g. per 100 ml. and to adjust the pH to 7.0 with 2N-NaOH immediately after the solution was prepared. For the same reason 2, 4, 6-tripyridyl-1, 3, 5-triazine was dissolved in 0.05N-HCl rather than 0.1N-HCl.

The higher yields obtained by the technique reported here may be of significance when assessing the effects of chelation therapy in iron-loaded patients.

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REFERENCES

- Bothwell, T. H., and Mallett, B. (1955). *Biochem. J.*, **59**, 599.
Young, D. S., and Hicks, J. M. (1965). *J. clin. Path.*, **18**, 98.