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Automated Determination of Serum (Plasma) and Urine Iron: A Comparative Investigation of Chromogens Improved Tripyridyltriazine Micromethod

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Summary: Four different, well known colour reagents for iron determination were tested in a citrate buffer at pH 2.0 under the same automated standard conditions and compared with a manual reference method on human serum and plasma samples. Specific results were obtained only with the chromogen tripyridyl-s-triazine. A micromethod was developed, which is generally well suited for automated, direct serum iron determinations, with respect to good flow properties, simple reagent composition, high reagent stability, rapid colour development, stable colour complex and high specificity. This method was run on either a Gilford System 203 S, Gilford Impact 400 or a Greiner G-400 analyser and adapted to the Technicon SMAC II, Technicon RA 1000, Eppendorf Epos and Abbott Spectrum automated systems. The tripyridyl-s-triazine method permits the determination of ferrioxamine iron in urine after a simple sample pretreatment.

Introduction

The first automated method for serum iron measurement was developed on a continuous flow system with the chromogen tripyridyl-s-triazine (1). Subsequently, several other automated methods for serum iron determination have been introduced in the routine laboratory (2–9). These methods were mainly based on the colour reagents bathophenanthroline sulphonate

and ferrozine. The latter chromogen has a somewhat higher molar absorptivity than tripyridyl-s-triazine and bathophenanthroline sulphonate. Later, ferene-S, a chromogen with improved molar absorptivity and the same features as ferrozine, with respect to slow colour development and interference by copper (10), was introduced for serum iron determination (11). The relatively slow colour development in the ferrozine reaction was found to be a limiting factor in some analysers (6). Interference by copper can be eliminated with thiosemicarbazide, which forms an uncoloured complex with copper (10). Reaction of thiosemicarbazide with ascorbic acid impairs reagent stability. If the test is not performed at sufficiently acidic pH, interference by released haemoglobin iron may arise (10). Such difficulties may easily impair the specificity of the measurement, in particular in the clinically important, abnormally low range. We searched for an improved, automated method for serum iron determination, which overcomes these problems.

Materials and Methods

Chemicals

Bathophenanthroline sulphonate (4,7-diphenyl-1,10-phenanthroline disulphonic acid sodium salt), TPTZ (2,4,6-tripyridyl-s-triazine), ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine sodium salt) and ferene-S (3-(2-pyridyl)-5,6-bis(2-(5-furyl-sulphonic acid))-1,3,4-triazine disodium salt) were from Sigma Chem. Corp., Duishofen, FRG. Other analytical grade reagents were obtained from E. Merck Darmstadt, FRG. The iron standard was either from Boehringer Mannheim, FRG (17.9 µmol/l) or prepared from a stock solution, containing 895.5 µmol/l iron. The stock solution was made by dissolving reduced iron in approx 6 mol/l HCl, followed by dilution to 1 l with distilled water.

Serum and plasma samples

Control sera were obtained from E. Merck, Darmstadt, FRG (Seronorm), from Beckman, Munich, FRG (Decision Level 1–3) and from Behringwerke, Marburg, FRG (Kontrollogen-LP).

Serum or plasma samples from patients with normal and pathological iron values were used. Sampling was performed between 8 and 9 a. m. under fasting conditions. Tubes containing ammonium heparinate were used for obtaining plasma.

Instrumentation

In the present investigation, serum (plasma) iron and urine desferal iron determinations were performed either on a Gilford System 203 S, a Gilford Impact 400 Analyser (Gilford Instruments, Oberlin, OH, USA) or a Greiner G-400 analyser (Greiner Instruments GmbH, Birkenfeld, FRG). Only the tripyridyl-s-triazine method was adaptable to the Greiner G-400 (at 37 °C). Atomic absorption spectrophotometric determinations of iron in urine were performed on a Perkin-Elmer 2380 system (Perkin-Elmer Corp., Norwalk, Ct., USA).

Reagents

The reagent for sample blanks (reagent 1) consisted of 0.24 mol/l aqueous citric acid monohydrate (50 g/l + 1 drop of chloroform) and 5.6 mmol/l ascorbic acid. The colour reagent (reagent 2) consisted of 1.6 mmol/l of one of the reagents, tripyridyl-s-triazine, bathophenanthroline sulphonate, ferrozine or ferene-S dissolved in reagent 1. Total bilirubin was measured on a Greiner G-400 with the dichloroaniline method (kit from Greiner No. 4057).

Method for serum (plasma) examinations

The methods were run with sample and reagent blanks at 25 °C on the Gilford Systems with four different standards (2.2, 4.5, 9.0 and 17.9 µmol/l) and at 37 °C on the Greiner G-400 with or without standards. Details for the programming of these systems are listed in table 1. The measurements were performed at a wavelength of 593, 533, 562 and 593 nm with tripyridyl-s-triazine, bathophenanthroline sulphonate, ferrozine and ferene-S, respectively. The tripyridyl-s-triazine/citrate method was found to be linear up to at least 260 µmol/l. For the examination of urine samples, higher standards were used (see below). The systems were accepted for use when they showed linearity for the different standards (automatically checked) and/or a within-run precision of 2% or less in a control serum (Decision Level 2). Otherwise, a cleaning of the system with 0.1 mol/l HCl and/or a photometer check (lamp drift) was performed. After an initial cleaning with HCl, problems of contamination normally do not occur in selective analysers such as the Greiner G-400 and comparable systems.

Tab. 1. Data on the programming of the Gilford systems and the Greiner G-400.

Gilford	
WAWELENGTH	593
TRANSPORT TEMP.	25
CUVETTE TEMP.	25
SAMPLE VOLUME	40
VOLUME DISP. A	100
VOLUME DISP. B	300
VOLUME DISP. C	300
READ TIME	3
EQUIL. TIME	5
TIME A TO B	2
TIME A TO C	2
LAG TIME	300

Greiner G-400		
MODE	50	
N/U	39	0
PT1	030	030
PT2	030	030
FILTER	7000	0
R1	01	39
R2	10	38
A	5494	
B	−13	
VOLUME R1	400	
VOLUME R2	400	
SAMPLE VOLUME	30	

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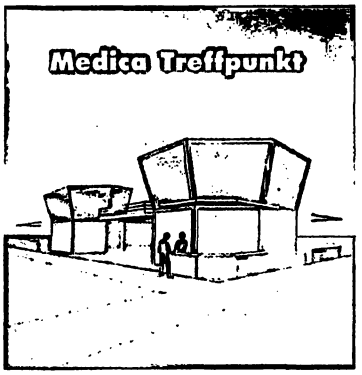

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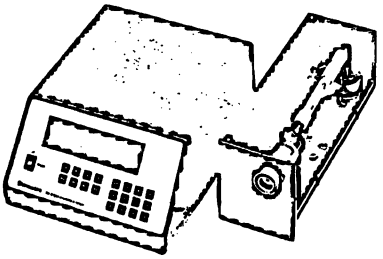

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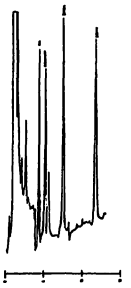

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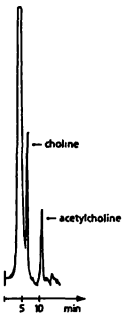

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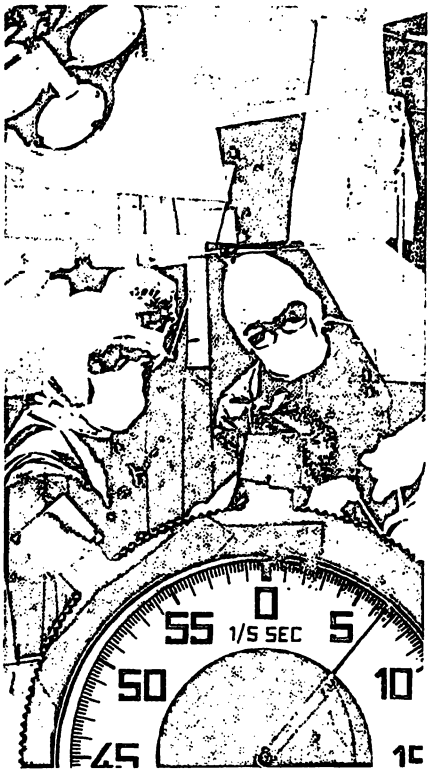

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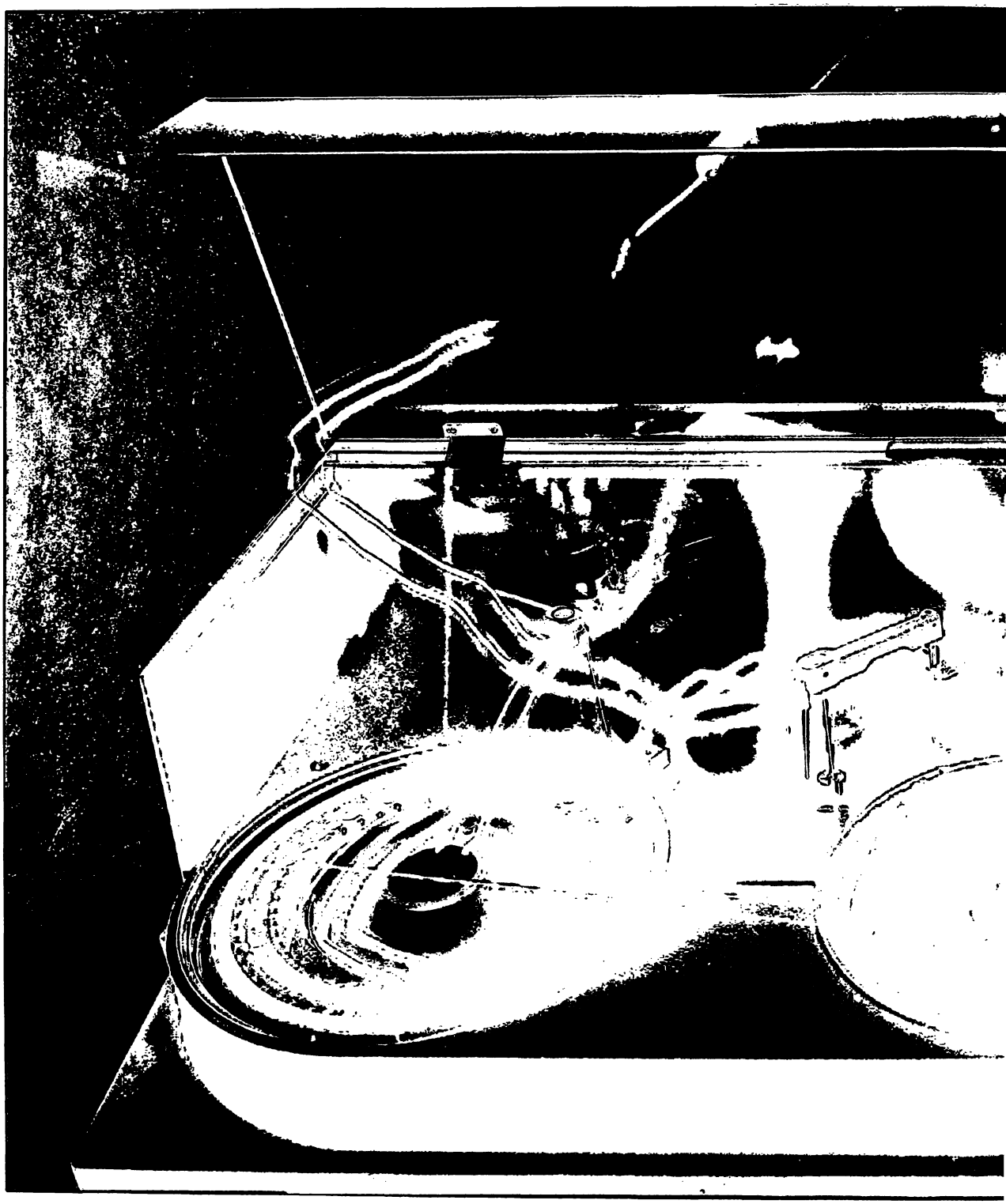

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Reference method

A manual reference method with the chromogen bathophenanthroline sulphonate with deproteinisation (kit from Boehringer Mannheim, FRG No. 124214), which is comparable with the recommended method of The International Committee for Standardization in Hematology (12) was used.

Methods for urine examination

Standards of 17.9, 90.0, 179.1, and 358.2 µmol/l and the same reagents and programming as for serum and plasma determinations (s. above) were used.

Pretreatment of urine samples

The urine samples were diluted 1 : 3.5 with a solution containing citric acid (0.57 mol/l) and ascorbic acid (0.48 mol/l); 0.5 ml of this diluted solution was placed in a capped micro reaction vessel and kept in a heat block (Eppendorf 3401, Eppendorf Gerätebau, Hamburg, FRG) at 56 °C for 15 min. After this pretreatment, the urine sample was assayed automatically as described above. If the total amount of ferrioxamine iron and transferrin iron in serum is to be determined, serum can be pretreated in the same manner.

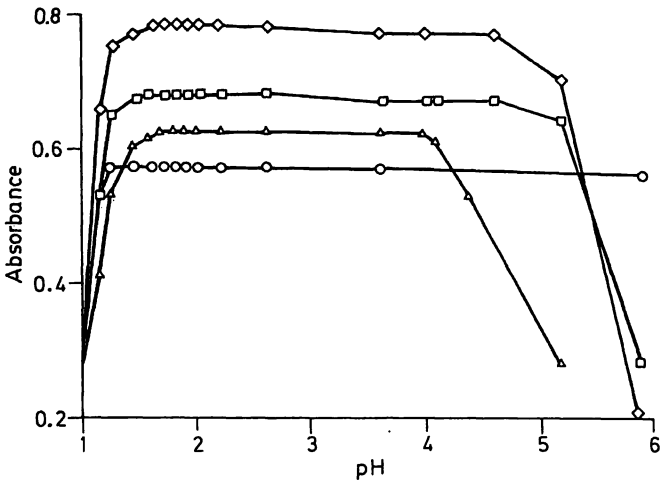

Fig. 1. Colour development and intensity of the four tested chromogens at different pHs by the use of citrate/HCl/NaOH buffer.
◇ = Ferene-S
□ = Ferrozine
△ = 2,4,6-Tripyridyl-s-triazine
○ = Bathophenanthroline sulphonate

Results

Investigation of different chromogens

The reaction time for total colour development was found to be 30 s for tripyridyl-s-triazine, 2 min for bathophenanthroline sulphonate and ferrozine and 1 h for ferene-S, examined at 25 °C and at a pH of 1.85 (see tab. 2). The four different chromogens were tested under the same standard conditions, using 0.24 mol/l citric acid/-HCl-NaOH buffer. Figure 1 indicates that tripyridyl-s-triazine has a constant colour intensity in the pH range between 1.6 and 4.0 and bathophenanthroline sulphonate in the pH range 1.3 to at least 6.0. The chromogens ferrozine and ferene-S were found to have a somewhat less constant colour intensity between pH 1.6 and 4.6 and between pH 1.7 and 4.6, respectively. All reagents were found to be stable for at least 2 months at 4–8 °C and 1 week at room temperature. The colour complexes of tripyridyl-s-triazine and ference-S were found to be stable for at least 72 h, and for bathophenanthroline sulphonate and ferrozine for at least 36 and 24 h, respectively. The interference of copper in the tripyridyl-s-triazine/citrate method was found to be less than 2.0% in a plasma with various concentrations of added copper chloride, as described in detail elsewhere (13).

Tab. 2. Some features of the four different chromogens under the same standard conditions (citrate buffer, pH 2.0).

Colour reagent	Reaction time (min)	Solubility	Molar absorptivity under test conditions (m^2/mol)
Tripyridyl-s-triazine	0.5	+	220660
Bathophenanthroline sulphonate	2.0	+	219210
Ferrozine	2.0	+	282360
Ferene-S	60.0	+	360020

Correlations with the reference method

Sixty different plasma samples from patients were tested with tripyridyl-s-triazine, bathophenanthroline sulphonate, ferrozine and ferene-S and the results compared with those from the reference method. In figure 2a, it is shown that a good correlation was obtained with the tripyridyl-s-triazine method. No interference was observed in this method in icteric, lipaemic or haemolytic plasmas. These findings show clearly that the tripyridyl-s-triazine/citrate method is specific and sensitive for iron determination in human blood. This method detects iron concentrations of less than 0.2 µmol/l. Distinctly turbid, lipaemic sera may impair precision and should be pretreated with a commonly available cleaning agent. Interference with the determination of haemoglobin iron arise at 37 °C on the Greiner system in plasmas with a haemoglobin content of 0.8 g/l or more. In the other systems, haemoglobin contents up to 5.0 g/l did not interfere, as demonstrated in table 3. In figure 2 it is shown

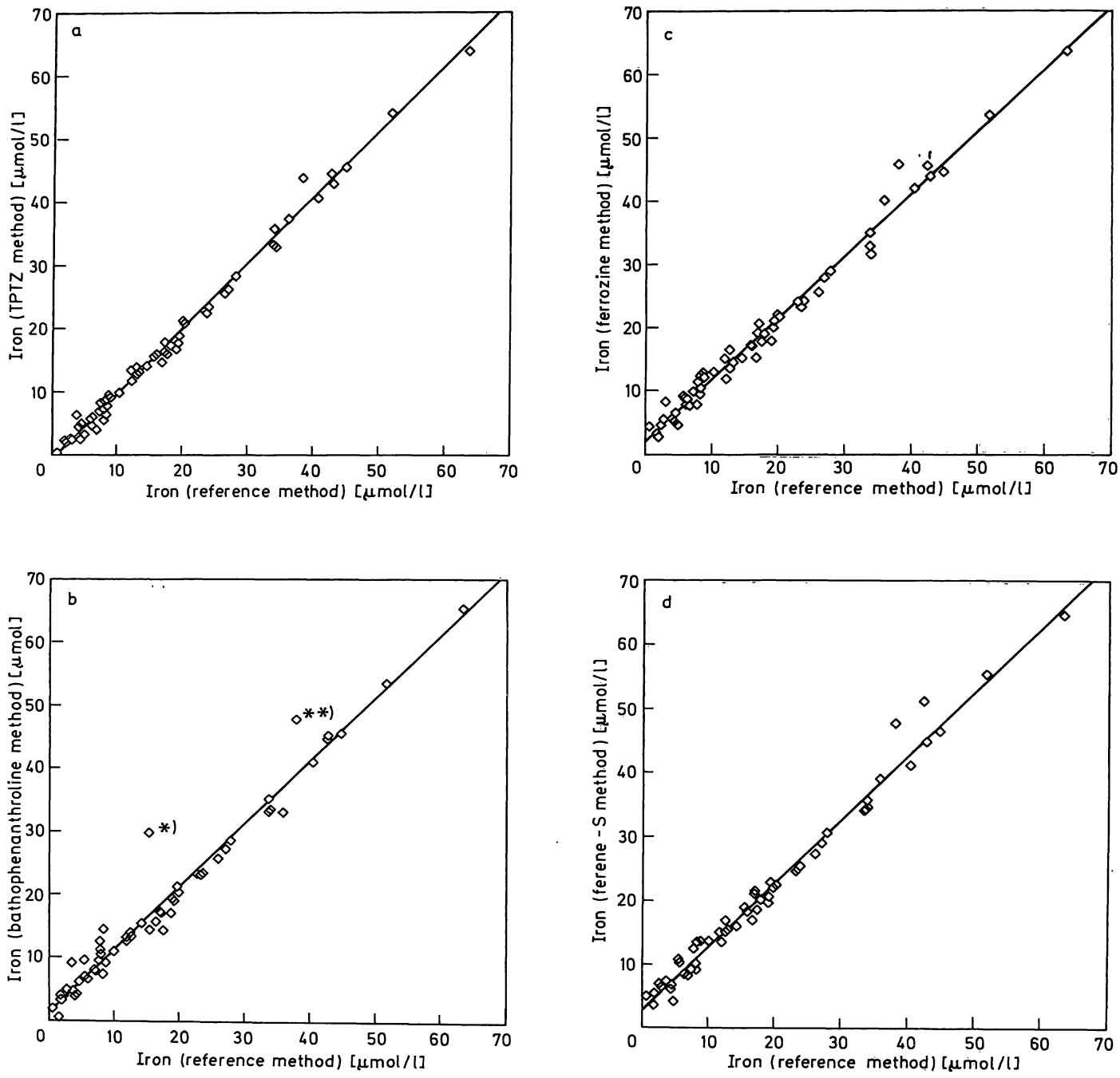

Fig. 2. Correlation data of the four different chromogens with the manual reference method in plasma samples from patients. The chromogens were tested under the same standard conditions (citrate buffer pH 2.0).

a) 2,4,6-Tripyridyl-s-triazine (TPTZ)
Total range: n = 60; r = 0.996; y = 0.1035 x − 0.508
Range 0.7−10.2 µmol/l: n = 25; r = 0.916; y = 0.948 x + 0.068
Range 10.3−63.4 µmol/l: n = 35; r = 0.995; y = 1.045 x − 0.854

b) Bathophenanthroline
Total range: n = 60; r = 0.983; y = 1.002 x + 1.187
Range 0.7−10.2 µmol/l: n = 25; r = 0.874; y = 1.154 x + 0.776
Range 10.3−13.4 µmol/l: n = 35; r = 0.974; y = 1.03 x + 0.093
*) plasma sample with a bilirubin content of 73.5 µmol/l.
**) plasma sample with a bilirubin content of 287.3 µmol/l.

c) Ferrocine
Total range: n = 60; r = 0.992; y = 0.977 x + 2.247
Range 0.7−10.2 µmol/l: n = 25; r = 0.898; y = 1.066 x + 2.078
Range 10.3−63.4 µmol/l: n = 35; r = 0.990; y = 1.005 x + 1.285

d) Ferene-S
Total range: n = 59; r = 0.992; y = 0.997 x + 2.835
Range 0.7−10.2 µmol/l: n = 24; r = 0.879; y = 1.016 x + 3.030
Range 10.3−63.4 µmol/l: n = 35; r = 0.989; y = 1.023 x + 1.933

Tab. 3. Determination of iron in haemolytic plasma samples. From a blood sample, erythrocytes were haemolysed in distilled water and diluted to different concentrations. Separate fractions of the plasma sample were mixed with the same volume of one of the haemolysate dilutions. The expected value was determined after addition of the same volume of distilled water to a plasma fraction. Haemoglobin was determined with the cyanmethaemoglobin method on a Gilford Stasar III spectral photometer (Gilford Instruments, Oberlin, OH, USA).

Hb concentration (g/l)	Expected value (µmol/l)	Found value (µmol/l)
0.62	12.2	12.5
1.25	12.2	12.2
2.50	12.2	12.0
5.05	12.2	12.3

Tab. 4. List of results (µmol/l) of the four tested methods under the same standard conditions in plasma samples in the pathologically decreased range.

	Reference method	Tripyridyl-s-triazine	Bathophenanthroline sulphonate	Ferrozine	Ferene-S
1	0.70	0.40	2.00	4.50	5.00
2	1.80	2.30	0.50	3.40	3.90
3	2.00	2.00	3.90	2.70	5.60
4	2.30	2.00	3.20	4.70	–
5	2.70	2.50	4.10	5.70	7.00
6	3.00	2.50	5.00	8.40	6.40
7	3.80	6.40	9.10	5.60	7.50
8	3.90	4.50	4.80	5.70	6.80
9	4.30	2.50	3.90	5.00	6.40
10	4.50	5.20	4.50	6.60	7.20
11	4.80	3.40	6.10	5.00	4.30
12	5.70	5.70	9.70	9.50	10.90
13	5.90	5.00	7.20	9.10	10.60
14	6.10	6.10	6.80	8.10	8.60
15	6.60	3.90	7.50	7.70	8.60
16	7.20	7.00	7.90	9.90	9.30
17	7.20	8.10	7.90	9.00	9.30
18	7.70	5.70	8.20	7.90	9.30
19	7.90	8.40	9.70	11.50	12.50
20	8.20	6.60	12.70	12.90	13.30
21	8.20	7.90	11.30	9.70	9.50
22	8.20	9.00	10.60	10.70	10.40
23	8.60	8.60	14.50	13.10	13.30
24	9.00	9.30	9.30	12.20	13.60
25	10.20	9.90	11.10	13.10	13.80

that the other chromogens have a considerably positive error, particularly in the pathologically decreased range, as also demonstrated in table 4. In plasmas with iron concentrations above 10.2 µmol/l the correlation with the reference method was found to be improved, and the difference between the 4 different chromogens was found to be almost balanced. Two plasma samples with elevated total bilirubin concentration were found to have a falsely positive error in the bathophenanthroline sulphonate/citrate method at pH 2.0. These plasma samples (fig. 2 b * and **) had bilirubin concentrations of 73.5 and 278.3 µmol/l, respectively, indicating that bilirubin interferes with this method. The latter finding was confirmed in a pooled plasma from 12 different icteric patients. In this pooled plasma, the following data were obtained: total bilirubin = 155.6 µmol/l, iron concentration in the tripyridyl-s-triazine method = 17.6 µmol/l, iron concentration in the bathophenanthroline sulphonate method = 24.7 µmol/l, positive error in the bathophenanthroline sulphonate method = 40.3%. By increasing (decreased plasma/reagent ratio) the pH in the direct bathophenanthroline sulphonate method from pH 2.0 to pH 4.0, the interference by bilirubin was abolished. Thus, the bathophenanthroline sulphonate/citrate method seems to be specific for iron measurement at the latter pH. No significant difference in the iron concentration could be detected between human serum and plasma.

Tab. 5. Data on the intra-assay precision of the tripyridyl-s-triazine method in different control sera.

Control serum	Charge No.	Assigned value[1] (µmol/l)	n	Found mean value (µmol/l)	SD	CV (%)
Decision L 1 (Beckman)	C 401202	21.1	30	22.5	0.25	1.11
Decision L 2 (Beckman)	C 401203	28.8	30	32.2	0.30	0.93
Decision L 3 (Beckman)	C 401204	41.5	30	47.0	0.34	0.72
Kontrollogen-LP (Behringwerke)	623208	35.5[2]	30	41.2	0.89	2.17
Seronorm (E. Merck)	162	26.3	30	26.4	0.48	1.82

[1] bathophenanthroline sulphonate without deproteinization.
[2] ferrozine without deproteinization.

Accuracy and precision in control sera

The best precision in the tripyridyl-s-triazine method was obtained in non-lyophilized sera with a CV ranging from 0.72 to 1.11% (within one run), as shown in table 5. The day-to-day variation and mean accuracy for Seronorm were 2.4% and + 0.5%, respectively.

Adaptation to other automated systems

The tripyridyl-s-triazine/citrate method was adapted to a Technicon SMAC II and Technicon RA 1000 (13), Eppendorf Epos and Abbot Spectrum automated analysers. The method can be run at any commonly used temperature. In the latter two systems, some modifications are necessary[1]).

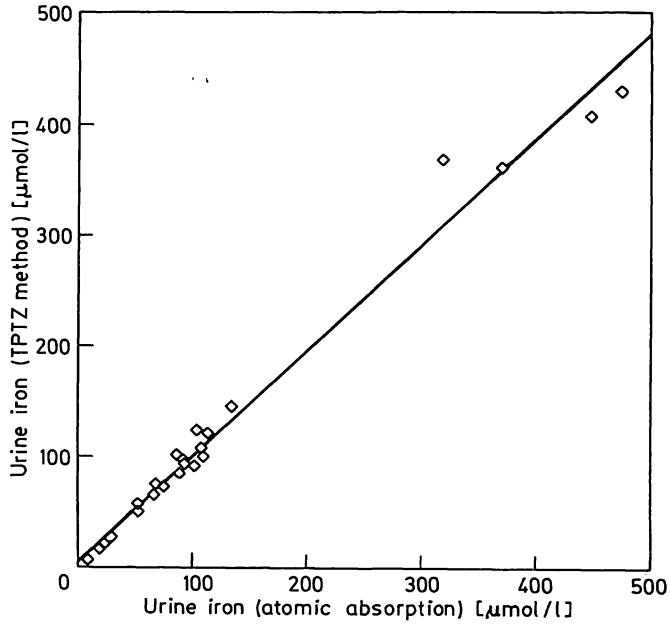

Fig. 3. Correlation data of the 2,4,6-tripyridyl-s-triazine (TPTZ) method with the atomic absorption spectrophotometric method in urine samples.
n = 26; r = 0.992; y = 0.950 x + 5.834

Ferrioxamine iron in urine

In order to release all desferal-bound iron during sample pretreatment at 56 °C, different molarities of citric acid and ascorbic acid were investigated. At 0.57 mol/l citric acid and 0.48 mol/l ascorbic acid, all ferrioxamine-bound iron was released for the tripyridyl-s-triazine reaction, as determined in urines with known ferrioxamine iron content. This pretreatment can also be used for serum samples, if both ferrioxamine iron and transferrin iron are to be determined.

[1]) Data on the programming of the Eppendorf Epos and Abbott Spectrum systems are available from the author.

The correlation data between the tripyridyl-s-triazine method and the atomic absorption method in urine samples is demonstrated in figure 3. These findings are in accordance to the data obtained in iron-free urine samples after addition of desferal and iron (II) chloride at various concentrations (not shown).

Discussion

The present investigation clearly shows that our tripyridyl-s-triazine/citrate method is specific and sensitive for direct iron measurements in human blood, and even in plasmas with iron values of 0.2 µmol/l or less. The high specificity of tripyridyl-s-triazine is consistent with an earlier report (14). Moreover, the simple reagent composition, high reagent stability and the instant colour development in the tripyridyl-s-triazine method are features of great practical importance, which facilitate an adaptation to other automated systems. It has previously been pointed out that tripyridyl-s-triazine, in contrast to ferrozine, is quite insoluble in water (14–15). This seems to be the main reason why tripyridyl-s-triazine has found only limited use. Under our standard conditions, at an acidic pH, tripyridyl-s-triazine was totally soluble within few minutes.

The reaction time of 1 h found with ferene-S is somewhat longer than reported by *Eskelinen* (11). Colour development within approx. 2 min in the bathophenanthroline sulphonate- and ferrozine methods and within approx. 30 s for tripyridyl-s-triazine is therefore an obvious advantage. In our automated systems, a relatively good precision was obtained with ferene-S, even after an incubation time of only 5 min. The high sensitivity of ferene-S is only of limited value under these conditions. On the other hand, a reaction time of more than 5 to 10 min limits the usefullness of a method in an automated system. The positive error in the ferrozine and ferene-S method indicates interference by copper, in accordance with an earlier report (11). We have no explanation at present for the interference of bilirubin at pH 2.0 in the direct bathophenanthroline sulphonate/citrate method. In view of this interference, the bathophenanthroline sulphonate method is not suitable as a micromethod under our standard conditions. In contrast to bathophenanthroline sulphonate, tripyridyl-s-triazine is available at low cost.

Both the reducing and chelating effect of citric acid and the rapid release of iron from transferrin at the low pH are advantageous. The coloured ferroin complexes also seem to be stabilized in our method, giving a constant colour development in a relatively wide

pH-range, in contrast to HCl-glycine-Tween buffer at the same pH (16). By the use of less sensitive photometric equipment, the latter finding enables a decrease in the ratio, sample/reagent volume, and hence an increase in pH.

The wide linear range of the ferrioxamine iron determination in urine samples with a high iron content is advantageous. During desferal treatment, an increase in the serum iron concentration occurs (17). Without pretreatment of the serum samples, our method will release some ferrioxamine iron. Since desferal partly binds transferrin iron (18), a decrease in the serum transferrin iron during desferal treatment can be expected. The quantity of released ferrioxamine iron may vary widely under different standard conditions. Direct serum iron determinations during desferal therapy might thus be rather uncertain. In contrast to an earlier report (19), we found no evidence for the sensitivity of tripyridyl-s-triazine to desferal under our standard conditions. Pretreatment of samples permits the determination of the total ferrioxamine iron and transferrin iron in serum.

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