

A robust procedure for the automated measurement of total serum bilirubin using potassium ferricyanide

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SUMMARY. Bilirubin is oxidized to biliverdin by 1.0 mmol/L potassium ferricyanide in the presence of caffeine. The absorbance decline at 480 nm with blanking at 660 nm is proportional to bilirubin concentration. The assay is linear up to 1000 $\mu\text{mol/L}$ and is suitable for the measurement of bilirubin in adult and neonatal patients. The assay is accurate and precise and is not significantly affected by haemolysis up to 5.0 g/L haemoglobin. Reagents and calibration are stable for at least 3 months.

Additional key phrases: total bilirubin; ferricyanide; stable reagent; interferences; intralipid

A widely used procedure for the measurement of total bilirubin in serum is based on the reaction of bilirubin with diazotized sulphanilic acid described by Jendrassik and Groff in 1938.¹ As diazobenzene sulphanilic acid is relatively unstable a number of commercial companies have replaced sulphanilic acid with 2,5 dichloraniline which has the advantage of yielding a more stable diazo derivative and is, therefore, more convenient when applied to automated analysis.² However, these methods are subject to interference due to indican and can demonstrate significant overestimation in patients with renal failure.²

Unlike most analytes of interest in clinical chemistry bilirubin demonstrates a characteristic absorbance with a peak at about 450 nm. A number of studies have demonstrated the effectiveness of utilizing this absorbance in direct spectrophotometric assays.³⁻⁵ However, while suited for neonatal samples they lack specificity in the analysis of adult samples.⁶

On oxidation of bilirubin to biliverdin the optical peak at 450 nm disappears. This absorbance change is the basis of several assays utilizing bilirubin oxidase (E.C.1.3.3.5.).⁷

Bilirubin may more conveniently be oxidized by potassium ferricyanide which has been used to eliminate the interference of this chromogen in the estimation of serum creatinine by the Jaffé reaction.^{8,9} This study demonstrates the

effectiveness of measuring the absorbance change of bilirubin in caffeine/benzoate due to oxidation by potassium ferricyanide, as a convenient procedure yielding accurate and precise results in the measurement of total serum bilirubin.

MATERIALS AND METHODS

Chemicals

Bilirubin, mixed isomers, from bovine gallstones was obtained from Sigma Chemical Co. Ltd (Dorset, UK). Intralipid was obtained from Kabivitrium, Cahill May Roberts (Dublin, Ireland). All other chemicals were of Analar grade and supplied by BDH Chemicals (Dorset, UK).

Reagents

Caffeine/benzoate, sulphanilic acid and diazo
These were formulated in accordance with the recommendations of Doumas.¹⁰

Caffeine/benzoate

Dissolve 56 g of anhydrous sodium acetate, 56 g of sodium benzoate and 1.0 g disodium EDTA in 500 mL distilled water. Add 37.5 g of caffeine stir until dissolved and dilute to 1 L.

Sulphanilic acid

Dissolve 5.0 g of sulphanilic acid in 500 mL of water, add 15.0 mL of concentrated HCl and dilute to 1 L, stable for 6 months at room temperature.

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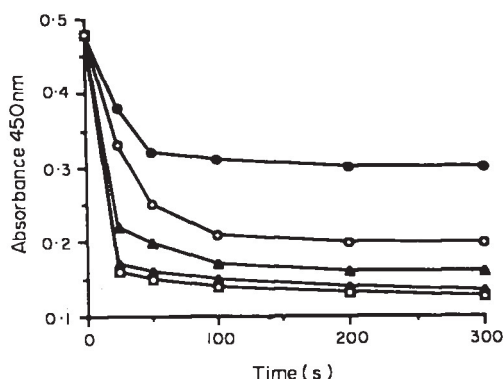


FIGURE 1. Effect of increasing potassium ferricyanide (mmol/L), in caffeine, on absorbance of icteric sample (total bilirubin 362 μ mol/L, conjugated bilirubin 244 μ mol/L). $\bullet$ = 0.25; $\circ$ = 0.5; Δ = 0.75; $\blacktriangle$ = 1.0; $\square$ = 2.0.

Sodium nitrite

5.0 g/L in water, stable for 2 weeks at 4°C.

Diazo

Just before use, mix 1 vol of nitrite with 40 vol of sulphanilic acid.

Ferricyanide

5.0 mmol/L potassium ferricyanide dissolved in 154 mmol/L sodium chloride (stable for 3 months at 4°C).

Methods

The effect of ferricyanide concentration on the rate of oxidation of bilirubin (Fig. 1) was measured on the Baker Encore centrifugal analyser (Allentown, PA, USA). Spectral scans

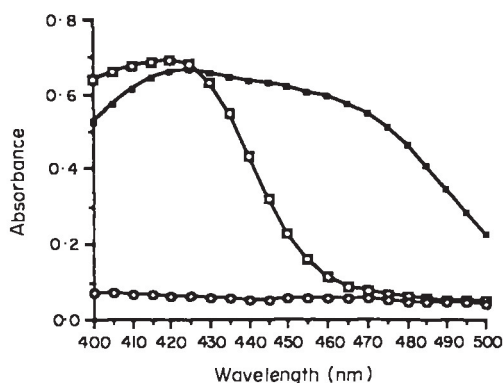


FIGURE 2. Spectral scan of icteric pool, 1/10 (bilirubin = 135 μ mol/L) and 1.0 mmol/L potassium ferricyanide in caffeine. $\blacksquare$ = Bilirubin/caffeine; $\square$ = ferricyanide/caffeine; $\circ$ = caffeine.

(Figs 1 and 2) were performed on the Shimadzu UV-VIS-160 recording spectrophotometer (Koyota, Japan).

Bilirubin assays

All assays were carried out on the Hitachi 704 discrete analyser (Tokyo, Japan) at 37 °C with the absorbance difference between analytical and blanking wavelengths (analytical/blanking) reported.

Diazo/caffeine benzoate procedure

Plasma (10 μ L) was added to 360 μ L of caffeine reagent. After 5 min an initial measurement was taken at 546/600 nm. Diazo reagent (90 μ L) was then added and the absorbance of the azobilirubin measured at 546/600 nm, after a further 5 min.

2,5 Diphenyldiazonium (DPD) procedure

This method was employed using the Boehringer Mannheim GmbH kit Catalogue Number 977144 and the manufacturer's instrument application for the Hitachi 704 was followed.

Increasing haemolysis

A mix of non-icteric whole blood samples were centrifuged and plasma separated. An aliquot of the packed cells was placed in an ultrasonic bath for 5 min to effect lysis of cells. Varying amounts of haemolysate were then added to an icteric plasma pool. The haemoglobin was quantified as the cyanmethaemoglobin derivative on the Technicon H1.

Increasing lipemia

Aliquots of a 20% intralipid solution were added to the icteric plasma pool to mimic lipaemia.

In both instances compensation was made for the dilutional effects by appropriate calculation.

Linearity

A stock solution of 10 mmol/L bilirubin dissolved in dimethylsulphoxide (4.7 mol/L) and sodium carbonate (70 mmol/L) was prepared. A pool of samples with bilirubin levels of less than 6 μ mol/L, subsequently exposed to indirect sunlight for 8 h was used as diluent. The plasma pool was spiked to a level of 1000 μ mol/L and serially diluted.

Calibration

All assays were calibrated using Beckman Decision 111 (Beckman Instruments Brea, CA, USA). With the diazo/caffeine benzoate and the

ferricyanide procedures the set point quoted for the Jendrassik Grof method (70 $\mu\text{mol/L}$, Lot No. M007093) was used while for the DPD method the set point quoted for DPD (74 $\mu\text{mol/L}$, Lot No. M007093) was used.

Imprecision

Two quality control samples were assayed consecutively 20 times to establish the within-run imprecision. To quantify the between run imprecision these were again run in duplicate each day for 20 days and the variation calculated from the second result in accordance with the recommendations of the European Committee for Clinical Laboratory Standards (ECCLS).¹¹

Development of ferricyanide assay

Figure 1 shows the absorbance change at 450 nm of an icteric sample (bilirubin 362 $\mu\text{mol/L}$) diluted 1/30 in caffeine/benzoate reagent containing varying ferricyanide concentrations. At concentrations of 1.0 and 2.0 mmol/L there is a rapid decrease in absorbance particularly in the first 50 s.

As ferricyanide also has a significant absorbance at 450 nm, the relative absorbance of bilirubin and ferricyanide in caffeine was monitored between 400 and 500 nm (Fig. 2). Bilirubin absorbance declines only 30% at 480 nm (wavelength available on Hitachi 704) while the ferricyanide peak is at a minimum. The 480 nm also has the advantage that oxidation of haemoglobin by ferricyanide in haemolysed samples causes only a slight decrease in absorbance at this wavelength (Fig. 3). Since haemoglobin and methaemoglobin show an isobestic point at 660 nm this was chosen as the blanking wavelength (Fig. 3).

The ferricyanide automated assay was therefore set up as follows. Sample (10 μL) was first

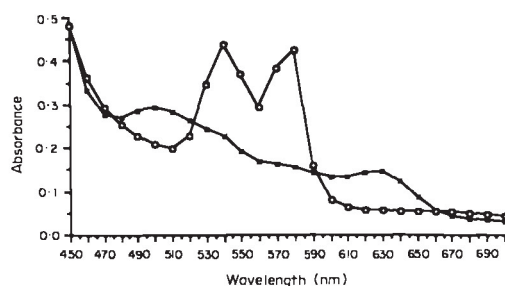


FIGURE 3. Absorbance of haemolysed plasma pool, 1/10, in caffeine and in caffeine containing 1.0 mmol/L potassium ferricyanide (bilirubin 10 $\mu\text{mol/L}$, Hb 5 g/dL). ■; ○ = ferricyanide/caffeine.

TABLE 1. Instrument settings for the ferricyanide bilirubin method on the Hitachi 704

	Bilirubin
Assay code	2 Point-14-31
Sample volume	10 μL
Reagent 1 (volume)	360 μL
Reagent 2 (volume)	90 μL
Wavelength (analytical)	480 nm
Wavelength (blanking)	660 nm
Calibration method	Linear (0) (0)

added to 360 μL of caffeine/benzoate (Reagent 1) and the absorbance difference at 480 and 660 nm measured after 5 min. Potassium ferricyanide (90 μL , 5 mmol/L; Reagent 2) effecting a final ferricyanide concentration of 1.0 mmol/L, as then added and the absorbance difference at 480/660 nm again measured after a further 5 min. Concentration of bilirubin as derived from the difference between both bichromatic measurements. Instrument parameters are presented in Table 1.

RESULTS

Linearity

Figure 4 shows virtually no deviation from linearity up to 1000 $\mu\text{mol/L}$ with an absorbance change of 0.66 milli absorbance units/ $\mu\text{mol/L}$ of bilirubin. The corresponding absorbance changes for the diazo/caffeine benzoate and DPD methods (for similar sample volume fraction) were 0.74 and 0.45 milli absorbance units/ $\mu\text{mol/L}$ of bilirubin, respectively.

Calibration stability

The absorbance changes (480/660 nm at 0.0 and 70.0 $\mu\text{mol/L}$ bilirubin (calibration points) were

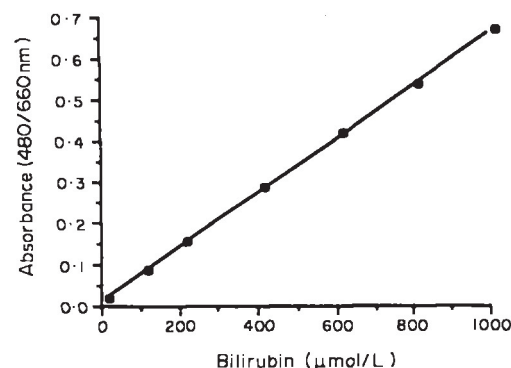


FIGURE 4. Linearity and absorbance sensitivity of ferricyanide bilirubin assay.

+6.0 and -39.0 milli absorbance units, respectively. During the first 3 months of operation there was no detectable variation in these absorbances therefore calibration could be carried out using a fixed factor.

TABLE 2. Imprecision of ferricyanide bilirubin method ($n=20$)

Mean ($\mu\text{mol/L}$)	Within-run (CV%)	Between-run (CV%)
19.3	1.85	2.11
69.7	0.95	1.17

TABLE 3. Bilirubin values of lyophilized materials assayed by three methods

Material	All methods (mean)	Bilirubin $\mu\text{mol/L}$		
		Diazo/caffeine benzoate	DPD	Ferricyanide
Corning SN (H)	11	11	11	10
Corning SA (H)	72	73	71	72
Boehringer cfas (H)	67	72	61	71
Roche P (A)	33	32	31	32
Diamed A (A)	69	69	71	71
Wellcontrol SVRS (A)	82	83	83	83
Nycomed PC (A)	348	359	341	351

H = Human based.

A = Animal based.

DPD = Diphenyldiazonium.

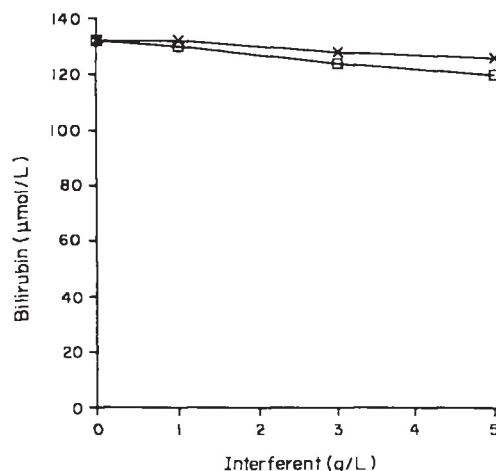


FIGURE 5. Effects of haemolysis and lipaemia on the ferricyanide bilirubin assay. x = Haemoglobin; □ = intralipid.

Imprecision

The within-run and between-run variation at 19.3 (which approximates the upper limit of the reference range) and 69.7 $\mu\text{mol/L}$ were acceptable (Table 2).

Assay of lyophilized materials

Seven commercial lyophilized materials were assayed by the three methods (Table 3). The proposed ferricyanide method showed good agreement with the diazo/caffeine/benzoate method and the all-method mean values quoted by the various manufacturers (Table 3). The close agreement of the animal based control materials with the diazo/caffeine/benzoate method indicates that carotenoids do not interfere with the ferricyanide method (Table 3).

Effects of haemolysis and lipaemia

The relative effects of increasing haemolysis and lipaemia are presented in Fig. 5. As shown the method is not significantly affected by up to 5 g/L

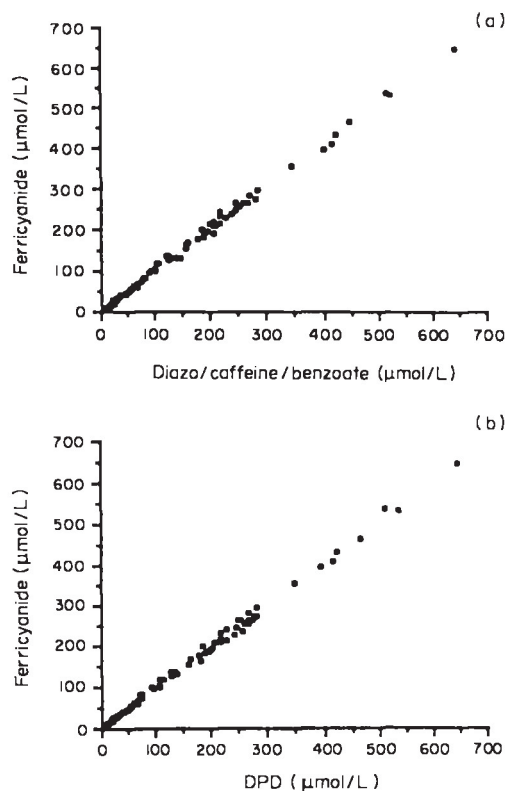


FIGURE 6. Correlation of potassium ferricyanide with diazo/caffeine benzoate (a) and DPD (b) bilirubin procedures. (a) $Y = 0.362 + 1.02 x$; $r = 0.998$; $Syx = 6.09$. (b) $Y = -0.135 + 1.00 x$; $r = 0.998$; $Syx = 6.66$.

haemoglobin. A slight negative interference was evident at higher concentrations of Intralipid.

Correlation

Twenty nonicteric, 70 adult icteric and 30 neonatal icteric samples were assayed by the 3 methods. All samples were free of visual haemolysis. Figure 6 shows the proposed ferricyanide procedure gives very good agreement with both the diazo/cafeine benzoate and DPD procedures.

DISCUSSION

A recent survey of widely used bilirubin methods on automated analytical systems has demonstrated extensive interference due to haemolysis.¹² The interference was not consistently related either to haemoglobin or bilirubin concentrations. This presents a serious limitation particularly with the analysis of icteric neonatal samples in which haemolysis frequently co-exists. Since diagnosis and treatment of jaundice in neonatology is very dependent on accurate measurement of serum bilirubin it behoves the laboratory to select a procedure minimally affected by contaminating haemoglobin.

The potassium ferricyanide oxidation of bilirubin to biliverdin overcomes many of the difficulties associated with bilirubin measurement. (1) Interference due to haemolysis is virtually eliminated (Fig. 5), the assay is therefore applicable to the measurement of neonatal samples which frequently present with haemolysis. (2) Reagents and calibration are stable for at least 3 months simplifying calibration and other performance requirements. (3) The reagent contains no expensive ingredients. (4) Linearity of the assay is adequate over the entire pathophysiological range.

The reaction of delta bilirubin (covalently bound bilirubin) with the diazo/cafeine/benzoate method is slow and can effect an underestimation in samples containing this fraction when short reaction times are used.¹³ Sufficient time (i.e. 5 min) was allowed in the two diazo procedures to ensure that delta bilirubin was fully quantitated. The decline in optical density at 480 nm in the presence of ferricyanide is rapid and shows no further decrease after 100 (Fig. 1). This, plus the fact that at no time in the diverse selection of patient samples assayed were obviously discrepant results found with the ferricyanide procedure compared to the two diazo methods that measure all forms of bilirubin indicates that the covalently bound bilirubin is also oxidized by ferricyanide.

This assay has now been in routine use on the Hitachi 704 for 9 months. During this time no

methodological difficulties or clinical discrepancies were apparent. In external quality assessment schemes the ferricyanide values have shown consistent agreement with the all method mean values derived. In the United Kingdom External Quality Assessment Scheme a Mean Running Variance Index Score (MR VIS) of 14 was achieved for the first 9 months of operation.

In summary, the ferricyanide oxidation of bilirubin in caffeine forms the basis of a robust stable reagent procedure for the automated measurement of total bilirubin in serum.

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