A Simplified Procedure for Eliminating the Negative Interference of Bilirubin in the Jaffé Reaction for Creatinine

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The negative interference by bilirubin in the measurement of creatinine by the Jaffé procedure is well documented. We describe a procedure for the elimination of interfering bilirubin by oxidation to biliverdin through the addition of potassium ferricyanide before the alkaline picrate reagent. We detected no negative interference with the addition of as much as 600 \( \mu \text{mol/L} \) bilirubin. The procedure shows good agreement with a recently described blank rate method for analyzing icteric samples (Clin Chem 1991;37:1460–1).

Negative interference due to bilirubin in the Jaffé procedure for serum and (or) plasma creatinine is well documented (1–4). This interference is evident with both conjugated and unconjugated bilirubin, is independent of creatinine concentration, and is directly related to bilirubin concentration (5).

Many approaches have been adopted to minimize this interference. A commercial reagent has been shown to be effective for the measurement of icteric samples (5), but it requires initial blank monitoring at 10 s, which limits its application. The reagent also contains sodium dodecyl sulfate, which tends to precipitate when reagents are stored at lower temperatures. High alkaline picrate concentrations have also been shown to be effective, but the reaction time is quite short and requires continuous measurement (5). Potassium ferricyanide also has been incorporated into the Jaffé procedure (6), but it is unstable in the working reagent (7) and needs to be added to the reagent at the time of analysis. More recently, blank rate procedures involving subtraction of the rate of decline in absorbance of the icteric sample in the alkaline reagent from the increasing absorbance in the mixed picrate reagent have been used with Hitachi analyzers (8, 9).

In this study we demonstrate the effectiveness of direct addition of potassium ferricyanide to patients' plasma samples to effect rapid oxidation of bilirubin to biliverdin, thereby removing interference in the Jaffé procedure.

Materials and Methods

Materials

Bilirubin from gallstones was obtained from Sigma Chemical Co. Ltd., Poole, UK. All other chemicals were of Analar grade from British Drug House, Poole, UK. Alkaline reagent, NaOH 200 mmol/L, was stable indefinitely when sealed from the atmosphere; picric acid reagent, 25 mmol/L, and 30% Brij 35 surfactant, 1 mL/L, were stable for 3 months at room temperature. Combined picrate solution—5:1 (by vol) alkaline reagent/picric acid reagent—was stable for at least 5 days at 4°C. Potassium ferricyanide solutions were made up in 154 mmol/L sodium chloride and were stable for at least 3 days at 4°C.

Methods

Aliquots of 100 mmol/L bilirubin dissolved in 100 mmol/L NaOH were added to a plasma pool. The pH of the pool did not change by more than 0.1 pH units, and the addition of an equivalent amount of NaOH to the plasma pool had no detectable effect on the assay. The dilutional effect of the added bilirubin was compensated for by appropriate calculation.

Three assay procedures were used with the Hitachi 704 (Tokyo, Japan).

Rate method. Twenty microliters of plasma was mixed with 350 \( \mu \text{L} \) of alkaline reagent for 5 min, after which 70 \( \mu \text{L} \) of picrate reagent was added (creatinine kit, cat. no. 818426; Boehringer, Mannheim, FRG). The rate of bichromatic absorbance change (505 nm analytical wavelength, 570 nm blanking wavelength) was measured 60 s after picrate was added for a further 60 s.

Blank rate method. Twenty microliters of sample was incubated with 350 \( \mu \text{L} \) of alkaline reagent (9). After 4 min, the rate of absorbance decline (505 nm analytical wavelength, 570 nm blanking wavelength), which is linear with time, was measured for 60 s. Seventy microliters of picrate reagent was added, and after 60 s the rate of absorbance change was measured for a further 60 s. Concentration was calculated by subtracting the rate of absorbance decline in the alkaline reagent from the rate of absorbance increase after picrate was added.

Ferricyanide (combined reagent) method. Twenty microliters of plasma was mixed with 20 \( \mu \text{L} \) of ferricyanide, Reagent 1 (2 mmol/L). After 5 min, 400 \( \mu \text{L} \) of combined picrate reagent, Reagent 2, was added, reducing the ferricyanide concentration to 91 \( \mu \text{mol/L} \). Sixty seconds after the addition of mixed picrate, the rate of absorbance change (505 nm analytical wavelength, 570 nm blanking wavelength) was measured for a further 60 s.

Results

To evaluate the effect of ferricyanide addition on bilirubin, a nonicteric plasma pool was supplemented with 400 \( \mu \text{mol/L} \) bilirubin and added to different concentrations of ferricyanide. Figure 1 represents the absorbance change of the supplemented pool at 450 nm (sample volume proportion = 6%). As shown, at ferricy-
The unsupplemented and supplemented pools were then assayed for creatinine by both the rate and ferricyanide procedures. Assay by the rate method demonstrated different rates of absorbance change, yielding creatinine concentrations of 59 and 100 μmol/L, respectively. With the ferricyanide procedure, the unsupplemented and supplemented pools showed similar increased rates of absorbance change, particularly during the measuring period of 60 to 120 s, and gave values of 91 and 96 μmol/L, respectively.

The effect of added bilirubin was subsequently investigated by the three procedures and is illustrated in Figure 2.

There is a marked reduction in the apparent creatinine concentration with increasing bilirubin by the more widely used rate method. Both the ferricyanide and the blank rate procedures demonstrated very slight increases with added bilirubin. The creatinine concentrations for the unsupplemented pool by the rate and ferricyanide procedures were 101 and 91 μmol/L, respectively. This difference was also apparent when 100 nonicteric samples were compared by both procedures, i.e., y (ferricyanide) = −10.6 + 0.998 x (rate method), \( S_{yx} = 4.3 \) (creatinine range = 44 - 1740 μmol/L). This difference was due to starting the reaction with combined picate reagent, as opposed to initial preincubation with alkaline reagent, and was independent of ferricyanide presence (data not shown). In an external quality-assessment program the overall mean values for creatinine concentrations of two samples were 117 and 437 μmol/L (n = 1322). These samples yielded values of 129 and 439 μmol/L by the rate method, and values of 115 and 432 μmol/L by the combined reagent procedure (the presence or absence of ferricyanide showed no detectable difference with these latter values). The closer agreement of the combined reagent procedure to the overall mean value may reflect the number of laboratories using a combined reagent approach.

Six samples with different bilirubin concentrations were measured for creatinine by the three procedures (the limited range of creatinine concentrations was dictated by the bilirubin content of samples). Table 1 demonstrates close agreement of both the ferricyanide and blank rate methods; the rate method shows a reduction in the apparent creatinine concentrations similar to that seen in Figure 2.

**Discussion**

A recent approach to eliminate bilirubin interference in the Jaffé reaction for serum creatinine was an off-line preincubation of icteric samples with bilirubin oxidase (10). This approach is tedious and involves the expense of the enzyme. The approach presented here could be used both on- and off-line, and does not involve additional cost.

The ferricyanide and blank rate procedures demonstrated assay values for icteric samples that were −10 μmol/L per 100 μmol/L of bilirubin higher than the rate procedure. These findings are consistent with the observations of Osselaer and Lievens (9). Although newer Hitachi analyzers have the capability of carrying out blank rate protocols, older Hitachi models and many other instruments lack this capability.

Ferricyanide was first used at a concentration of 15 μmol/L (6). This was increased by Knapp and Mayne (7) to 50 μmol/L with no adverse effects on the assay. We used a final concentration of 91 μmol/L and observed no interference or effect on analytical sensitivity. The procedure can be applied to a wide range of analyzers, and could be particularly useful when longer reaction times and lower picate concentrations are used and when the interference by bilirubin is more substantial (5).

In conclusion, we demonstrated that the simple addition of potassium ferricyanide to plasma samples as part
of the analytical procedure is very effective in eliminating the negative interference of bilirubin in the Jaffé reaction for plasma creatinine estimation.

References