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A Bichromatic Technique for Serum Iron on the CentrifiChem 400

To the Editor:

Centrifugal analyzers give the best performance in terms of analytical time when only one rotor is needed for each determination. With the CentrifiChem 400, for iron quantitation, a blank run is required as well as a test run, because of the possible presence of interfering substances. This involves the preparation of two rotors, a time-consuming procedure that makes use of the CentrifiChem disadvantageous for this determination.

Trying to avoid this disadvantage, I developed a bichromatic procedure similar to that described by Kelly et al. (*1*) for total-bilirubin determination.

For this purpose I chose Dow Diagnostic reagents: the iron reduced by ascorbic acid at low pH (pH = 1.7) forms a magenta complex with ferrozine. Interference by copper, which also reacts with the chromogenic agent, is eliminated by the presence of thiosemicarbazide. At this pH, copper in fact reacts completely and specifically with thiosemicarbazide to form a stable uncolored complex (*2*).

To investigate the problem, I used two different approaches: a conventional CentrifiChem iron method, in which test and blank require separate rotor preparation, and a bichromatic procedure detailed in Table 1.

After 3 s, as suggested by Kelly et al. (*1*), switch the Blank control to Hold, change the filter to 550 nm, and activate the logic-reset button on the internal memory panel.

A regression analysis of results for samples from 85 patients assayed with the conventional (x) and bichromatic (y) method gave the equation $y = 0.95x + 0.37$ ($r = 0.9904$). I evaluated also the within-run precision of the method by assaying two serum samples 25 times in one assay, and the between-run precision by assaying them on 15 separate days (Table 2). The precision in both cases was acceptable.

These results were obtained by discarding sera that were opalescent or icteric for unconjugated bilirubin. Such samples give an absorption spectrum that is unsuitable for the described technique. Under the test conditions they in fact have an absorbance value smaller at 620 nm than at 550 nm, so the iron concentration calculated by the bichromatic technique is higher than that calculated by the conventional technique.

Figure 1 shows absorption spectra of the following sera: (*a*) normal serum; (*b*) opalescent serum (triglycerides: 3.30 mmol/L); (*c*) icteric serum (total bilirubin: 282.1 μmol/L; conjugated: 191 μmol/L); (*d*) icteric serum with high unconjugated bilirubin (total bilirubin: 188 μmol/L; conjugated: 17.1 μmol/L).

It is apparent from the spectral scans that the blanks of an opalescent serum or of a serum with a high unconjugated-bilirubin concentration differ from the normal serum blank, making these samples unsuitable for the bichromatic technique.

My data suggest that the described procedure is applicable to the CentrifiChem in iron quantitation of all sera,

Table 1. Instrument Setting for the Bichromatic Method

Sample, μL	50
Sample and diluent, μL	99
Reagent, μL	250
Wavelength, nm	620 (→550)
T_0, s	3
Δt, min	6
Blank	Auto (→Hold)
Mode	Term
Print	Concn
Factor/Std	Std. value
Temp	25 °C

Table 2. Precision of the Proposed Technique

	$\bar{x}$ Concn, μmol/L	SD	CV, %
Within-run (n = 25)			
Control A	17.82	0.97	5.44
Control B	34.36	1.34	3.89
Between-run (n = 15)			
Control A	18.04	1.16	6.43
Control B	34.69	1.54	4.44

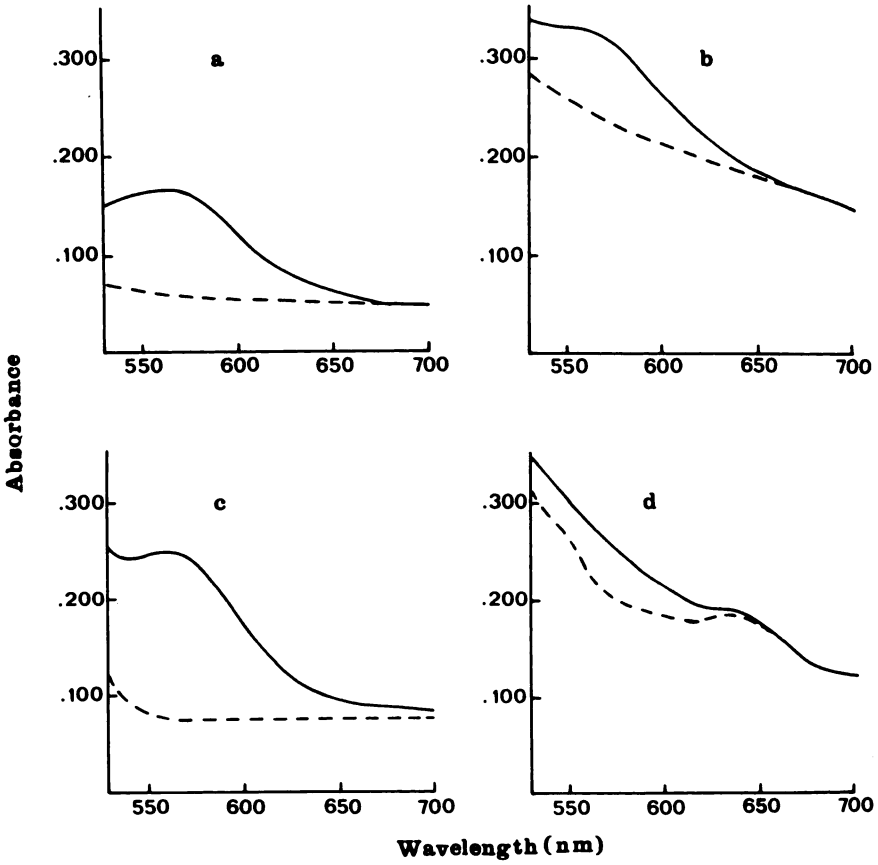

Fig. 1. Absorption spectra of blanks (- - - -) and samples (—)
(*a*) normal serum; (*b*) opalescent serum; (*c*) icteric serum; (*d*) icteric serum with unconjugated bilirubin

except unclear and icteric ones with very high unconjugated bilirubin concentrations.

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Comments on Interference from Bilirubin in a System for HDL-Cholesterol Determination

To the Editor:

In a recent issue some criticism about our work on HDL-cholesterol determination (*1*) has been expressed in a Letter by Zoppi and De Luca (*2*). In our paper, we did not describe a "method" for suppressing interference from bilirubin in the Barham–Trinder reaction, but merely an optimized system in which bilirubin does not interfere "chemically" with the determination of HDL-cholesterol in highly-diluted samples. We have never suggested that uric acid be determined at pH 6.1, as Zoppi and De Luca seem to imply. Rather, we clearly pointed out that the optimal pH for our cholesterol oxidase is 6.5 and that uric acid does not interfere up to at least 200 mg/L. Accordingly, "drawbacks" *a* and *c* cannot be ascribed to our system.

We assessed interference from endogenous bilirubin by using 39 samples with bilirubin content up to 140 mg/L. The modest bias between test and reference values appeared to be ascribable to random error. We also took into consideration the results of a test of interference by added bilirubin, in which a modest positive bias was not significant for bilirubin at 100 mg/L, but was significant for 150 mg/L. The critical value we proposed—100 mg/L—comes from a honest and cautious interpretation of our data and is meant to avoid any risk of overestimation owing to the color contribution of bilirubin.

When Zoppi and De Luca say that "at pH values lower than 7, bilirubin should be less prone to oxidation than at alkaline pH," they emphasize an advantage of our system rather than a drawback.

These authors propose the use of aminopyrine, but cannot actually explain its action. It is well-known that aminopyrine reacts with *p*-quinones (*3*), with hydrogen peroxide and peroxidase (*4*, *5*), and in Trinder-type systems devised for detection of hydrogen peroxide (*6*).

We think that a satisfactory theory about the mechanism of the Trinder reaction is needed. Then we shall be able to understand the mechanisms of interferences. Until then, every device we can find to improve the performance of reagents will be empirical.

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Combined Enzymic–Jaffé Method for Creatinine in Serum

To the Editor:

We read with great interest the articles of Masson et al. (*1*, *2*) on the combined Jaffé method for creatinine, involving creatinine amidohydrolase and creatine kinase. Since 1980 we have also used the alkaline picrate reaction in association with the fully enzymic method of Wahlefeld et al. (*3*), with reagents produced by Boehringer Mannheim Diagnostica (cat. no. 166413).

Our procedure is as follows: Into each of two tubes place 0.1 mL of serum. To the first (test) add 0.5 mL of the buffered substrate in double the concentration specified by the manufacturer and to the second (blank) 0.5 mL of the same substrate containing in addition creatinine amidohydrolase, 5000 U/mL. Mix well and incubate for 20 min at 20–25 °C. Add to each tube 0.5 mL of alkaline picrate reagent, prepared by mixing four volumes of picric acid (50 mmol/L) and one volume of sodium hydroxide (1.6 mol/L) just before use.

The color produced is most intense at 20 min and remains stable for at least an hour. Measure the absorbance of the test vs the blank in a 10-mm cuvet, at 495 nm.

When creatinine standards in creatinine-free human serum or in a 50 g/L solution of human albumin are used, the absorbance is linearly related to concentration from 0.050 to 1.500 *A* for a creatinine content of 50 to 1500 μmol/L. The precision of the procedure is excellent: 10 analyses of serum specimens containing 60, 130, 600, 750, and 1050 μmol/L showed, within-day, CVs ranging from 0.8 to 2% and, day-to-day, from 2.4 to 3.2%. Analytical recovery is complete, ranging from 98.5 to 101% for 50, 250, and 500 μmol of creatinine added per liter to sera containing creatinine concentrations of 60, 130, 600, and 750 μmol/L.

On comparing our observations with those of Masson et al. (*1*, *2*), our procedure is superior in regard to linearity, sensitivity, precision, and recovery. This is due mainly to the failure of the method of Masson et al. to achieve complete conversion of creatinine. These authors claim that this conversion is 95%, but in our hands the conversion of creatinine in their method reached 90–95% for creatinine concentrations only up to 100 μmol/L, and varied from 85 to 90% for higher concentrations up to 500 μmol/L, the maximum that could be determined by their method. In contrast, according to our procedure with the fully enzymic method of Wahlefeld et al. (*3*), creatinine conversion is complete in any concentration up to 1500 μmol/L.

Moreover, our procedure, involving the use of alkaline picrate reaction in association with the fully enzymic method of Wahlefeld et al. (*3*), is simpler and has a greater range of linearity and greater sensitivity, precision, and reliability than their original method (*3*), in which creatinine is determined by measuring the oxidation of NADH to NAD^+ at 340 nm.

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