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TOTAL AND UNBOUND BILIRUBIN DETERMINATION USING AN AUTOMATED PEROXIDASE MICROMETHOD

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INDEXING WORDS

peroxidase micromethod; automated assay; unbound bilirubin; total bilirubin; hyperbilirubinemia; bilirubin-albumin binding; kernicterus; neonate; pediatric chemistry

SYNOPSIS

The peroxidase micromethod has been automated to measure total and unbound bilirubin. The apparatus consists mainly of a spectrophotometer monitored by a programmable computer. A comparison with the Alkali Azobilirubin Blue method showed a highly significant correlation of 0.976 for total bilirubin. The correlation coefficient between the automated and the manual peroxidase micromethod was 0.974 for unbound bilirubin. The addition of hemolysates caused minimal effects on the values obtained for total and unbound bilirubin, even at levels of hemoglobin above those usually encountered in sera of infants. An adequate control of the temperature is essential to secure precision and accuracy in the measurements. The analysis requires only 25 µl of serum. The apparatus is portable, easy to operate, and the results are obtained almost immediately. All of these make it possible its routine use in the nursery.

INTRODUCTION

The report in 1952 by Hsia et al.[12] that infants with kernicterus were frequent when total bilirubin in serum exceeded 20 mg/dl, and rare

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when less than 20 mg/dl, led to adopt the standard practice of performing an exchange transfusion upon elevation of serum bilirubin concentrations above 20 mg/dl. However, bilirubin encephalopathy has since been observed at serum bilirubin levels well below this critical value, specially in sick, low birth weight premature infants.[10] Thus, total bilirubin alone is not a reliable measure to predict risk of kernicterus.[15] On the other hand, the hypothesis that unconjugated bilirubin not bound to serum albumin (free bilirubin or unbound bilirubin) is responsible for the neurotoxicity attributed to bilirubin[20] led to the search of methods to evaluate the determinants of bilirubin-albumin binding and unbound bilirubin concentrations in serum. The most accepted methods currently used are the peroxidase oxidation and the Sephadex column elution techniques. The advantage of the peroxidase method over the latter is that it is more sensitive and requires smaller quantities of sera.[7] Moreover, this method[14] can, also, estimate the bilirubin-binding capacity, that is, the number of primary binding sites available, and the binding affinity or the strength of the bound.[26] The peroxidase method has proved its validity for estimation of unbound bilirubin concentrations in solutions of bilirubin and purified albumin.[2] Constant efforts are being made now to improve its accuracy and precision[1, 4] and, at the same time, to establish its practical use in relation to prediction of risk of kernicterus.[6, 9, 19]

In 1977, Nakamura and Lee[18] described a more practical technique to assess unbound bilirubin (UB) in sera of neonates with unconjugated hyperbilirubinemia based on the peroxidase method.[14] They employed glucose and glucose oxidase instead of hydrogen peroxide. Unbound bilirubin is oxidized to colorless compounds by peroxidase derived from glucose by mediation of glucose oxidase. Total bilirubin (TB) concentration was determined spectrophotometrically from the absorbance and unbound bilirubin from the initial oxidation velocity of bilirubin.[18] The entire analysis required a spectrophotometer with an attached recorder and additional calculations limiting its use as a bedside or nursery routine procedure. These have been obviated by the automation of the method.

This report describes the automation of unbound bilirubin determination using the peroxidase micromethod.[18] To evaluate this method, we assessed the precision, accuracy, interferences due to hemolysis and turbidity, the effect of temperature, and the range of linearity of the automated method, comparing the results with the manual procedure.

MATERIALS AND METHODS

Reagents

Versatol Pediatric, containing 20.5 mg/dl of bilirubin, and 5.5 g/dl of protein was purchased from General Diagnostics (Division of Warner-Lambert Co., Morris Plains, New Jersey 07950). Standards of bilirubin containing human serum albumin were obtained as lyophilized material from Junsei Chem. Co., Ltd. (Tokyo, Japan) and were reconstituted adding 3 ml of 0.1 mol/L phosphate buffer pH 7.4. A solution of bilirubin for peroxidase rate constant determination (K) was prepared by dissolving 11.7 mg of crystalline bilirubin (Sigma Chem. Co., St. Louis, MO 63178) in 2 ml of 0.1 mol/L NaOH, adding 0.2 ml of 0.05 mol/L EDTA as preservative, and finally, diluting to 100 ml with distilled water. Working Standards of bilirubin (Sigma) in human serum albumin (Albumin Human FR V powder-refrigerate-ICN Nutritional Biochemical, Cleveland, O. 44128) were prepared by us at various concentrations and bilirubin-albumin ratios. All bilirubin solutions were prepared in the dark, kept in an ambar bottle and protected from light in dark boxes until their use.

Lyophilized glucose oxidase (GOD, EC 1.1.3.4) and peroxidase (POD, EC 1.11.1.7) were obtained from Junsei Chem. Co. and reconstituted by adding 3 ml of 0.1 mol/L phosphate buffer pH 7.4 to provide a level of 0.33 mg/ml of each one. For standard assay with pure bilirubin, 500-fold diluted GOD/POD was employed.

Bilirubin Kit-N, which used the Alkali Azobilirubin Blue method,[16] was purchased from Nippon Shoji Kaisha Ltd. (Osaka, Japan). Intrafat 10 g % w/v was purchased from Daygo Eiyo Chem. Co. (Osaka, Japan).

Hemoglobin-Test Wako, which used the cyanmethemoglobin method, was purchased from Pure Chemical Industries, Ltd. (Osaka, Japan).

Phosphate buffer 0.1 mol/L, pH 7.4, and glucose 5 g % w/v. were purchased from Junsei Chem. Co.

Before their use, all the materials were allowed to stand at room temperature. The buffer solution was warmed to 25 °C, except when another temperature was indicated.

Procedure

1. Within-run precision

Total and unbound bilirubin determinations were performed on 12 consecutive replicates of working bilirubin standards containing approxi-

mately 22 mg/dl of bilirubin, 3.2 g/dl of albumin and a bilirubin/albumin molar ratio (r) of 0.83. Their mean values and coefficients of variation (CV) were calculated.

2. Between-day precision

Pooled sera were obtained from hyperbilirubinemic infants undergoing exchange transfusion, frozen at -20 °C and stored in aliquots. Single measurements of total and unbound bilirubin were made on the pooled sera day to day during 10 days. Their means and CV were calculated. The reproducibility by different operators was, also, studied.

3. Effect of hemolysis

Hemolysate was prepared washing erythrocytes (from fresh blood of volunteer adults) three times with saline, adding to the packed cells an equal volume of distilled water, and then removing the cell debris by centrifugation. Hemolysate was added at different concentrations to working bilirubin standards and to sera to study the effect of hemolysis on bilirubin measurements. Hemoglobin concentration was assayed with the Hemoglobin-Kit Test.

4. Effects of turbidity

Working bilirubin standards and pooled sera were made turbid by the addition of Intrafat at different concentrations, and then were analyzed for total and unbound bilirubin.

5. Effects of temperature

To study the effects of temperature on the velocity of bilirubin oxidation, buffer solutions were incubated in a water bath to attain the thermal equilibrium desired (15 to 35°C). Room temperature was controlled by air conditioning.

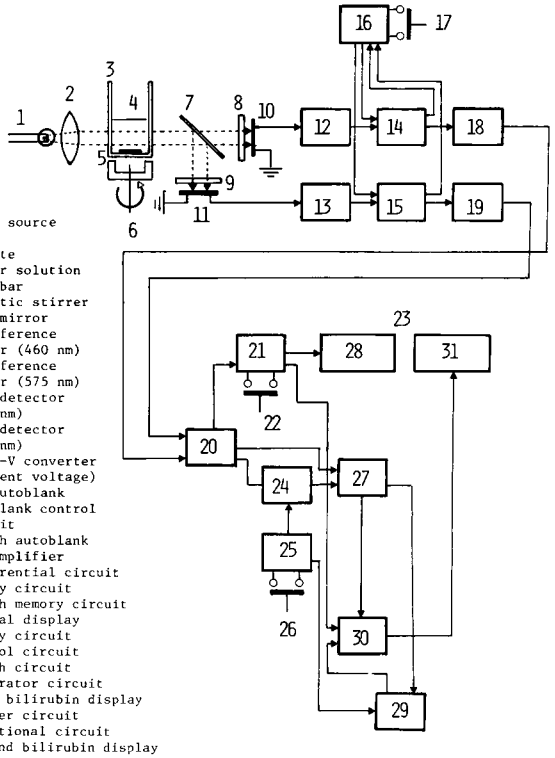
6. Comparison with the manual methods

To compare the automated method with the manual peroxidase micromethod, unbound bilirubin measurements were made on sera of icteric infants. Total bilirubin values by Alkali Azobilirubin Blue method were compared with those by the automated method. A linear regression was performed by the method of least squares, and the correlation coefficient was calculated.

Serum samples were obtained usually by heel puncture from infants of our hospital and from outpatient infants and measured immediately. Some samples were stored at 4 °C and analyzed within 24 hours.

Apparatus

The automated measurements of total and unbound bilirubin are done using an experimental device (UB Analyzer) manufactured by Arrows Co. (Osaka, Japan). The apparatus consists mainly of a spectrophotometer interfaced with a small computer (see Fig. 1). The enzyme standardization is performed as described for the manual method.[18] The activity of the enzyme solution used is furnished to the operational circuit (Fig. 1, No. 30) previously programmed to calculate the unbound bilirubin concentration. The volumes of the reagents used for the manual method are scaled down proportionately to one-half. One ml of buffer solution is put into the cuvette and 25 µl of the sample is added. The cylindrical cuvette (No. 3) is monitored in situ by a double beam spectrophotometer: 460 nm for the peak of bilirubin (No. 10) and 575 nm for the peak of hemoglobin (No. 11), and the concentration of total bilirubin is displayed on the screen (No. 28) after the elimination of the effect of hemoglobin by a differential circuit (No. 20). Twenty-five µl of glucose solution and 25 µl of GOD/POD solution are, then, added to the cuvette. The switch (No. 26) to initiate the function of the control circuit (No. 30) is pressed immedi-



(1) Light source
(2) Lens
(3) Cuvette
(4) Buffer solution
(5) Iron bar
(6) Magnetic stirrer
(7) Half mirror
(8) Interference filter (460 nm)
(9) Interference filter (575 nm)
(10) Photodetector (460 nm)
(11) Photodetector (575 nm)
(12)(13) I-V converter (current voltage)
(14)(15) Autoblank
(16) Autoblank control circuit
(17) Switch autoblank
(18)(19) Amplifier
(20) Differential circuit
(21) Memory circuit
(22) Switch memory circuit
(23) Digital display
(24) Memory circuit
(25) Control circuit
(26) Switch circuit
(27) Comparator circuit
(28) Total bilirubin display
(29) Counter circuit
(30) Operational circuit
(31) Unbound bilirubin display

ately. When the output of the differential circuit (No. 20), which decreases with the degradation of bilirubin, corresponds to the value of the memory circuit (No. 24)--that is, 80% of the total bilirubin--the comparator circuit (No. 27) and the UB operational circuit (No. 30) start to function. Finally, the value of unbound bilirubin is shown on the display (No. 31).

RESULTS

Within-run precision

For the automated method, using working standard solutions of bilirubin-albumin, the CV for TB and UB were 2.25% and 3.27%, respectively. For the manual method these values were 2.55% and 6.73% (Table 1). Different operators using the apparatus made no great difference on its performance (Table 2).

Table 1 Within-run precision: comparison between the manual and the automated peroxidase micromethod.

Method	Total bilirubin(mg/dl)		Unbound bilirubin(μg/dl)	
	Mean ± S D	CV, %	Mean ± S D	CV, %
Manual	22.74 ± 0.58	2.55	1.18 ± 0.08	6.78
Automated	21.75 ± 0.49	2.25	1.22 ± 0.04	3.27

The working bilirubin standard solution had a bilirubin-albumin molar ratio (r) of 0.83. Each mean value represents the average of 12 determinations.

Table 2 Comparison of operators using the automated peroxidase micromethod.

Operator	Total bilirubin(mg/dl)		Unbound bilirubin(μg/dl)	
	Mean ± S D	CV, %	Mean ± S D	CV, %
A	22.74 ± 0.13	0.58	0.833 ± 0.031	3.72
B	22.38 ± 0.23	1.03	0.828 ± 0.018	2.17
C	21.33 ± 0.43	2.01	0.781 ± 0.027	3.46

Samples of working bilirubin standard solutions were determined 10 times by each operator.

Between-day precision

The CV for TB and UB values determined consecutively during ten days on pooled sera were always less than 5% (Table 3).

Table 3 Between-day precision of the automated peroxidase micromethod.

Determination	Total bilirubin(mg/dl)		Unbound bilirubin(μg/dl)	
day	Mean ± S D	CV, %	Mean ± S D	CV, %
0	17.07 ± 0.27	1.58	0.398 ± 0.011	2.67
1	16.72 ± 0.49	2.93	0.389 ± 0.011	2.92
3	16.82 ± 0.53	3.15	0.419 ± 0.017	4.06
5	16.43 ± 0.72	4.38	0.400 ± 0.010	4.75
7	16.74 ± 0.73	4.36	0.395 ± 0.008	4.56
10	16.48 ± 0.92	5.58	0.372 ± 0.013	3.49

The samples were obtained from pooled sera of exchanged-transfused blood, apportioned into 0.5 ml aliquots and frozen at -20°C. Each mean value represents the average of 10 determinations.

Effects of hemolysate

Hemolysates with hemoglobin concentrations up to 5 g/dl, caused no significant effects on the values of TB (Fig. 2). At hemoglobin concentrations higher than 1 g/dl, with bilirubin-albumin molar ratios of 1.0 and 1.5, a progressive increase of UB was observed.

Effects of turbidity

Intralipid concentrations equal or higher than 100 mg/dl caused progressive increase in the values of TB measured in working bilirubin standard solutions. The same effect was observed when determinations were made in pooled sera at concentrations of Intralipid as low as 50 mg/dl. Bizarre results were obtained with respect to UB (Table 4).

Effects of temperature

The variations in rate of bilirubin oxidation and UB concentrations were calculated from the oxidation velocity with varying temperatures of buffer. An S-shaped curve (Fig. 3) was obtained showing an in-

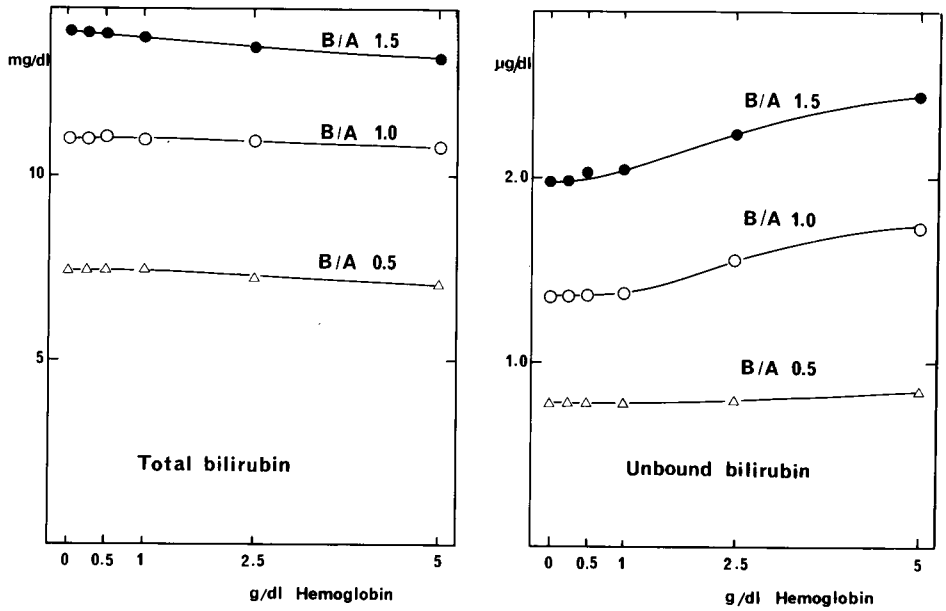


Fig. 2 Effect of hemolysates on the determination of total and unbound bilirubin using the automated peroxidase micromethod. Hemoglobin values correspond to their concentrations in the samples. Each point is the mean of 3 determinations. B/A: Bilirubin-albumin molar ratio.

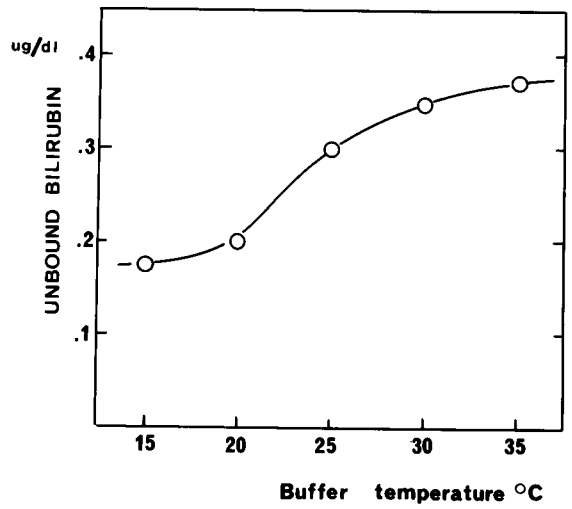


Fig. 3 Effect of temperature on unbound bilirubin determination using the automated peroxidase micromethod. Each point is the mean of 6 determinations of working bilirubin standards.

Table 4 Effect of Intrafat on total and unbound bilirubin concentrations measured with the automated peroxidase micromethod.

Concentrations of Intrafat mg/dl	Working bilirubin std.		Pooled serum	
	T B mg/dl	U B µg/dl	T B mg/dl	U B µg/dl
0	24.5	0.95	13.7	0.33
5	24.4	0.93	13.7	0.34
10	24.5	0.94	14.0	0.34
20	24.3	0.92	14.0	0.33
50	24.2	0.94	14.7	0.35
100	25.6	0.98	15.0	0.35
200	26.8	0.93	16.5	0.39
400	28.7	0.91	19.2	0.45

The values represent the mean of 3 determinations in each case.

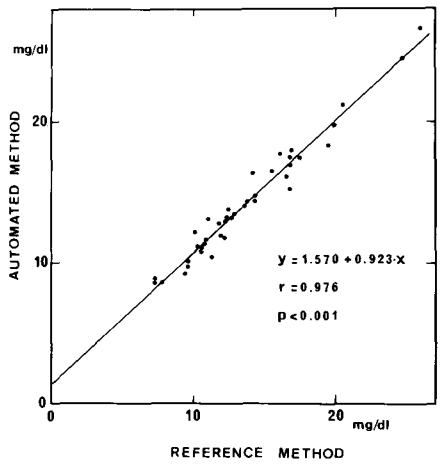


Fig. 4
Correlation between the Alkali Azobilirubin Blue method and the automated peroxidase micromethod: determination of total bilirubin in sera of 44 icteric infants.

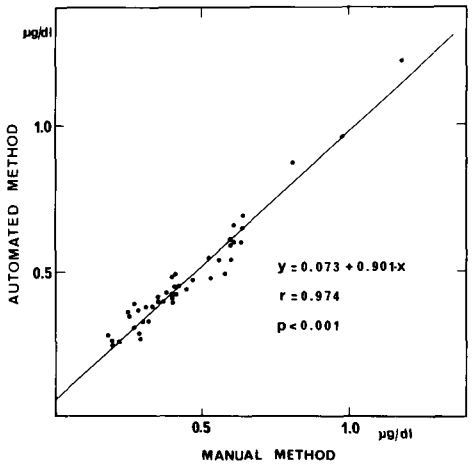


Fig. 5
Correlation between the manual and the automated peroxidase micromethod: determination of unbound bilirubin in sera of 40 icteric infants.

crease in the velocity of bilirubin oxidation and, thus, an increase in UB levels with increasing temperatures. A more pronounced effect was observed when the room temperature was changed to 25, 30 and 35°C (data unpublished).

Comparison between the manual and the automated methods

Fig. 4 shows the linear regression for TB measurements correlating the Alkali Azobilirubin Blue method and the automated peroxidase micromethod. The correlation coefficient was 0.976 indicating a highly significant correlation between the two methods.

Fig. 5 shows the linear regression relating the automated and the manual peroxidase micromethods for UB measurements. The correlation coefficient was 0.974 indicating a highly significant correlation between the two methods.

DISCUSSION

This first study to determine total and unbound bilirubin by the automated peroxidase micromethod shows results that excel the usual standard manual techniques. The newly developed method is precise and shows minimal interferences due to hemolysis. Only a minimum amount of sera (25 µl) is necessary, being half the volume required by the manual method. The values desired are obtained almost instantly on the digital screen. The apparatus itself has been designed to be portable and simple to operate facilitating its routine use in the nursery.

Since the majority of the samples obtained from heel or fingertip puncture are frequently hemolyzed, it is important that such interference be as negligible as possible in relation to bilirubin determination.[16] Michaëlsson and Sjölin reported that hemoglobin concentrations of the samples taken with due routine precautions did not exceed 0.7 g/dl.[17] The interference is chemical in nature and the spectral error does not account for this interference.[5] It has been recently demonstrated that oxyhemoglobin is the species of hemoglobin in erythrocyte hemolysates that inhibits the diazo reaction[22] and interferes with the Jendrassik-Groff method.[23] Moreover, Waud et al.[25] have reported that TB measured by direct spectrophotometry, which corrects for the presence of hemoglobin, is not affected by hemolysis. Our results are in accord with these findings having obtained minimal interference even at levels of hemoglobin above those usually encountered in sera of infants. On the other hand, Broder-

sen and Bartels demonstrated that hemoglobin catalyses the oxidation of bilirubin with hydrogen peroxide,[3] acting as an accelerating factor, a process which is markedly inhibited by tert-butyl-p-hydroxyanisole.[4] This explains, at least in part, the increase of UB concentrations in bilirubin-albumin solutions with hemolysates containing Hb concentrations above those commonly found in serum samples.

Turbidity of the samples is another factor that has to be considered when Intrafat is administered in the nursery, specially in sick, low birth weight infants.[21] Intrafat per se does not affect the binding of bilirubin to albumin. After its administration it is metabolized by the body to non esterified fatty acids, which at high concentrations, can displace bilirubin from the binding sites of albumin.[2] The alterations in the values of TB and UB observed at high concentrations seem to be of spectral nature since turbidity is observable at first glance. In hyperbilirubinemic sera it is hard to distinguish between mild and moderate turbidities due to Intrafat, so it is recommendable to previously clear the samples in a high-speed centrifuge at 15,000 rpm for about 10 minutes.[21]

This method is influenced by changes in temperature,[11] therefore precautions must be taken to secure precision in the results. The S-shaped curve obtained in our experiment is the result, not only of the increase in peroxidase activity with increasing temperatures,[8] but also, of the changes in the binding affinity of human serum albumin to bilirubin. Jacobsen demonstrated a decrease in the binding affinity of HSA with increasing temperatures and salt concentrations suggesting that hydrogen bonds and salt linkages are the main factors in the binding of bilirubin to its primary site on human serum albumin.[13]

In 1979 Wennberg et al.[27] described an automation of the peroxidase method using a Gilford 3500 Computer Directed Analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074). However, the manual method on which it is based is somewhat different from ours. We think that our apparatus has some advantages over theirs. It has been designed specially for UB determinations. With little training it can be operated by any member of the medical staff. It is portable and it is quick. It presents the values for TB and UB automatically due to its built-in microcomputer which performs all the calculations. We want to emphasize again the fact that the automated method requires only a minimum volume of serum (25 µl), which is specially important in sick low birth weight infants.

In a previous report, we have discussed the relation between serum

levels on UB and phototherapy.[19] There have been few tentative studies suggesting values of UB in relation to risk of kernicterus.[11] However, no critical level of UB has been established yet at which an exchange transfusion or phototherapy is indicated to prevent kernicterus.

Even though there still remain to be evaluated the predisposing factors (e.g. acidosis, hypoxia) presumably associated with the development of bilirubin encephalopathy,[24] we believe that in the near future, investigating a significative number of infants, it will be possible to establish the correlation between UB levels and risk of kernicterus and criteria to warrant an exchange transfusion. We are sure that the device described here will facilitate ensuing studies and will be helpful in the clinical management of neonatal jaundice.

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