Clinica Chimica Acta, 79 (1977) 411—417

CCA 8750

MICRODETERMINATION OF UNBOUND BILIRUBIN IN ICTERIC NEWBORN SERA: AN ENZYMATIC METHOD EMPLOYING PEROXIDASE AND GLUCOSE OXIDASE

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(Received March 7th, 1977)

Summary

An enzymatic assay method for the microdetermination of unbound bilirubin in newborn icteric sera is described. Unbound bilirubin is oxidized to colorless compounds by peroxidase in the presence of hydrogen peroxide derived from glucose by the mediation of glucose oxidase. In this method, the bilirubin is not significantly degraded before the addition of peroxidase, in contrast to the method using hydrogen peroxide. The oxidation rate is determined by spectrophotometry and chloroform extraction is eliminated.

The unbound bilirubin concentration can be determined fromthe initial oxidation velocity of total bilirubin. The Michaelis constant, K_M, was approximately 20 μM. The coefficient of variation for icteric serum determination was 4.4—6.5%. The concentration of unbound bilirubin was reduced after five days of storage at —20° C.

The bilirubin-albumin binding affinity was studied with purified albumin and adult serum. The dissociation constants were 2×10^{-8} M and 5×10^{-9} M, respectively, at bilirubin/albumin molar ratios below 1.0.

Clinically, serum samples from 75 icteric newborn infants were analysed, and the sera of premature infants were found to have remarkably high levels of unbound bilirubin compared to those of fullterm infants. The sera of a Rhesus immunization infant and an ABO incompatibility infant were remarkably higher than that of the nonhemolytic icteric sera. The unbound bilirubin concentration was also affected, in an in vitro study, by the addition of hemolysate.

Introduction

The level of serum total bilirubin does not provide a fully reliable guide for evaluating the risk of bilirubin encephalopathy, particularly in low birth-

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weight infants with nonhemolytic hyperbilirubinemia. Several reports published during the past few years have mentioned the occurrence of kernicterus at total bilirubin levels not previously considered dangerous for newborn infants [1,2,3].

It has been shown in vitro and in vivo that the toxicity of bilirubin is due uniquely to the protein-free, unconjugated bilirubin fraction (unbound bilirubin). Until recently, we employed the Sephadex method for determining the level of unbound bilirubin and we demonstrated that this technique is valid for evaluating unbound bilirubin clinically and as an indicator for exchange transfusion [3,4]. However, the method is not really adequate for clinical purposes due to its insufficient sensitivity, and the time requirement for column preparation.

Jacobsen [5,6] has reported a sensitive enzymatic method for the measurement of unbound bilirubin in icteric newborn sera. Here, we attempt to adapt this enzymatic assay into a more practical technique for the evaluation of icteria sera. We employ glucose and glucose oxidase instead of hydrogen peroxide. The bilirubin-albumin binding affinity, and the validity of the technique are considered.

Materials and methods

Principle

Brodersen and Bartels [7] have observed that bilirubin can be oxidized by peroxidase in the presence of hydrogen peroxide. Excess albumin greatly inhibits the oxidation of bilirubin, due to albumin-bilirubin complex formation. Jacobsen [5] concluded that bilirubin and albumin equilibrate very rapidly compared to the rate at which unbound bilirubin is oxidized.

It first order reaction conditions pertain in the bilirubin oxidation caused by peroxidase, we have $v_o = -dA/dt = k \cdot B \cdot$ [peroxidase] (B, bilirubin concentration), where v_o is the rate of bilirubin disappearance over the measured time interval, and k is the rate constant. Thus, the unbound bilirubin concentration can be calculated from the oxidation velocity.

Reagents

1. Phosphate buffer, 0.1 M, pH 7.4.
2. Bilirubin stock solution. 11.7 mg of bilirubin (Sigma) dissolved in 0.2 ml of NaOH, 0.1 M and 0.1 ml of ethylene diaminetetraacetate, 0.1 M, were diluted to 10 ml by the addition of distilled water. This stock solution was stable for several hours in a dark chamber.
3. Albumin stock solution. 27.6 mg of human serum albumin, Fraction V (Nutritional Biochemicals Co.), was dissolved in 10 ml of phosphate buffer, 0.1 M, pH 7.4.
4. Glucose solution. 0.5 g of glucose was dissolved in 10 ml of distilled water.
5. Glucose oxidase solution. 10 mg of glucose oxidase of fungal origin (Grade I, 210 U/mg, Boehringer Mannheim) was dissolved in 1.0 ml of phosphate buffer, 0.1 M, pH 7.4.
6. Peroxidase solution. A 10 mg/ml preparation of horseradish peroxidase (suspension in 3.2 M ammonium sulfate solution) was obtained from Boehrin-

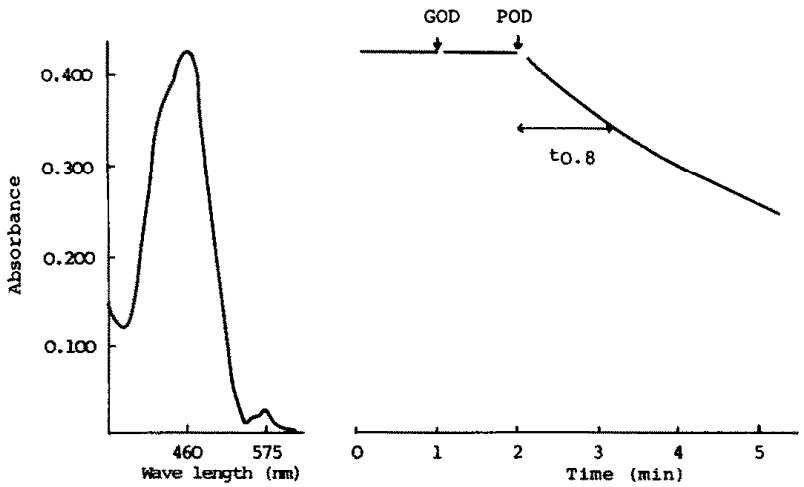


Fig. 1. Absorption spectrum of diluted newborn icteric serum, and the decrease in absorbance at 460 nm after addition of peroxidase.

ger Mannheim and diluted to 1 mg/ml by the addition of phosphate buffer for the assay of icteric sera. The standard assay of albumin-free bilirubin oxidation was performed with a more dilute peroxidase solution (1 μg/ml). For the experiments on bilirubin-albumin binding, the concentration of peroxidase solution was varied according to the molar ratio of bilirubin to albumin.

Procedure

50 μl of serum were added to 2.0 ml of phosphate buffer in a cuvette of 10-mm path length. The absorbance at 460 nm (absorbance maximum of albumin-bound bilirubin) and 575 nm (peak absorbance of heme) were recorded. 20 μl of glucose solution and 10 μl of glucose oxidase solution were added to the sample and the absorbance at 460 nm was recorded after one minute to determine any decrease (Fig. 1). 10 μl of peroxidase solution was then added and the mixture agitated with a stirring rod. The decrease in absorbance at 460 nm was again recorded with an Hitachi spectrometer (Model 101) coupled with an Hitachi linear recorder. The assays were carried out at room temperature.

Calculations

The total bilirubin concentration was determined by direct spectrophotometry at 460 nm for the albumin-bound bilirubin, where the extinction coefficient was 49 000. The effect of heme on the absorbance at 460 nm was eliminated by employing the formula: $A_{460} - 0.8 \times A_{575}$. Under these experimental conditions where bilirubin oxidation follows first order kinetics, the rate constant, k, was determined by measuring the oxidation velocity of bilirubin in the absence of albumin. The initial velocity, v_o, was estimated from the time required for a decrease of 20 percent in the initial bilirubin concentration. The unbound bilirubin concentration was calculated from the initial velocity of bilirubin degradation and the ratio of the peroxidase concentration to that in the standard assay of the albumin-free bilirubin solution.

$$\text{Unbound bilirubin (mg\%)} = \frac{[\text{peroxidase}]_{st} \cdot t_{0.8\ st}}{[\text{peroxidase}] \cdot t_{0.8}} \times B_0$$

[peroxidase]$_{st}$, peroxidase concentration in the standard assay; [peroxidase], peroxidase concentration in the assay of icteric sera; B_o, initial concentration of total bilirubin).

Results

Reaction kinetics. A kinetic study of bilirubin oxidation was carried out using 2.5—40 μM bilirubin solutions. The Michaelis constant, K_M, was approximately 20 μM and the V_{max} was 26 nmol/min (Fig. 2).

Bilirubin-albumin binding. The albumin (Fraction V) and normal adult serum binding properties were investigated by increasing the amount of bilirubin (1 μM—16 μM) to a constant albumin concentration (10 μM), and the data were plotted on a Scatchard plot (Fig. 3). The first dissociation constant for albumin fraction V was 2×10^{-8} M; that for normal adult serum (5×10^{-9} M) was considerably higher than that of pure albumin solution.

Accuracy and limitations of the method. Determinations of the unbound bilirubin concentration of icteric newborn serum were replicated six times in each of seven samples (Table I). The coefficient of variation was 4.4—6.5%. The control serum was divided and frozen in a refrigerator, and the effect of storage was investigated for ten days. The total bilirubin concentration did not vary significantly during this time, but the unbound bilirubin concentration decreased remarkably after five days of storage.

Effect of hemolysis of icteric sera. The effect of hemolysis on the total bilirubin and unbound bilirubin concentrations was investigated by adding different concentrations of hemolysate to newborn icteric serum (Fig. 4). The total bilirubin concentration was constant in spite of the addition of different

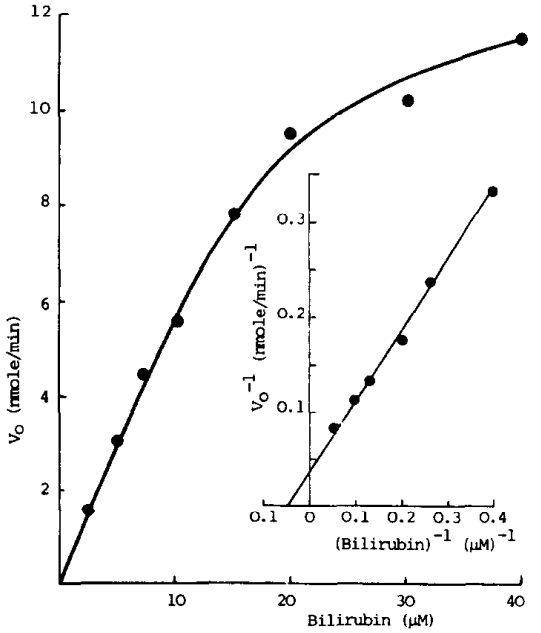


Fig. 2. Oxidation of aqueous bilirubin in the presence of peroxidase.

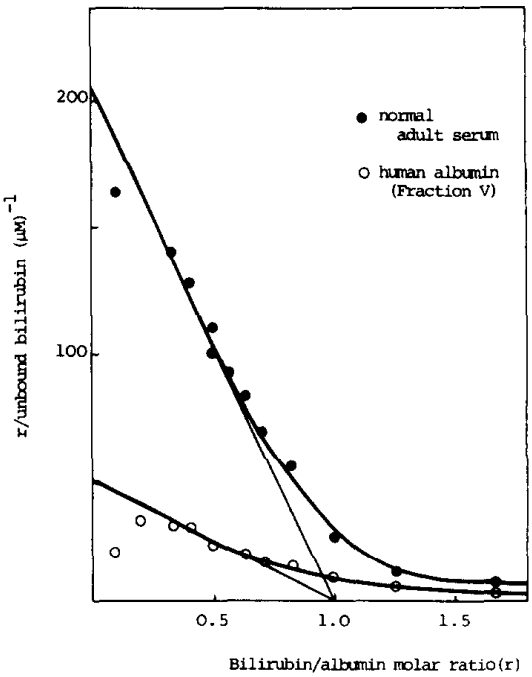


Fig. 3. Scatchard plot of bilirubin-albumin binding.

TABLE I

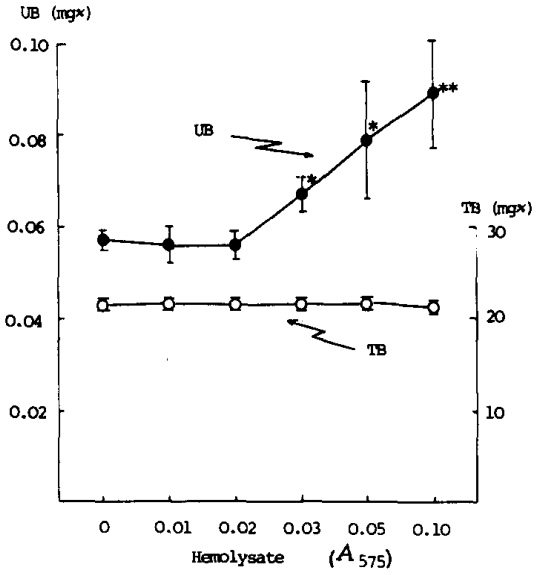
PRECISION OF THE PROPOSED METHOD

Storage time (days)	Total bilirubin (mg %)		Unbound bilirubin (μg %)	
	Mean ± S.D. *	C.V. (%)	Mean ± S.D. *	C.V. (%)
0	21.13 ± 0.28	1.3	25.74 ± 1.42	5.5
1	21.17 ± 0.18	0.9	23.95 ± 1.29	5.4
2	21.39 ± 0.14	0.7	26.49 ± 1.26	4.8
3	21.13 ± 0.24	1.1	25.70 ± 1.12	4.4
5	21.31 ± 0.14	0.7	21.07 ± 1.37	6.5
7	21.19 ± 0.10	0.5	14.97 ± 0.87	5.8
10	21.09 ± 0.03	0.2	12.50 ± 0.74	5.9

* Mean ± S.D. of six replicated determinations in each case.

concentrations of hemolysate, when using a collecting formula. The unbound bilirubin concentration did not vary when A_{575} of the hemolysate was below 0.02, but when A_{575} of the hemolysate was over 0.02, the unbound bilirubin concentration increased progressively. In clinical assays, the absorbances at 575 nm does not exceed 0.02 in nonhemolytic icteric sera.

Unbound bilirubin concentrations in icteric sera. We analysed 75 icteric sera from 35 premature infants and 40 fullterm infants within seven days of birth. The unbound bilirubin concentrations ranged from 0.002 mg% to 0.089 mg%



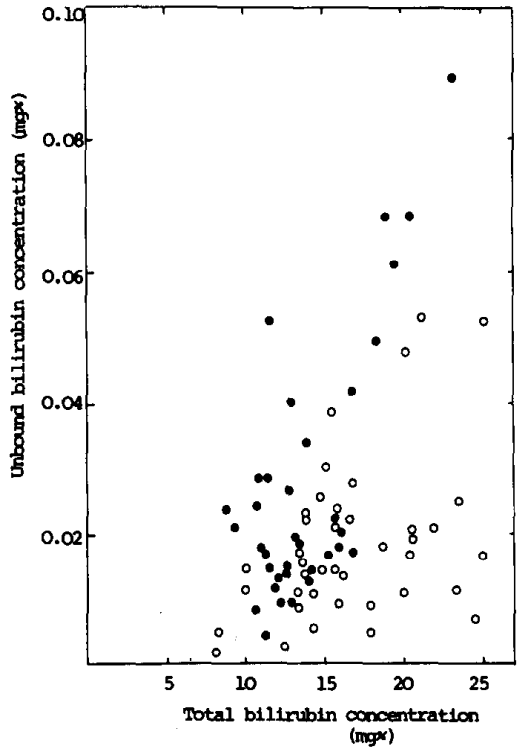


Fig. 4. Effect of hemolysis on the total bilirubin (TB) and unbound bilirubin (UB) levels by the addition of different concentrations of hemolysate. *$p < 0.01$; **$p < 0.001$.

Fig. 5. The concentration of unbound bilirubin and total bilirubin in the icteric sera of 35 premature infants (●) and 40 fullterm infants (○).

TABLE II

EFFECT OF EXCHANGE TRANSFUSION ON UNBOUND BILIRUBIN

Patient	Gestational age (weeks)	Birth weight (g)	Cause of icterus	Time of analysis (days after birth)	Before exchange transfusion		After exchange transfusion	
					Total bil. (mg%)	Unbound bil. (mg%)	Total bil. (mg%)	Unbound bil. (mg%)
1. T.A.	37	3110	Rh immunization	3	23.2	0.089	13.8	0.031
2. T.S.	33	1400	ABO incompatibility	3	19.0	0.068	12.7	0.014
3. T.B.	26	635	Nonhemolytic	2	11.6	0.053	8.8	0.015
4. B.O.	34	2785		5	19.6	0.039	10.3	0.005
5. A.K.	35	1700		5	16.0	0.021	7.7	0.006
6. H.A.	35	2550		4	20.5	0.068	12.0	0.019
7. I.R.	37	2265		3	19.5	0.061	10.0	0.022
8. N.A.	38	2600		7	20.2	0.048	8.9	0.007
9. H.A.	39	3160	Enclosed hemorrhage	5	21.2	0.053	13.3	0.015
10. M.A.	39	3760		3	23.5	0.025	15.4	0.009
11. S.A.	40	4355		4	25.2	0.053	13.3	0.014

(Fig. 5), and exceeded 0.05 mg% in seven infants; viz., one premature infant with Rhesus immunization, one premature infant with ABO incompatibility, three premature infants with nonhemolytic hyperbilirubinemia (total bilirubin concentration, 11.6 mg%—20.5 mg%) and two fullterm infants with nonhemolytic hyperbilirubinemia (total bilirubin concentration, 21.2 mg% and 25.2 mg%). All of these seven infants were subjected to exchange transfusion (Table II).

High unbound bilirubin levels were observed in some cases even when the total bilirubin level was lower than 15 mg%. In particular, the unbound bilirubin levels in premature infants were remarkably high when compared with those of fullterm infants.

Discussion

By the Sephadex gel filtration method, it was not possible to measure unbound bilirubin when the bilirubin was mixed with albumin or adult serum in vitro at molar ratios of bilirubin-albumin of less than 1.0, due to the limited sensitivity [8]. The peroxidase method permits the determination of unbound bilirubin under these conditions and under usual clinical conditions (i.e. before the high affinity albumin binding sites are saturated) [5,6].

In Jacobsen's method [6], the unbound bilirubin was oxidized by peroxidase in the presence of ethyl hydroperoxide or hydrogen peroxide and a decrease in the absorbance at 460 nm on adding ethyl hydroperoxide without peroxidase was observed. In this study, we used glucose and glucose oxidase as the donor of hydrogen peroxide. We observed no decrease in the absorbance at 460 nm after adding glucose and glucose oxidase in usual clinical assays of icteric sera, and the oxidation of bilirubin took place just after the addition of peroxidase.

In vitro experiments were designed with purified albumin and adult serum at bilirubin-albumin molar ratios of less than 1.0, and the dissociation constants

were calculated to be: 2×10^{-8} M in purified albumin and 5×10^{-9} M in adult serum. However, the unbound bilirubin concentrations in icteric newborn sera were considerably higher than expected from these in vitro results, since the bilirubin/albumin molar ratios in icteric newborn sera were always lower than 1.0 and even below 0.4. These findings are in agreement with observations made by the Sephadex gel filtration method [4]. This suggests that in icteric newborn serum not only do competitive factors play a role in the bilirubin-albumin binding, but they also exert their effect at a different intensity from child to child, since unbound bilirubin was detected with great individual variability over the measured range of total bilirubin concentrations.

The infants suffering from Rhesus immunization and ABO incompatibility showed high unbound bilirubin concentration, due probably to the remarkably competitive effect of hematin, as demonstrated by the Sephadex method [4]. An in vitro study also showed that unbound bilirubin increased with on adding hemolysate.

Premature infants generally showed higher unbound bilirubin concentrations, relative to their total bilirubin concentrations, than fullterm infants. This observation suggests a possible basis for the occurrence of kernicterus in premature infants at total bilirubin levels which have so far not been considered dangerous for newborn infants.

We observed that when serum was stored at $-20°$C there was a ramerkable decrease in the unbound bilirubin fraction after five days but no decrease in total bilirubin. Clinical assays of bilirubin should therefore be carried out promptly or without storage of serum. Our method is relatively easy to perform and a single determination requires only five minutes. By its use, early evaluations of unbound bilirubin levels should allow the physician to predict the risk of kernicterus and to plan his clinical procedures accordingly.

Acknowledgement

We would like to thank Prof. T. Matsuo, Head, Department of Pediatrics, Kobe University, School of Medicine, for his thoughtful review of the manuscript.

References

1 Stern, L. and Denton, R.L. (1965) Pediatrics 35, 483
2 Gartner, L.M., Snyder, R.N., Chabon, R.S. and Bernstein, J. (1970) Pediatrics 45, 906
3 Zamet, P., Nakamura, H., Perez-Robles, S. and Larroche, J.Cl. (1976) Birth Defects: Original Article Series, Vol. XII, No. 2, 236
4 Nakamura, H. and Lardinois, R. (1972) Biol. Neonate 21, 400
5 Jacobsen, J. (1969) FEBS Lett. 5, 112
6 Jacobsen, J. and Wennberg, R.P. (1974) Clin. Chem. 20, 783
7 Brodersen, R. and Bartels, P. (1969) Eur. J. Biochem., 10, 468
8 Chunga, F. and Lardinois, R. (1971) Acta Pediatr. Scand. 60, 27