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Effect of Storage and Freezing on Unbound Bilirubin Measurement

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Abstract

Background—Unbound bilirubin (B_f) is a better predictor of bilirubin toxicity than total serum bilirubin (TSB) in the postnatal period. The Food and Drug Administration approved peroxidase test for B_f analysis is not currently available in clinical laboratories. The samples are often stored at varying temperature and for varying duration before B_f analysis.

Methods—We evaluated the effect of storing blood samples up to 1 month at -20°C and -80°C on B_f measurement. In a subset of samples collected for neonates enrolled in a prospective B_f study, TSB ($\mu\text{mol/l}$) and B_f ($\mu\text{mol/l}$) were measured by the peroxidase test and then the samples were immediately frozen at -20°C ($n=10$) or -80°C ($n=10$) for either 2 weeks or at -20°C ($n=16$) or -80°C ($n=10$) for 1 month. After the designated freezing period, the samples were thawed and TSB and B_f measured again.

Results—There was no effect of storage for 2 weeks at either -20°C or -80°C on B_f measurement. There appears to be no effect on B_f after storage at -80°C for a month. However, there was significant decrease in B_f after storage at -20°C for a month.

Conclusions—The results of this study suggest that blood samples for B_f measurement should be analyzed within 2 weeks after collection and storage at -20°C .

Keywords

unbound bilirubin; premature infants; temperature; storage; peroxidase

Introduction

Total serum bilirubin concentration (TSB) is currently used for the management of neonatal jaundice. Emerging evidence suggests that the fraction of TSB not bound to albumin, referred to as the unbound bilirubin concentration (B_f), may be a better predictor of bilirubin-induced neurotoxicity than TSB.[1-3] A semi-automated, Food and Drug Administration approved method based on the peroxidase method has been most widely used to measure B_f [4-6] but is not routinely available in clinical laboratories.[7] Current bilirubin research is aimed at

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evaluating the usefulness of B_f . [1,2] A multicenter neonatal NICHD phototherapy study including B_f measurements for logistics reasons required storage of samples before analysis. [4] Indirect evidence in that study suggested binding did not change after sample storage at -20°C for several months, but to our knowledge the impact of temperature and duration of storage on sample bilirubin binding has not been systematically evaluated. In anticipation that this will be an issue in future studies of bilirubin binding in newborn jaundice, we conducted a prospective study evaluating the effect of temperature and time on B_f measurement.

Materials and methods

Study Population

Blood samples from premature infants at the University of Rochester Medical Center participating in the National Institutes of Health funded B_f study were used after approval by the Institutional Research Review Board of the University of Rochester Medical Center. Informed consent was obtained from the parents of subjects.

B_f measurement

Blood samples obtained during the first 2 postnatal weeks were collected in amber serum separator tubes to protect bilirubin from light exposure. Baseline TSB ($\mu\text{mol/l}$) and B_f ($\mu\text{mol/l}$) were measured in the serum by the peroxidase test using an unbound bilirubin (UB) analyzer (Arrows Company, Japan). 2 peroxidase concentrations were used to avoid underestimating the equilibrium B_f . [4] A subset of samples ($n = 46$) with enough remaining serum ($> 50\ \mu\text{l}$) to repeat the test, were placed within minutes in either a -20° or -80°C freezer for up to 1 month. At periodic intervals, the samples were thawed in dark for a follow-up TSB and B_f measurement. The bilirubin/albumin equilibrium association or affinity binding constant (K , $1/\mu\text{mol}$) was calculated using the TSB, equilibrium B_f (B_{feq}) calculated from the 2 steady state B_f when necessary, and serum albumin measured using the Bromocresol green method. [4] Serum albumin concentrations were assumed to be the same at baseline and follow-up period for each sample.

Statistical analyses

Statistical Analyses were performed using Stata 9 (Stata Corporation, College Station, TX). Baseline B_f and the K were compared with follow-up B_f and K at each temperature and duration using the Wilcoxon signed-rank test for non-parametric data and a $P < 0.05$ considered statistically significant.

Results

Forty-six paired blood samples were evaluated at baseline and follow-up (Tables 1 and 2). There was no significant effect of blood sample storage for 2 weeks at -20°C on B_f (0 % change in mean B_f , $n = 10$); however a significant decrease in B_f (17 % decrease in mean B_f) occurred after storage for 1 month at -20°C ($n = 16$) compared to baseline B_f . K after storing samples for 1 month at -20°C was significantly greater than baseline K (21% increase in mean K). There was no significant difference between B_f (10% increase in mean B_f) and K at baseline and after storing for 2 weeks at -80°C ($n = 10$). Similarly, there was no significant difference (5% increase in mean B_f) between B_f at baseline and after storage for 1 month at -80°C ($n = 10$). However, there was significant decrease in K (34% decrease in mean K) after storage for one month at -80°C due to a decrease in TSB (18% decrease in mean TSB) at that time.

Discussion

Our data suggest that B_f measurement is dependent on the duration and temperature at which samples are stored. B_f was not affected by storage for 2 weeks at either -20° or -80° C. However, longer storage at -20° C may be associated with a significant decrease in B_f and when more than 2 weeks of storage are needed, -80° C should be used with the caveat that after a month TSB may decrease. Our results are important to consider when designing studies investigating B_f or whenever B_f cannot be measured proximate to the time the sample is drawn.

The finding of decreased stability of B_f at -20° C is similar to that reported previously except the maximum duration of stability reported was 5 days.[8] However, the decrease in B_f in that study might in part be accounted for by differences in protection of samples from ambient light, use of a single peroxidase concentration, and less rigorous standardization of reagents compared with those used with the UB Analyzer.

It is unclear why K decreased after storage for a month at -80° C. It may be due to a fall in TSB over that time, but if so B_f should also decrease unless the TSB is converted into products that bind similarly to native bilirubin.[9] Some albumin denaturation might also occur over time. TSB decreased equally after storage for 2 weeks at -80° C and -20° C. However, there was a differential decrease in TSB after storage for one month with significant decrease observed after storage at -80° C compared to at -20° C. The reason for this differential decrease in TSB is unclear but could likely be the effect of small group sizes or measurement error. Inconsistency in TSB measurement using other standardized methods has been previously reported [10,11] Although we found significant decrease in TSB concentration after one month storage, this information is rarely used clinically as samples are usually not stored for TSB measurement.

The limitation of the study is that the results are based on small different group sizes. Secondly, the results on binding affinity are based on single albumin measurement at baseline and not on repeat albumin measurements after storage. Regardless, the results of this study suggest that for best results in clinical research studies bilirubin binding be measured as soon as possible after sampling but within 2 weeks when blood samples are stored at -20° . Further studies would be needed before storage longer than one month at -80° C can be justified.

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Abbreviations

B_f	unbound bilirubin
TSB	total serum bilirubin
B_{feq}	equilibrium B _f
K	bilirubin/albumin equilibrium association or affinity binding constant
UB analyzer	unbound bilirubin analyzer

Table 1
Effect of temperature on unbound bilirubin after 2 weeks storage of blood samples

	Baseline Mean (SD) Median (range)	Follow-up Mean (SD) Median (range)	% Change in Mean	<i>P</i> [*]
-20° C Freezer (n =10)				
Unbound bilirubin concentration (μmol/l)	0.009 (0.003)	0.009 (0.004)	0%	NS
	0.009 (0.005 - 0.015)	0.007 (0.005 – 0.018)		
Total serum bilirubin concentration (μmol /l)	150 (34)	137 (31)	-9%	0.03
	147 (108 -196)	137 (103 - 198)		
Binding constant (liter/μmol)	55 (17)	52 (20)	- 5%	NS
	56 (39 - 98)	47 (27 - 106)		
-80° C Freezer (n =10)				
Unbound bilirubin concentration (μmol/l)	0.009 (0.002)	0.010 (0.003)	+ 10%	NS
	0.010 (0.005 - 0.012)	0.010 (0.005 - 0.016)		
Total serum bilirubin concentration (μmol /l)	169 (31)	152 (27)	-10%	0.03
	183 (108 - 202)	154 (108 - 202)		
Binding constant (l/μmol)	69 (18)	58 (34)	-16%	NS
	63 (50 -102)	50 (34 - 153)		

* Wilcoxon signed-rank test

Table 2
Effect of temperature on unbound bilirubin after one month storage of blood samples

	Baseline Mean (SD) Median (range)	Follow-up Mean (SD) Median (range)	% Change in Mean	P*
-20° C Freezer (n =16)				
Unbound bilirubin concentration ($\mu\text{mol/l}$)	0.012 (0.005) 0.010 (0.006 - 0.028)	0.009 (0.005) 0.008 (0.004 -0.027)	- 17%	0.002
Total serum bilirubin concentration ($\mu\text{mol/l}$)	162 (44) 154 (106 - 256)	155 (44) 145 (89 - 256)	- 4%	NS
Binding constant (liter/ μmol)	56 (17) 57 (11 - 82)	68 (27) 68 (12 - 116)	+ 21%	0.03
-80° C Freezer (n = 10)				
Unbound bilirubin concentration ($\mu\text{mol/l}$)	0.008 (0.004) 0.007 (0.004 -0.017)	0.009 (0.003) 0.007 (0.005 - 0.017)	+ 5%	NS
Total serum bilirubin concentration ($\mu\text{mol/l}$)	167 (32) 161 (120 - 222)	137 (31) 145 (97 - 159)	- 18%	0.005
Binding constant (liter/ μmol)	90 (35) 78 (35 - 132)	59 (16) 56 (34 -78)	- 34%	0.01

Wilcoxon signed-rank test