

Fluorescence Sensor for the Quantification of Unbound Bilirubin Concentrations

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BACKGROUND: Hyperbilirubinemia in jaundiced neonates is routinely assessed by use of total serum bilirubin. However, the unbound or free form (B_f), not total bilirubin, crosses the blood–brain barrier and can be neurotoxic. Although the peroxidase-mediated oxidation of bilirubin can be used to measure plasma concentrations of B_f , this measurement is relatively complex and the assay is not routinely used. We describe a fluorescence sensor for quantifying B_f in plasma.

METHODS: Our method uses a mutated fatty acid binding protein labeled with the fluorescent molecule acrylodan (BL22P1B11), whose fluorescence is quenched upon binding bilirubin. Another configuration (BL22P1B11-Rh) was developed that uses BL22P1B11 together with the fluorophore rhodamine B, which responds by a change in the ratio of its fluorescence.

RESULTS: The “ B_f probes” were calibrated with aqueous solutions of bilirubin and yielded similar bilirubin dissociation constants [$K_d = 16$ (1.5) nmol/L]. We used the probes to determine B_f concentrations in equilibrium with human serum albumin (HSA) and in human plasma samples supplemented with bilirubin. We obtained equivalent B_f values in both systems, and the B_f probe results were in agreement with the peroxidase assay. B_f measurements revealed that bilirubin–HSA binding was well described by 2 sites with K_d values of 15.4 (1) nmol/L and 748 (14) nmol/L. We measured B_f concentrations in the range expected in jaundiced neonates with a mean CV of approximately 3%.

CONCLUSIONS: The BL22P1B11-Rh probe provides accurate plasma sample B_f concentrations with a single measurement, in 1 min with either a handheld B_f meter or a laboratory fluorometer.

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Bilirubin is a product of hemoglobin turnover that is poorly soluble in water and is largely associated with albumin in plasma. The small fraction of total plasma bilirubin (B_T)⁴ that is present in the aqueous phase, the unbound or free fraction (B_f), is able to permeate the blood–brain barrier and is neurotoxic at sufficiently high concentrations (1). Under normal conditions, total serum bilirubin is maintained at low concentrations by regulating bilirubin production and excretion. In approximately 60% of neonates, however, the mechanisms of bilirubin excretion are not developed enough to keep up with production, resulting in bilirubin accumulation and jaundice (2). This imbalance is considered either benign or possibly beneficial and, for most newborns, resolves spontaneously (3, 4). Concentrations of B_f can rise to concentrations that are neurotoxic, however, resulting in deficits ranging from reversible hearing defects to the more severe and possibly fatal neurological sequelae of kernicterus (1).

Early intervention with phototherapy or exchange transfusion can possibly reverse bilirubin-mediated neurotoxicity (2, 5, 6). Guidelines for intervention depend principally on total bilirubin concentrations, taking into account gestational age and risk factors (7). Fundamental biochemical and clinical evidence, however, predicts that B_f rather than B_T will more accurately correlate with bilirubin-mediated neurotoxicity (1, 8–10). It would be expected therefore that B_f would be superior to B_T for identifying neonates at risk for bilirubin neurotoxicity (11).

The only method currently used for determining B_f is based on the horseradish peroxidase-mediated oxidation of unbound bilirubin (12). Implementation of the peroxidase assay is available with an FDA-cleared instrument (Arrows Ltd.), but adoption of this method for the general screening of jaundiced newborns has been limited because of issues that complicate accurate B_f determinations (13, 14). Most importantly, the Ar-

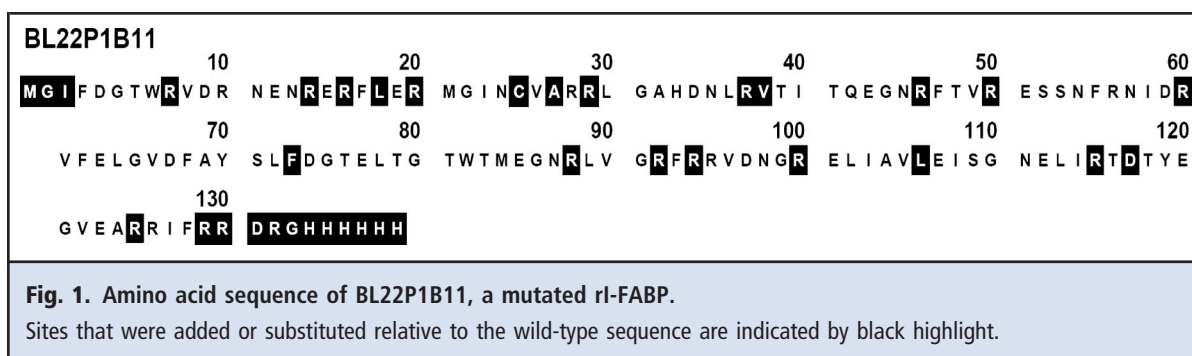
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⁴ Nonstandard abbreviations: B_T , total bilirubin; B_f , free bilirubin; rI-FABP, rat intestinal fatty acid binding protein; FFA, free fatty acid; FFA_u, unbound FFAs; BL22P1B11-Rh, BL22P1B11 mixed with rhodamine B; HSA, human serum albumin; UV, ultraviolet; K_d , dissociation constant.



rows method requires multiple measurements with relatively large sample volumes, and corrections are needed for interferents and diluent conditions (13, 14).

We report here the development of a new method for measuring B_f that uses a fluorescently labeled fatty acid binding protein mutant (B_f probe) that allows direct monitoring of the equilibrium B_f concentration.

Methods

B_f PROBES

Bilirubin probes were derived from a rat intestinal fatty acid binding protein (rI-FABP). FABPs are small, 15-kDa water-soluble proteins that bind long-chain free fatty acids (FFAs) with high affinity and are found in the cytosol of most cells (15). The method used to generate, purify, and characterize the B_f probes is essentially that described previously for unbound FFA (FFA_u) probes (16) and is described in detail in Supplemental Data, which accompanies the online version of this article at <http://www.clinchem.org/content/vol58/issue5>.

Briefly, combinatorial mutagenesis of residues within the rI-FABP binding pocket generated libraries of binding mutants. Conjugation with acrylodan, an environmentally sensitive fluorophore, converted the mutant rI-FABP into probes that were screened for their response to a panel of FFA_u. Among these probes, approximately 10% did not respond sufficiently to FFA_u and were screened for their response to unconjugated bilirubin. A bilirubin-sensitive hit from this screen was selected for additional mutagenesis and yielded the B_f probe BL22P1B11 (Fig. 1). BL22P1B11 was expressed by use of the pET system in *E. coli* BL21(DE3) and the protein was purified by affinity, sizing, and anion exchange chromatography. The purified protein was reacted with acrylodan, and the reaction product was purified with hydrophobic interaction and sizing chromatography. This process led to the B_f probe BL22P1B11. We generated the BL22P1B11-Rh probe by adding enough rhodamine B

to BL22P1B11 probe stock to obtain a rhodamine B-to-BL22P1B11 mol/L ratio of 2:1. The BL22P1B11 and BL22P1B11-Rh probes used in these studies were resuspended from lyophilized powders. Both B_f probes are available from FFA Sciences.

BILIRUBIN

We prepared bilirubin stock solutions daily by dissolving a known mass of bilirubin (Frontier Scientific) in 10 mmol/L NaOH, under argon and shielded from light. The calculated bilirubin concentrations ranged from 35 to 47 mg/dL (600–800 $\mu\text{mol/L}$). Absorbance scans for dilutions of each new bilirubin stock in 10 mmol/L NaOH revealed an absorbance peak at 440 nm and a mean absorptivity of 54 958 $\text{mol/L}^{-1}\text{cm}^{-1}$ (CV 3.8%, 46 measurements). We used an absorptivity of $55 \times 10^3 \text{ mol/L}^{-1}\text{cm}^{-1}$ at 440 nm to confirm the concentrations of the subsequent daily bilirubin stocks. Concentrated bilirubin stocks were used within 90 min to prepare human serum albumin (HSA)–bilirubin complexes or were diluted to 25 $\mu\text{mol/L}$ with 10 mmol/L NaOH for use in free bilirubin titrations. We prepared bilirubin–HSA complexes at molar ratios between 0.1 and 1.4 by adding a 35–47 mg/dL (600–800 $\mu\text{mol/L}$) bilirubin stock to a 600–660 $\mu\text{mol/L}$ HSA (Sigma) stock in HEPES buffer (20 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Na_2HPO_4). All complexes had a final HSA concentration of 300 $\mu\text{mol/L}$. Pooled human adult plasma (Golden West Biologicals), HSA concentration 620 $\mu\text{mol/L}$, was supplemented with bilirubin by use of the same procedure as for the bilirubin–HSA complexes.

ABSORBANCE AND FLUORESCENCE INSTRUMENTATION

We made absorbance measurements using either a CARY 300 (Varian) or a BioMate 3 (Thermo Spectronic) ultraviolet (UV)–visible spectrophotometer. We performed fluorescence measurements as described in Huber et al. (16) with a Spex FluoroLog-3 spectrofluorometer (JY Horiba) using either 10-mm-pathlength polystyrene cuvettes or multiwell plates with a MicroMax plate reader. We also performed flu-

orescence measurements using a FFA_u meter (16) modified for bilirubin by replacing the emission filters with ones centered at 525 and 580 nm (B_f meter). Measurements were at 22 °C, with excitation at 375 nm, and emission intensities were measured at 525 and 575 nm [I(525) and I(575), respectively] for the Spex-FluoroLog or at 525 and 580 nm for the B_f meter. The fluorescence of a sample in the absence of probe (blank) was subtracted from the fluorescence of the sample plus probe.

BILIRUBIN- B_f PROBES BINDING ISOTHERMS

We determined binding isotherms in HEPES buffer by measuring the change in fluorescence of the B_f probes in response to increasing bilirubin concentrations. We used separate cuvettes for each bilirubin concentration to avoid sample heating, photobleaching, and protein denaturation resulting from excessive stirring. The B_f probes were resuspended from lyophilized stocks and were used at a final concentration of 0.4 $\mu\text{mol/L}$. This probe concentration allowed the titrations to reach saturation at low bilirubin concentrations while still providing a reliable fluorescence signal. Total bilirubin concentrations ranged from 0.003 to 0.047 mg/dL (0.05–0.8 $\mu\text{mol/L}$) for BL22P1B11 and 0.003 to 0.082 mg/dL (0.05–1.4 $\mu\text{mol/L}$) for BL22P1B11-Rh. The excitation wavelength was 375 nm for both probes, with fluorescence intensities monitored at 525 nm for BL22P1B11 and at both 525 and 575 nm for BL22P1B11-Rh.

FITTING THE BINDING ISOTHERM DATA FOR BL22P1B11

Before fitting a model to the BL22P1B11 titration data, we corrected the I(525) intensity for inner-filter absorbance at the excitation wavelength due to bilirubin absorbance at 375 nm according to

$$I_{525}^{\text{Corr}} = I(525)10^{B_T \epsilon(375) \frac{d}{2}} \quad (1)$$

where B_T is the total bilirubin concentration, $\epsilon(375)$ is the absorptivity of bilirubin bound to albumin at 375 nm (11 900 $\text{mol/L}^{-1}\text{cm}^{-1}$), and d is the cuvette pathlength (see online Supplemental Data). Inner-filter corrections at the 525-nm emission were <1% for the B_T concentrations used.

We fitted corrected titration data for BL22P1B11 with a single-site binding model to determine the equilibrium dissociation constant (K_d) according to

$$\frac{I(525)}{I_o} = 1 - \frac{(K_d + B_T + P_T) - \sqrt{(K_d + B_T + P_T)^2 - 4B_T P_T}}{2P_T}, \quad (2)$$

where $I(525)$ is the blank-adjusted fluorescence intensity of the probe, I_o is the intensity of the probe in the absence of bilirubin, P_T is the total probe concentra-

tion, K_d is the dissociation constant, and B_T is the total bilirubin concentration (see online Supplemental Data for derivations).

FITTING THE BINDING ISOTHERM DATA FOR BL22P1B11-RH

To generate a more robust assay that avoided excitation inner-filter effects, we created a ratio probe by mixing BL22P1B11 with the soluble fluorophore rhodamine B. The response of this mixture, denoted BL22P1B11-Rh, to bilirubin was a change in the ratio of fluorescence emissions at 525 nm (acrylodan) to 575 nm (rhodamine B) due to the quenching of the 525-nm fluorescence. Because the fluorescence of both acrylodan and rhodamine B were reduced to the same degree by bilirubin inner-filter absorbance at 375 nm, the ratio [$R = I(525)/I(575)$] was not affected. The titration of BL22P1B11-Rh with bilirubin was described by the following single-site model (see online Supplemental Data):

$$R = -\frac{\sqrt{(P_T^2 + (2K_d - 2B_T)P_T + K_d^2 + 2B_T K_d + B_T^2) \times (rR_o^2 - R_o) + (P_T + K_d - B_T)rR_o^2 + (-P_T + K_d + B_T)R_o}}{2(B_T r R_o^2 - (P_T + K_d + B_T)rR_o + P_T)}, \quad (3)$$

where R is the blank-adjusted fluorescence ratio [$I(525)/I(575)$], R_o is the ratio in the absence of bilirubin, r is the $I(575)/I(525)$ ratio for BL22P1B11 in the absence of rhodamine B, and P_T , B_T , and K_d are the same as in Eq. 2. The value of r is instrument dependent and was measured to be 0.171 for the FluoroLog 3 and 1.05 for the B_f meter.

B_f DETERMINATION WITH BL22P1B11, BL22P1B11-RH, AND PEROXIDASE

Procedural details for B_f measurements with the probes are in online Supplemental Data. We measured free bilirubin concentrations (B_f) in HSA–bilirubin complexes or bilirubin-supplemented plasma with Eq. 4 for BL22P1B11 and Eq. 5 for BL22P1B11-Rh:

$$B_f = K_d \left(\frac{I_o}{I(525)} - 1 \right) \quad (4)$$

and

$$B_f = K_d \left(\frac{(R - R_o)}{(RrR_o - R)} \right). \quad (5)$$

These equations apply for samples in which B_f is buffered by the HSA-binding equilibrium and therefore not perturbed by the presence of the probe. B_f was also measured by the peroxidase method (12) (see online Supplemental Data).

BILIRUBIN-HSA BINDING ISOTHERM

We used BL22P1B11-Rh to measure equilibrium B_f values in sets of bilirubin–HSA complexes. Each set used an HSA concentration from 6 to 24 $\mu\text{mol/L}$, with

bilirubin-to-HSA molar ratios ranging from 0 to 1.4. Although excitation inner-filter effects do not affect BL22P1B11-Rh, emission inner-filter corrections caused by differential bilirubin absorbance at 525 and 575 nm can be large at exceptionally high bilirubin concentrations, especially in longer-pathlength (10-mm) cuvettes. Using $[\epsilon(525) - \epsilon(575)] = 800 \text{ mol/L}^{-1}\text{cm}^{-1}$ in Eq. 1, the maximum correction in B_f was approximately 3% for 10-mm cuvettes and 1% for the smaller (5-mm) cuvettes of the B_f meter. The bilirubin–HSA binding isotherms were well described by the following equilibrium model:

$$B_T = B_f + \frac{B_f A_T}{K_d A_1 + B_f} + \frac{B_f A_T}{K_d A_2 + B_f} + \frac{B_f P_T}{K_d P + B_f} \quad (6)$$

where A_T is the total HSA concentration, $K_d A_1$ and $K_d A_2$ are the HSA–bilirubin dissociation constants for 2 independent HSA-binding sites, P_T is the total B_f probe concentration, $K_d P$ is the probe–bilirubin dissociation constant, and B_T is the total bilirubin concentration. Eq. 6 describes the total bilirubin concentration as the free plus HSA- and probe-bound bilirubin concentrations. We fitted this implicit equation in B_f to the bilirubin–HSA binding isotherms using MLAB software (Civilized Software) to determine $K_d A_1$ and $K_d A_2$.

STATISTICS

We performed nonlinear least-squares fits for probe calibration and analysis of HSA-binding isotherms with Origin 8.5 (OriginLab) and MLAB, respectively. Fits from which K_d values were determined exhibited R^2 values >0.98 . The K_d and B_f values are means (SDs).

Results

BINDING AFFINITIES OF BILIRUBIN TO THE B_f PROBES

We determined the dissociation constants for the BL22P1B11–bilirubin and BL22P1B11-Rh–bilirubin complexes by titrating the B_f probes with free bilirubin in HEPES buffer at pH 7.4. We determined the K_d values by fitting the BL22P1B11 data with Eq. 2 and the BL22P1B11-Rh data with Eq. 3 (Fig. 2). The mean K_d values and the corresponding SDs were 15.8 (2.7) nmol/L ($n = 8$) for BL22P1B11 and 16.6 (1.2) nmol/L ($n = 16$) for BL22P1B11-Rh (Fig. 2 and Table 1). Titrations of BL22P1B11-Rh at pH 7.0 and 8.0 revealed no substantial difference from the results at pH 7.4 (Table 1), which indicates that probe function and bilirubin solubility remain relatively constant over the extremes of the physiologic pH range.

Our interpretation of the binding isotherms requires that both the B_f probe and unconjugated bilirubin

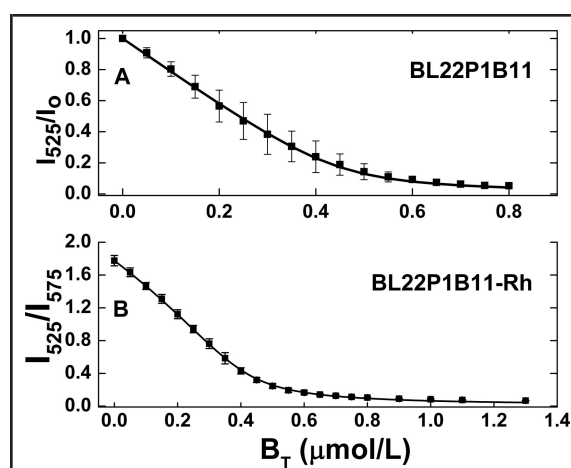


Fig. 2. Representative bilirubin titration data for BL22P1B11 and BL22P1B11-Rh.

(A), BL22P1B11 results corrected for inner-filter absorbance were fitted with Eq. 2, yielding a K_d of 15.3 nmol/L. (B), BL22P1B11-Rh results fitted with Eq. 3 yielded a K_d of 15.9 nmol/L. Each data point is the mean and SD of triplicate measurements.

are monomers in solution. We used absorbance to assess bilirubin solubility by monitoring the absorptivity and λ_{\max} with increasing bilirubin concentration. We prepared serial dilutions of bilirubin at concentrations between 0.0058 and 0.23 mg/dL (0.1 and 4 $\mu\text{mol/L}$) from bilirubin stocks in HEPES buffer, pH 7.4. A Beers law plot of the dilution series showed that the absorbance was directly proportional to bilirubin concentration up to approximately 1.5 mg/dL (2.5 $\mu\text{mol/L}$) bilirubin, a concentration where the ideal and observed values deviated (see online Supplemental Data, Supplemental Table 1, and Supplemental Fig. 1). The total free bilirubin concentrations used for probe calibrations did not exceed 0.84 mg/dL (1.4 $\mu\text{mol/L}$).

Although BL22P1B11-Rh binds unconjugated bilirubin with high affinity ($K_d = 16 \text{ nmol/L}$), it binds ditauobilirubin, a model for conjugated bilirubin

Table 1. BL22P1B11-Rh K_d values at 3 pH values.^a

	pH		
	7.0	7.4	8.0
n^b	6	16	6
Mean K_d (SD)	16.2 (1.2)	16.6 (1.2)	15.2 (0.9)

^a K_d values were calculated by fitting free bilirubin titration data measured in HEPES buffer at pH 7.0, 7.4, or 8.0.

^b Independent binding isotherms used for determining the mean K_d .

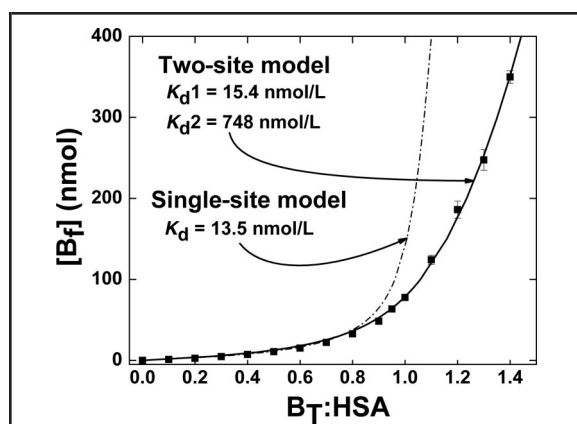


Fig. 3. Bilirubin-HSA binding isotherms.

BL22P1B11-Rh was used to measure B_f in bilirubin-HSA complexes with B_T /HSA from 0.1 to 1.4. Symbols are means and SDs of measurements, and lines are models. Isotherms are with B_T /HSA < 0.9 or B_T /HSA to 1.4 and with HSA 6 $\mu\text{mol/L}$.

(17), with a K_d of 300 nmol/L (see online Supplemental Data and Supplemental Fig. 2) and FFAs with K_d values of > 2500 nmol/L (see online Supplemental Data and Supplemental Table 2). Weak binding of FFAs is important, because FFA_u and B_f are both present in blood at nmol/L concentrations (18, 19). Moreover, evidence from 460-nm irradiation of bilirubin suggests that the probe may be relatively insensitive to photooxidized bilirubin and/or photoisomers of bil-

irubin (see online Supplemental Data and Supplemental Fig. 3).

THE BILIRUBIN-HSA BINDING ISOTHERM

We obtained bilirubin-HSA binding isotherms by using BL22P1B11-Rh to measure the equilibrium values of B_f in bilirubin-HSA complexes. Sets of complexes had constant HSA concentrations but increasing molar ratios of bilirubin to HSA. We measured 13 separate isotherms with HSA = 6 $\mu\text{mol/L}$ and bilirubin:HSA ratios up to 1.4. A single-site model with a $K_d = 14$ (1) nmol/L fit the data well for bilirubin:HSA ratios < 0.8 (Fig. 3). For bilirubin:HSA ratios ≥ 1.0 , however, the measured B_f values were substantially smaller than predicted by the single-site model (Fig. 3). All the binding isotherm data including bilirubin:HSA ratios up to 1.4 were well described with a 2-site model (Eq. 6) comprising a high-affinity site with $K_d = 15.4$ (1) nmol/L and a low-affinity site with $K_d = 748$ (14) nmol/L (Fig. 3).

We used complexes of bilirubin-HSA to assess the imprecision of B_f determined with BL22P1B11 and BL22P1B11-Rh. We carried out 4 measurements for each complex in a series of complexes, with HSA = 24 $\mu\text{mol/L}$, that generated B_f concentrations [0.06–4.7 $\mu\text{g/dL}$ (1–80 nmol/L)] spanning the concentrations expected in jaundiced neonates (1). The CV was smaller for BL22P1B11-Rh, which had mean values of 2.6% and 3.6% for the FluoroLog-3 and B_f meter, than for BL22P1B11, with corresponding values of 8.6% and 7.6% (Table 2). Moreover, the CVs trended smaller for larger B_f .

Table 2. B_f in bilirubin-HSA complexes.^a

B_T :HSA	BL22P1B11-Rh				BL22P1B11			
	FluoroLog		B_f meter		FluoroLog		B_f meter	
	B_f , nmol/L	CV, %	B_f , nmol/L	CV, %	B_f , nmol/L	CV, %	B_f , nmol/L	CV, %
0.10	1.5	9.3	1.7	6.8	1.0	31.1	1.0	29.7
0.20	3.4	2.0	4.0	3.3	3.0	20.7	3.2	11.0
0.30	5.9	2.1	6.8	5.8	5.9	8.7	4.3	6.0
0.40	9.4	1.4	10.4	2.2	9.1	2.9	8.9	5.1
0.50	15.1	1.7	15.4	5.0	13.6	1.3	14.4	4.1
0.60	20.3	0.8	22.0	3.8	21.2	4.7	22.0	3.9
0.70	35.0	1.9	36.9	0.8	26.3	4.1	28.2	1.7
0.80	54.0	2.3	57.4	1.7	47.2	0.7	46.4	3.5
0.90	78.1	2.3	79.9	2.7	79.6	2.8	80.0	3.5

^a Probes were 0.4 $\mu\text{mol/L}$, HSA was 24 $\mu\text{mol/L}$, and total bilirubin increased from 2.4 to 22 $\mu\text{mol/L}$. Complexes were the same for each probe and fluorometer. Measurements were performed in quadruplicate using 10×10 mm cuvettes (2 mL) and glass cuvettes of 5 mm diameter (200 μL) for the FluoroLog and B_f meter, respectively. Multiply by 0.059 to convert nanomoles per liter to micrograms per deciliter.

Table 3. B_f in bilirubin supplemented plasma and HSA complexes.^a

B_T :HSA	Bilirubin-supplemented plasma						Bilirubin-HSA complexes			
	FluoroLog		B_f meter		Peroxidase		FluoroLog		B_f meter	
	B_f	SD	B_f	SD	B_f	SD	B_f	SD	B_f	SD
0	1.8	0.1	1.3	0.1						
0.1	4.5	0.2	3.4	0.4	4.9	2.4	2.2	0.02	2.0	0.1
0.3	10.8	0.02	10	0.1	10.3	0.3	8.5	0.1	7.7	0.1
0.5	22.1	0.3	20.2	0.3	22.1	2.4	17.8	0.1	17.3	0.5
0.7	44.5	1.0	40.7	0.8	43.5	3.9	41.1	0.3	40.0	0.5
0.9	105.9	0.1	102.3	2.8	83.4	5.4	96.9	4.4	91.0	1.4

^a B_f was measured with the peroxidase method and BL22P1B11-Rh, using the FluoroLog 3 and B_f meter. Values in nmol/L are mean and SD for duplicate or triplicate (peroxidase) determinations. HSA concentrations of plasma and bilirubin-HSA complexes were 24 μ mol/L.

B_f IN BILIRUBIN-SUPPLEMENTED ADULT PLASMA

We measured B_f using BL22P1B11-Rh and the peroxidase assay in adult plasma supplemented with bilirubin. Virtually identical results were obtained with BL22P1B11-Rh and the peroxidase method for all supplemented concentrations, except for the 0.9 sample, for which the BL22P1B11-Rh was 25% larger than the peroxidase value (Table 3). We also measured B_f in bilirubin:HSA complexes prepared concurrently with the bilirubin-supplemented plasma samples; they yielded values similar to those of plasma (Table 3).

Discussion

We developed a new method for measuring B_f that uses an extensively mutated FABP conjugated with the fluorescent molecule acrylodan. The performance of this fluorescent probe (BL22P1B11) was enhanced by mixing it with rhodamine B to generate a ratiometric assay system, BL22P1B11-Rh. The ratiometric assay does not require determination of the total bilirubin concentration to correct for inner-filter absorbance (Eq. 1) and is capable of determining B_f concentrations with a mean CV of approximately 3% (Table 2) over the range of concentrations expected in plasma from jaundiced newborns. In addition, inner-filter corrections due to hemoglobin may be required for samples with substantial hemolysis (not in this study) but would be markedly smaller for BL22P1B11-Rh than for BL22P1B11 (see online Supplemental Data).

B_f probes were calibrated by titration with bilirubin in near neutral pH solutions. We observed that formation of the bilirubin-probe complex results in abrogation of the probe's acrylodan fluorescence, presumably through resonance energy transfer due to the overlap of acrylodan emission and bilirubin absorbance spectra. The titrations were well modeled as mo-

nomeric bilirubin binding to a single site on the B_f probes with high affinity [$K_d = 16$ (1.2) nmol/L]. In particular, no spectroscopic evidence was found for bilirubin aggregation in aqueous solutions, with pH values ranging from 7.0 to 8.0 and bilirubin concentrations ranging from 0.006 to about 1.46 mg/dL (0.1–2.5 μ mol/L). Previous studies have reported substantially lower bilirubin solubility levels as well as a substantial dependence of solubility on pH. However, it was also proposed that bilirubin may be supersaturated after preparation but remain soluble long enough to complete the measurements (20). We also observed that high-concentration [0.23 mg/dL (4 μ mol/L)] bilirubin stocks will precipitate over the course of several days but do not show visible signs of precipitation in the first several hours after stock preparation. Although bilirubin dimers have been reported at pH 10 (21), the effect on B_f measurements should not be large given the low affinity of these dimers ($K_d = 1.5$ μ mol/L) compared with the B_f probes and HSA. Further evidence that the bilirubin is initially monomeric and the probe calibrations are accurate is provided by agreement between the B_f probe and peroxidase method results for bilirubin-supplemented plasma (Table 3).

BL22P1B11-Rh-based measurements of B_f in bilirubin-supplemented human plasma were in good agreement with the peroxidase method and with values obtained by use of bilirubin:HSA complexes (Table 3). These results indicate that BL22P1B11-Rh is relatively insensitive to the other metabolites commonly found in adult blood. For example, the B_f probes are not sensitive to FFA_u (see online Supplemental Data and Supplemental Table 2), whose concentrations are normally about 1 nmol/L but in pathologic conditions can increase greatly in adults and neonates (16, 22–24). BL22P1B11-Rh is also largely insensitive to ibuprofen and indomethacin, drugs commonly used to treat ne-

onates (see online Supplemental Data and Supplemental Table 3). Conjugated bilirubin interferes with the determination of B_f with the peroxidase method (13). Although we did not examine the effect of the physiologically important glucuronide-conjugated bilirubin, the relatively weak binding of ditaurobilirubin to BL22P1B11-Rh ($K_d = 300$ nmol/L) (see online Supplemental Fig. 2) compared to unconjugated bilirubin ($K_d = 16$ nmol/L) suggests that the probe may discriminate against other conjugates. Photoisomers may also interfere with B_f measurements, especially during phototherapy (25). Because photoisomers are the predominant product of irradiation at 460 nm (25), the observed decrease in bilirubin concentrations (see online Supplemental Fig. 3) suggests that BL22P1B11-Rh lacks sensitivity to photoisomers. These characteristics, together with the insensitivity of BL22P1B11-Rh to pH over ranges found in patient serum (7.0–8.0), indicate that the probes should provide reliable B_f concentrations for a wide range of serum conditions.

Plasma B_f concentrations depend on the molar ratio of B_T to HSA modulated by metabolites and drugs that may alter bilirubin binding to HSA (23, 26–29). Neonatal HSA concentrations depend on gestational age, ranging from 280 to 470 μ mol/L (30). In neonates receiving Intralipid, FFA concentrations increase severalfold, resulting in substantial displacement of bilirubin from HSA and, therefore, large increases in B_f concentrations (23, 26, 27). This variability in bilirubin-binding capacity can result in patients having similar plasma B_T but different B_f . Moreover, when the bilirubin-binding capacity of HSA is greatly reduced by large bilirubin:HSA ratios and/or displacement by metabolites or drugs, the dilution of patient samples before measurement can reduce B_f concentrations and underestimate B_f . Because the BL22P1B11-Rh probe is relatively insensitive to inner-filter effects, it should be possible to obtain accurate B_f values with smaller sample dilutions than used in the present study.

Potential study limitations include the possibility that bilirubin isomers other than IX- α (25, 31) and/or bilirubin metabolites (32) interfere with B_f measurements. The ditaurobilirubin and bilirubin irradiation experiments suggest that the probes may be relatively insensitive to physiologic bilirubin conjugates and photoisomers (see online Supplemental Data). Given

the preference of FABP proteins for the less-soluble FFAs (15), we speculate that the B_f probe binds bilirubin IX- α with higher affinity than its more soluble derivatives and photoisomers (32). Another limitation is the use of adult plasma supplemented with bilirubin instead of neonatal plasma. Fetal albumin has a faster bilirubin dissociation rate constant than adult albumin (33), although the 2 proteins are indistinguishable (34). The adult–neonate difference in rate constants is still unexplained but might be related to FFA–albumin interactions (33). Neonates have higher plasma FFA concentrations than adults, and this may promote bilirubin dissociation leading to increased B_f (19, 22, 23, 27). Infusion of intralipid may reduce the 375-nm excitation intensity through increased light scattering and might yield erroneously high B_f values by BL22P1B11 but not BL22P1B11-Rh. Hemolysis is more likely in neonates and may interfere with BL22P1B11, but less so with BL22P1B11-Rh measurements (see online Supplemental Data).

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