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Fluorescence Excitation Spectrum of Bilirubin in Blood: A Model for the Action Spectrum for Phototherapy of Neonatal Jaundice†

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Abstract

A recent report (Lamola et al. 2013 *Pediatric Research*, 74, 54–60) presents a semi-empirical model for facile calculation of an action spectrum for bilirubin photochemistry in vivo using the most current knowledge of the optics of neonatal skin. The calculations indicate that competition for phototherapy light by hemoglobin in the skin is the predominant factor that defines the spectrum of light absorbed by bilirubin. If the latter is correct, a valid physical analog of the calculated spectrum is the excitation spectrum of bilirubin in blood. The fluorescence excitation spectrum was recorded and, indeed, found to be very similar to the calculated spectrum. Both spectra exhibit maxima near 476 nm and widths at half height of about 50 nm. This result supports the conclusion that light between 460 and 490 nm is most effective for phototherapy of neonatal jaundice.

INTRODUCTION

Upon irradiation with blue light, unconjugated bilirubin bound to human serum albumin (HSA) undergoes efficient ($\Phi \approx 0.2$) reversible configurational ($Z \rightarrow E$) isomerization, and much less efficient ($\Phi \approx 0.002$) structural isomerization to a ring closed product called lumirubin. Both products are found in infants undergoing phototherapy for neonatal jaundice. There is evidence that the transformation to lumirubin is primarily responsible for the phototherapy effect. [Reviews by Ennever (1) and Maisels and McDonagh (2) provide original references.]

It was recognized very soon after the discovery of sunlight therapy for neonatal jaundice that blue light alone is effective and avoids the ultraviolet component of sunlight (3). A recent review (4) recommends light in the wavelength range 400 to 520 nm and peaked at 450 ± 20 nm. The recommendation of the American Academy of Pediatrics (5) is a more narrow range, 460 to 490 nm. Over the years the preferred light sources have been the so-called “super blue” fluorescent lamps. An example is the blue lamp Philips TL20W/52 (λ_{max} 450 nm) (Philips, Amsterdam, The Netherlands). This source has good spectral overlap with the visible absorption band of bilirubin/HSA. There have been, however, continuous investigations and literature reports concerning the question of the most effective wavelength range for phototherapy with no apparent resolution (2, 6). The advent of light emitting diode (LED) sources, with much more narrow band widths (≈ 25 nm) compared to fluorescent lamps (≈ 50 nm), highlights the need to define the optimum range. Presently phototherapy devices using LEDs have peak intensity near 460 nm, the absorption

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maximum of bilirubin bound to HSA. The rationale for using a light source with maximum intensity near 460 nm is, of course, consistent with the “first law of photochemistry”: in the absence of a photosensitizer, light must be absorbed by the moiety that undergoes unimolecular photochemistry. Yet, a very thorough study (7) demonstrated that a fluorescent source with peak intensity near 490 nm is significantly more effective than one with maximum intensity near 450 nm.

What phenomena may cause the *in vivo* action spectrum to be different from the absorption spectrum of bilirubin/HSA? In the recent literature two factors are most offered: the quantum yields of both the configurational and structural bilirubin photoisomerizations are wavelength dependent (8,9), and higher wavelength light “penetrates deeper into the skin” (for example see reference 7). Considered in the earlier literature but virtually ignored in the recent literature, is the competition for phototherapy light by strongly absorbing substances in the skin, namely hemoglobin and melanin (10, 11). It is well established that hemoglobin is the main absorber of visible light in the skin (12 and references therein) and that the optical density of hemoglobin, in the wavelength range of the main bilirubin absorption band, approaches 2 (99% absorption) within two or three mm below the skin surface. In addition, some light is back scattered out of the skin, and any melanin present would also present a significant competitive absorber of visible light (12, 13).

A recent report (14) presents a semi-empirical model for facile calculation of an action spectrum for bilirubin photochemistry *in vivo* based upon the most current knowledge of human neonatal skin optics, well-defined spectra, and the wavelength dependences of bilirubin photoisomerizations. The calculations indicate that competition for phototherapy light by hemoglobin in the skin is the overwhelming factor that defines the spectrum of the light absorbed by bilirubin. While both skin back scatter and melanin absorption do, of course, reduce the amount of light that would be otherwise absorbed by the bilirubin, their effects vary slowly across the wavelength region of interest and so the spectrum of light absorbed by bilirubin is negligibly altered. Calculated “action spectra” for various bilirubin/hemoglobin ratios and level of melanin were all peaked near 476 nm and were much narrower than the visible absorption band of bilirubin. The spectrum calculated for a specific bilirubin/hemoglobin ratio is shown in Figure 1. Inclusions of the wavelength dependences of the bilirubin photoisomerizations minimally alter the spectrum.

The calculations predict that the hemoglobin level in the infant’s blood should significantly affect phototherapy efficacy with higher hemoglobin content reducing efficacy. In addition the calculations predict that a narrow-band source, such as LEDs, peaked at 476 nm would be the most efficient for phototherapy, at the same time reducing the heat burden of the infant by avoiding absorption of useless light. Previous observations that light in a range of wavelengths longer than the maximum of the *in vitro* bilirubin absorption peak is more effective for phototherapy (6) are supported by the calculated action spectra. In fact, the calculations quantitatively predict the results of an exceptionally careful clinical study that showed that, for equal irradiance, a fluorescent light source peaked near 490 nm is significantly more effective than one peaked near 450 nm (7).

If the competition for phototherapy light by hemoglobin is, indeed, the overriding factor in defining the spectrum of light absorbed by bilirubin, an experimental model that corresponds to the semi-empirical calculation is the excitation spectrum for the fluorescence of bilirubin in blood. We report this excitation spectrum here.

MATERIALS AND METHODS

Excitation spectra of bilirubin in blood were recorded using a Horiba Jobin-Yvon Spex Fluorolog-3 fluorimeter run in the front-face mode because of the extremely high absorbance of the sample. The sample was contained in a 1 mm pathlength cuvette. The excitation slit was set at 5 nm and the emission slit was set at either 5 nm or 10 nm. Emission spectra of the sample excited at 460 nm were recorded to verify that the emission detected was indeed that of bilirubin. With the emission wavelength set at 530 nm, close to the maximum of fluorescence from bilirubin/HSA, excitation spectra were recorded from 400 to 510 nm. The scan time was sufficiently short and excitation light intensity sufficiently low such that negligible differences were observed in a repeat scan. However, to avoid changes due to photochemistry the sample was well mixed between scans. The temperature was ambient, near 23°C.

The sample utilized was an unused portion of a preparation made for the calibration of a bilirubin hematofluorometer (15) being used for another study at Stanford University School of Medicine under a protocol approved by the Institutional Review Board. The sample was prepared as follows. Blood was obtained by venipuncture from a healthy adult volunteer with informed consent. Red cells and serum were separated by centrifugation (buffy coat removed). The appropriate volume of serum was added to a small tube containing the disodium salt of bilirubin, prepared as described elsewhere (15), to give a target concentration of serum bilirubin concentration near 15 mg/dl, and mixed well. A volume of red cells was added to give a target hematocrit near 50% and the reconstituted blood was gently mixed and aerated for twenty minutes to achieve full oxygenation. The actual bilirubin concentration in the reconstituted blood specimen was 0.147 mmol/l (corresponding to 17.8 mg/dl serum) as measured using a hematofluorometer and the hemoglobin level, 2.44 mmol/l (hematocrit $\approx$ 48%), was determined using a HemoCue Hb 201⁺ hemoglobinometer (HemoCue, Angelholm, Sweden). A sample without added bilirubin served to define a baseline. The small amount of bilirubin, about 0.4 mg/dl, in the latter sample was ignored.

RESULTS

A fluorescence excitation spectrum obtained with the 5 nm slit width setting is shown in Figure 2 after subtracting the signal obtained from a blood sample without added bilirubin (not shown). Repeat spectra were closely similar as were spectra run with the 10 nm slit width setting. No correction was made for any wavelength dependent variation in fluorescence polarization.

The excitation spectra, as exemplified by the spectrum of Figure 2, are not very different from the action spectrum for bilirubin absorption of Figure 1. In particular the maxima of all the excitation spectra were between 474 nm and 480 nm, and the spectral widths at half height were similar, about 50 nm.

DISCUSSION

The fluorescence of bilirubin at clinically significant concentrations in whole blood excited with blue light at room temperature is extremely weak due to the high absorbance of the exciting light by hemoglobin and the low bilirubin fluorescence yield, $\approx$ 0.001 (15). Consequently the fluorescence excitation spectrum is expected to be somewhat noisy. It is assumed that the excitation spectrum represents the great majority of the bilirubin. That is, that almost all the bilirubin experiences a similar environment and there are not significant fractions of the bilirubin that are significantly different in fluorescent properties. At the

concentration of bilirubin employed, almost all the bilirubin should be bound to HSA in the specific binding site (15). We assume, therefore, that the fluorescence signals from the samples examined here reasonably reflect the probability of absorption of exciting light by the bilirubin in the sample. Taking this to the next step, the fluorescence excitation spectrum should then also reflect the spectrum of the initial rate of photochemistry and, consequentially, the initial rate of reduction of serum bilirubin during phototherapy (the clinical action spectrum) assuming validity of the thesis that competition for phototherapy light by hemoglobin is the dominant factor for defining the therapy action spectrum. While it would have been surprising if the fluorescence excitation spectra differed significantly from the calculated spectrum for light absorption by bilirubin, it is assuring that there is close agreement between the spectra. These results support the conclusion (11, 14) that light between 460 and 490 nm is most effective for phototherapy of neonatal jaundice and that light outside this range is primarily absorbed by hemoglobin and uselessly heats the infant.

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Figure 1.

The calculated relative fraction of light absorbed by bilirubin in a blood sample containing 2.44 mmol/l hemoglobin and 0.147 mmol/l bilirubin according to the method of reference 14. The calculated spectrum is normalized to 1.0 at the maximum.

Figure 2.

Fluorescence excitation spectrum (set to 1.0 at the maximum) of a blood specimen containing 2.44 mmol/l hemoglobin (hematocrit $\approx$ 48%) and 0.147 mmol/l bilirubin (17.8 mg/dl referenced to the serum volume). The emission wavelength was 530 nm and the slit widths setting was 5 nm.