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Note

Determination of the relative detector response for unstable bilirubin photoproducts without isolation

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Calibration factors or relative response ratios (RR) are an integral part of any quantitative chromatographic analysis. For each component, the relative response ratio is the response of the detector relative to an internal standard or a particular component whose absolute response is known [1]. For some detectors (e.g., refractive index detectors) the variation in RR is not large and the RR for each component can be assumed to be unity without introducing a significant error in the analysis. For UV—visible absorption detectors, the relative response ratios are a function of the extinction coefficients of the components, and these may vary widely. Therefore, quantitative analysis of mixtures using UV—visible detection requires knowledge of the RR for each component. In practice, this requires the analysis of a sample of known composition, most commonly prepared from solutions of pure components. However, if the components cannot be isolated or purified, this approach is not possible. We report here the determination of the RR for the photoproducts of 4*Z*,15*Z*-bilirubin IXα without isolating the unstable components.

4*Z*,15*Z*-Bilirubin IXα (ZZ) undergoes two types of photoisomerization reactions (Fig. 1). One is a *Z*-to-*E* configurational isomerization at either the 4 or 15 position, to yield the isomers 4*Z*,15*E*-bilirubin (ZE) and 4*Z*,15*E*-bilirubin (EZ; not shown in Fig. 1). The other reaction of 4*Z*,15*Z*-bilirubin is an intramolecular cyclization reaction involving the endo vinyl group on carbon 3 forming a structural isomer of bilirubin, named lumirubin (LR) [2, 3]. These products are formed in vivo in hyperbilirubinemic infants treated with phototherapy. Because much of our work requires quantitative measurement of these isomers in clinical samples, we needed to determine the RR for

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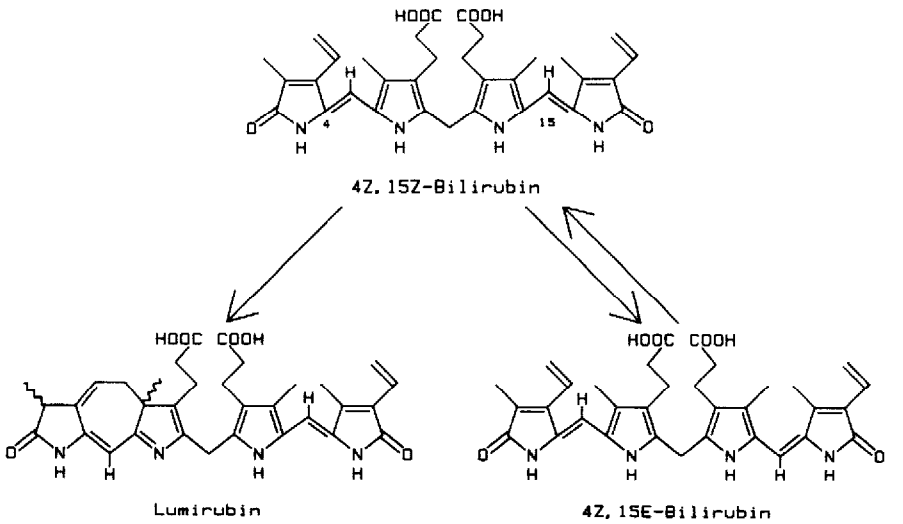

Fig. 1. Photoisomerization reactions of bilirubin IXα.

each bilirubin species. However, the *E* isomers (EZ and ZE) are unstable and revert to the native ZZ photochemically, thermally, or in the presence of acid, and therefore cannot be readily isolated or purified. Nevertheless, two factors allowed us to determine the necessary response ratios. One is the highly regioselective formation of ZE (with little or no EZ formation) when ZZ bound to human serum albumin is irradiated [4]. The second is that the rate of formation of ZE is much faster than the rate of formation of LR, so that one can obtain a mixture containing ZZ and ZE without a significant amount of LR.

EXPERIMENTAL

A Varian chromatograph (Model 5060, Sunnyvale, CA, U.S.A.) fitted with a Rheodyne injector (Model 7126, Cotati, CA, U.S.A.) was used with a diode-array detector (Hewlett-Packard Model 1040A, Palo Alto, CA, U.S.A.) equipped with a DPU multichannel integrator and a Hewlett-Packard 85B computer. The analysis was performed using an isocratic, reversed-phase ion-pair system with 0.1 *M* di-*n*-octylamine acetate in methanol (pH 7.6) as the eluent at a flow-rate of 0.7 ml/min through a C_{18} column (Zorbax-ODS column, 250 × 4.6 mm, 5 μm particle size, Dupont, Wilmington, DE, U.S.A.) fitted with a 0.5-μm in-line filter and a 40 × 4 mm guard column [5]. The absorbance of the effluent from the column was monitored at 450 nm. A typical chromatogram of an irradiated sample of ZZ bound to human albumin is shown in Fig. 2.

All work was done under photographic safe lights. 4*Z*,15*Z*-Bilirubin IXα (Sigma, St. Louis, MO, U.S.A.), purified as described by McDonagh and Assisi [6], was dissolved (3 m*M*) in freshly prepared 0.1 *M* sodium hydroxide. Human serum albumin (Sigma, fatty acid-free) was dissolved in 0.05 *M* 4-(2-hydroxyethyl)-1-piperazineethane sulfonate (HEPES) buffer (0.15 *M* sodium chloride, 1 m*M* Na_2EDTA, pH 7.4) degassed with argon and 1 ml of the 3 m*M* solution of ZZ in sodium hydroxide was added to make a final volume of 100 ml.

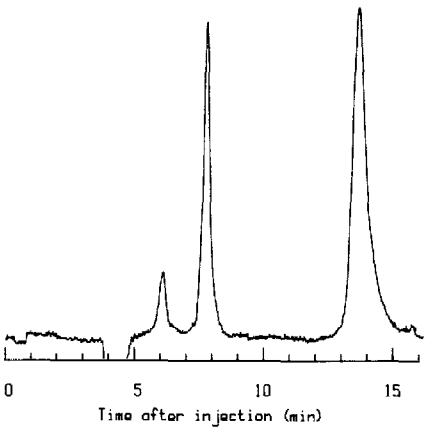

Fig. 2. HPLC analysis of a sample of bilirubin bound to human serum albumin irradiated for 30 min in vitro. Detection at 450 nm. Peak assignments: lumirubin, retention time (t_R) = 6.1 min; 4*Z*,15*E*-bilirubin, t_R = 7.8 min; 4*Z*,15*Z*-bilirubin, t_R = 13.7 min.

This solution, containing 30 μM ZZ and 60 μM albumin, was used as the stock solution.

Light from a 200-W high-pressure Hg lamp was passed through an interference filter (445 nm, half bandwidth 35 nm) and attenuated with a neutral-density filter (1.0 A.U.) to a fluence of approximately $6 \cdot 10^{-5}$ W/cm^2. Cuvettes containing samples of the stock solution were irradiated with stirring for 30, 60, 120 and 240 s. These samples contained increasing amounts of ZE with no detectable LR or EZ (lower limit of detectability $< 0.5\%$). Samples of the same stock solution were irradiated for longer periods (from 10 to 60 min) to produce solutions containing LR as well as ZE. Samples were injected directly into the high-performance liquid chromatographic (HPLC) system. Multiple HPLC assays, with sample injection employing the full-injection technique (20-μl loop) for quantitative reproducibility [7], were used to analyze the unirradiated and irradiated samples. Mean values of integrated peak areas for multiple analyses of the same sample (coefficient of variation $\leqslant 1.2\%$) were used for subsequent calculations.

RESULTS AND DISCUSSION

Chromatographic analysis of the briefly irradiated samples revealed that with increasing amount of ZE present there was a progressive decrease in the uncorrected sum of the integrated peak areas (ZZ plus ZE). Because ZE is produced from ZZ, this decrease in total integrated peak areas in the samples containing both ZE and ZZ from that measured for the sample containing only ZZ (the unirradiated sample) must be the result of a lower molar absorptivitiy at 450 nm for ZE. For each irradiated sample, we calculated a correction factor, which when multiplied times the peak area for ZE would result the total integrated area being unchanged from that measured for the unirradiated sample. For the four samples, this factor ranged from 1.39 to 1.47 with a mean of 1.43. The inverse of this factor, 0.70, is the relative response ratio of the detector to ZE (relative to ZZ). For convenience, this is abbreviated as RR_{ZE}^{450}, where the superscript represents the wavelength and the subscript represents the component.

A similar experiment was carried out with longer irradiation times to allow for formation of LR. These samples were then analyzed as above. With the previously determined RR for the ZE isomer, we were able to calculate that $RR_{LR}^{450} = 0.43$.

The equimolar absorbance spectra for ZZ, ZE and LR in the HPLC solvent are shown in Fig. 3. These spectra were calculated from digitized spectral data on each component obtained during chromatography using the diode-array detector. The spectral data from the detector were corrected to give equimolar spectra using the relative response ratios of ZE and LR at 450 nm. The relative response ratio for ZE and LR at any wavelength can be calculated from these spectra. For the greatest dynamic range of the system of analysis, the optimal wavelength of detection is the λ_{max} of the component present in the smallest concentration, or the λ_{max} of the component to which the detector is least sensitive. Choice of the optimal wavelength is especially important when the component with the smallest absorption is also the component present in the least amount, as is true in this case for LR. Thus, for this solvent system, the λ_{max} of LR, 437 nm, would be the optimal wavelength for detection. The necessary response ratios can be calculated from the spectral data in Fig. 3 ($RR_{LR}^{437} = 0.50$; $RR_{ZE}^{437} = 0.75$).

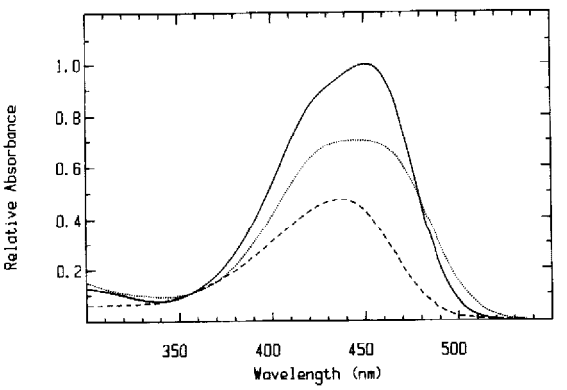

Fig. 3. Equimolar absorption spectra of 4*Z*,15*Z*-bilirubin (——), 4*Z*,15*E*-bilirubin (· · · ·), and lumirubin (— — — —) in HPLC solvent. Absorbance of 4*Z*,15*Z*-bilirubin at 450 nm set to 1.0.

The results presented in this paper illustrate that relative response ratios can be determined and employed in quantitative applications without isolation or purification of unstable components. Although the approach used here to determine RR is not universally applicable, the underlying principle may find use in similar systems, i.e., where the components that need to be quantified have a precursor—product relationship.

ACKNOWLEDGEMENTS

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