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Accurate and sensitive high-performance liquid chromatographic method for geometrical and structural photoisomers of bilirubin IXα using the relative molar absorptivity values

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Abstract

It has been reported that considerable differences exist between the relative molar absorptivity values of the geometrical and structural photoisomers of bilirubin. We have devised an accurate HPLC method for photoisomer quantification based on the following principle: the sum of both the integrated peak areas corrected by each factor for each photoisomer, and the integrated peak area of unchanged (ZZ)-bilirubin [(ZZ)-B] after an anaerobic photoirradiation, should be constant and equal to the integrated peak area of initial (ZZ)-bilirubin [(ZZ)-Bi] before photoirradiation. On this basis, the following equation can be used to determine each factor.

$$[(ZZ)\text{-Bi}] = \frac{[(ZE)\text{-B}]}{\alpha} + \frac{[(EZ)\text{-B}]}{\beta} + \frac{[(EZ)\text{-CB}]}{\gamma} + \frac{[(EE)\text{-CB}]}{\delta} + [(ZZ)\text{-B}]$$

α, β, γ and δ represent the factors used to correct the integrated peak areas of individual bilirubin photoisomers, and they are arranged in the order of the formula. It was demonstrated that the relative 455 nm molar absorptivity values for (ZZ)-bilirubin and all its geometrical and structural photoisomers, i.e., (ZZ)-bilirubin, (ZE)-bilirubin (EZ)-bilirubin, (EZ)-cyclobilirubin (=lumirubin) and (EE)-cyclobilirubin in the HPLC eluent, are, respectively, 1.0, 0.81 ($=\alpha$), 0.54 ($=\beta$), 0.47 ($=\gamma$) and 0.39 ($=\delta$). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Geometrical isomers; Structural isomers; Bilirubins

1. Introduction

Phototherapy for neonatal hyperbilirubinemia is an established procedure in neonatal medicine. Currently it has been believed that the principal mechanism for the removal of bilirubin from the body in the course of phototherapy consists of both configurational ((EE)-/(ZE)-/(EZ)-bilirubin) and structural photoisomerization ((EE)-/(EZ)-cyclobilirubin (E-/Z-lumirubin)) (Fig. 1) [1]. The bilirubin photoisomers were studied by Onishi et al. [2] utilizing a gradient of acetonitrile in phosphate containing tetrabutylammonium hydroxide on a Shimadzu PCH column. McDonagh et al. [3] were able to separate the configurational photoisomers on a C_{18} column with 0.1 *M* octylamine in methanol as solvent. However, both HPLC methods are insufficient for the separation of (ZZ)-bilirubin (IIIα, IXα, XIIIα)

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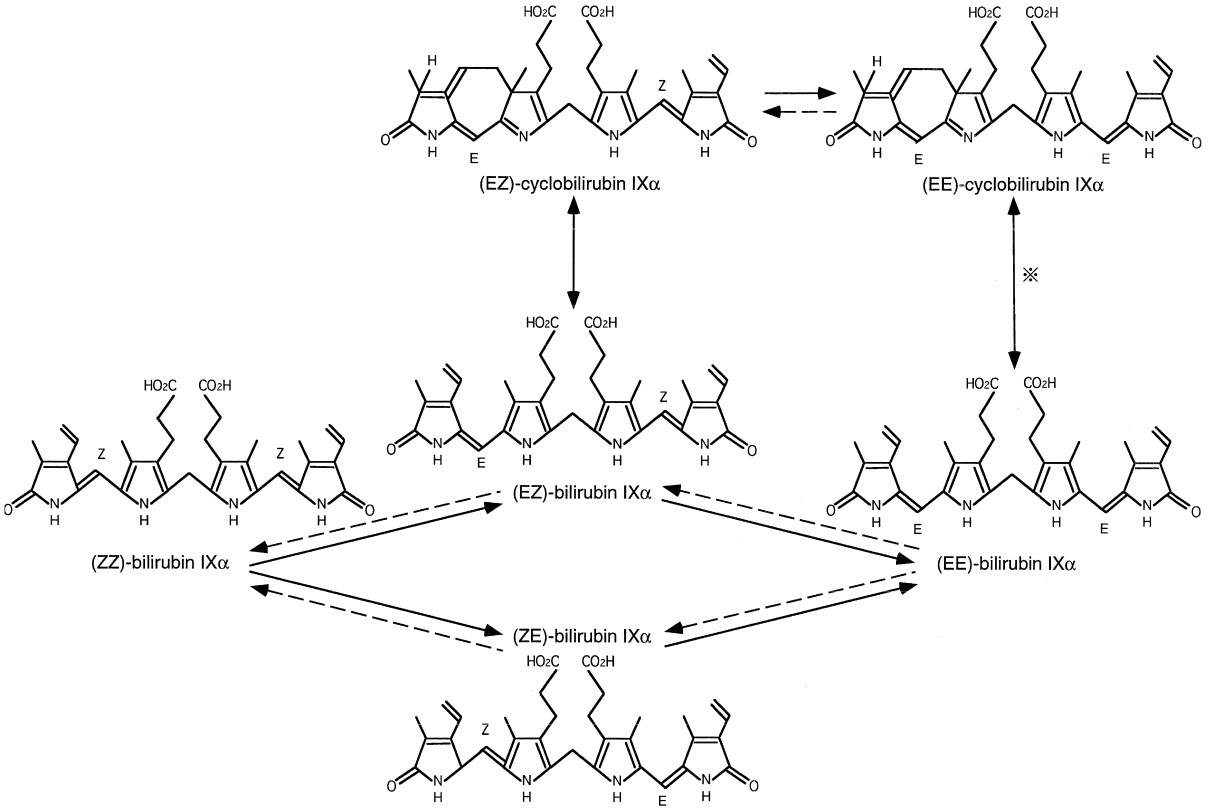

Fig. 1. Interrelationship of bilirubin and its photoisomers by kinetic study of the photochemical interaction (from Onishi et al. [1]) ←---- photochemical and thermal conversion; ← photochemical conversion only; theoretical pathway, but not confirmed. Steric configurations of the hydrogen atom and methyl group at C-2 and the methyl group at C-7 of (EZ)- and (EE)-cyclobilirubin are not shown.

and the photoisomers, the stability of these photoisomers and the quantitative analysis. Although these photoisomers were found to have different molar absorptivities compared with (ZZ)-bilirubin, each peak level was calculated integrated area or concentration with the assumption that molar absorptivity values of all photoisomers on HPLC scan were the same as that of (ZZ)-bilirubin. In practice, this requires the analysis of a sample of known composition, most commonly prepared from solutions of pure components. However, if these photoisomers cannot be isolated or purified, this approach is not possible. The considerable differences between the relative molar absorptivity values of (ZZ)-bilirubin, (ZE)-bilirubin and (EZ)-cyclobilirubin (lumirubin) which were used to correct the integrated peak area on the chromatograms, were calculated from absorption spectra of the various isomers which were normalized using a diode-array detector and the photochemical changes of bilirubin [4]. However, the values did not always agree [5]. It has become clear from the results to date that when (ZZ)-bilirubin was exposed to light sources in phototherapy [6], the production ratio of the bilirubin photoisomers was determined by these light sources [7], the exposure time [8], bilirubin solutions [9], and bilirubin binding protein [10]. The HPLC separation in the present report is more accurate and more efficient than the previously reported method [3,5].

2. Experimental

2.1. Chemicals

Bilirubin (Tokyokasei, Tokyo, Japan) was used

without further purification. Albumin (human) was obtained from Sigma (St. Louis, MO, USA, No. A-1887, Biochemicals, organic compounds and diagnostic reagents, 1996). Acetonitrile HPLC grade, *N*,*N*-dimethylformamide (JIS), sodium phosphate dibasic 12-water, sodium phosphate monobasic dihydrate, and phosphoric acid were purchased from Wako (Osaka, Japan), and dimethylsulfoxide from Dojin (Tokyo, Japan).

2.2. Instrumentation, chromatographic columns and supplies

A Shimadzu LC-3AFG liquid chromatograph (Kyoto, Japan) with an SPD-6AV detector and Chromatopac C-R1A was used for all HPLC procedures in combination with a radial compression unit (Waters RCM 8×10 Module, Waters Assoc., Milford, MA, USA). Separations were performed using a Nova-Pak C_{18}, 5-mm, radial compression cartridge (Waters C_{18}, Waters Assoc.).

2.3. Chromatographic conditions

A gradient elution technique was used for HPLC with acetonitrile–0.01 *M* sodium phosphate buffer (pH 5.5)–dimethyl formamide (50:300:650, v/v) as the primary eluent and acetonitrile–0.01 *M* sodium phosphate buffer (pH 5.5)–dimethylformamide (200:150:650, v/v) as the secondary eluent. The final pH of both primary and secondary eluents was adjusted to 5.5±0.05 with orthophosphoric acid. The best separation was achieved by using a linear gradient of 0–34% (v/v) for 17 min, and 34–100% (v/v) for 8 min at a flow-rate of 1.0 ml/min.

2.4. Stability of (ZZ)-bilirubin and photoisomers in HPLC eluents

(ZZ)-Bilirubin and the photoisomers were collected from a fraction separated by the HPLC. The collected fractions were kept in the dark at room temperature (20°C). The integrated peak area of each fraction was measured at 0, 10, 20, and 30 min using the HPLC. We performed three experiments, and the mean of each integrated peak area of (ZZ)-bilirubin and the photoisomers was applied to them.

2.5. Sample preparation for HPLC

Only for the human serum albumin–bilirubin solution, sample preparation was performed as follows: Human serum albumin–bilirubin solution–dimethylsulfoxide–acetonitrile (1:1:1, v/v) were vortex-mixed for 10 s and then centrifuged for 5 min at 1000 *g*. A portion (25 μl) of the supernatant was injected into the chromatograph.

2.6. Photochemical experiments

Except where noted otherwise, all the bilirubin aqueous, organic and protein matrix solutions were deoxygenated with pure (99.99%) N_2 before photoirradiation. Several 1.0 ml portions of the solution in Pyrex tubes were exposed to light. The light sources used for blue–white light and green light were four 20 W Toshiba, FL20BW and four 20 W National, BG160, lamps, respectively. At different periods of light exposure, the tubes were successively transferred into the dark.

Each portion was analysed by HPLC. Except for photochemical experiments, all manipulations were carried out in the dark or with a photographic safe light and at room temperature.

2.7. Calculation of correction factors

Under in vitro experimental conditions such as anaerobic photoirradiation, the photochemical changes other than geometrical and structural photoisomerization of (ZZ)-bilirubin, i.e., photooxidation, were essentially negligible. For this reason, the sum of both the integrated peak area corrected by each factor of each photoisomer and the integrated peak area of unchanged (ZZ)-bilirubin after an anaerobic photoirradiation should be constant and equal to the integrated peak area of initial (ZZ)-bilirubin before photoirradiation. On this basis, the following equation can be used to determine each factor.

$$[(ZZ)\text{-Bi}] = \frac{[(ZE)\text{-B}]}{\alpha} + \frac{[(EZ)\text{-B}]}{\beta} + \frac{[(EZ)\text{-CB}]}{\gamma} + \frac{[(EE)\text{-CB}]}{\delta} + [(ZZ)\text{-B}]$$

[(ZZ)-Bi] represents the integrated peak area of the

initial (ZZ)-bilirubin before photoirradiation; [(ZZ)-B] represents the integrated peak area of the unreacted residual (ZZ)-bilirubin after photoirradiation; [(ZE)-B] and [(EZ)-B] represent the integrated peak areas of the (ZE)-bilirubin and (EZ)-bilirubin, respectively, after photoirradiation; [(EZ)-CB] represents the integrated peak area of the (EZ)-cyclobilirubin after photoirradiation; [(EE)-CB] represents the integrated peak area of the (EE)-cyclobilirubin after photoirradiation; α and β represent the factors used to correct the integrated peak area of (ZE)- and (EZ)-bilirubin on the chromatogram, respectively; γ represents the factors used to correct the integrated peak area of (EZ)-cyclobilirubin on the chromatogram; δ represents the factor used to correct the integrated peak area of (EE)-cyclobilirubin on the chromatogram.

We performed three experiments, and the mean of each integrated peak area of (ZZ)-bilirubin and the photoisomers was applied to them.

3. Results

3.1. Optimization of chromatographic condition

(ZZ)-Bilirubin showed a linear relation between the peak area in the chromatogram and the amount of pigment injected for the range of concentrations tested, which was 0–200 mg/l. The sensitivity was 0.3 ng and the RSD was 2.2% on the five measurements. We confirmed that no significant change of (ZE)-bilirubin IIIα, IXα and XIIIα dissolved in the primary eluent occurred for 20 min at room temperature (20°C).

(ZE)[=(EZ)]-Bilirubin IIIα was eluted between (ZE)-bilirubin IXα and (EZ)-bilirubin IXα. (EZ)[=(ZE)]-cyclobilirubin XIIIα was eluted between (EE)-cyclobilirubin IXα and (EZ)-cyclobilirubin IXα. Therefore, no overlapping of peaks was observed among the photoisomers of (ZZ)-bilirubin IIIα, (ZZ)-bilirubin IXα and (ZZ)-bilirubin XIIIα (Fig. 2).

Moreover, the amounts of these geometrical and structural photoisomers derived from (ZZ)-bilirubin IIIα and (ZZ)-bilirubin XIIIα, respectively, were undetectable under the experimental conditions.

3.2. Peak assignments of bilirubin and photoisomers

(ZZ)-Bilirubin IIIα, IXα and XIIIα was obtained by HPLC of the ethyl anthranilate azopigments [11]. (ZE)-Bilirubin and (EZ)-bilirubin (IXα and IIIα) was achieved by thermal and photochemical reversion of each geometrical photoisomer to (ZZ)-bilirubin after collection of each peak [12,13], and the different production of the geometrical photoisomers when (ZZ)-bilirubin in organic solutions or human serum albumin–bilirubin complex was irradiated during 10–20 s by blue–white light [10]. (EZ)-Cyclobilirubin was identified by ^{1}H-NMR spectra and ^{13}C-NMR [13]. (EE)-Cyclobilirubin was obtained by thermal and photochemical reversion of the structural photoisomer to (EZ)-cyclobilirubin. (EZ)[(ZE)]-cyclobilirubin XIIIα was proved by photochemical change during the irradiation of blue–white light [14].

3.3. Stability of (ZZ)-bilirubin and photoisomers in HPLC eluents (Fig. 3)

(ZZ)- and (EZ)-bilirubin were stable at 30 min. (ZE)-Bilirubin was decreased at 2%/10 min. (EZ)-Cyclobilirubin and (EE)-cyclobilirubin were decreased at 10% and 15%/10 min, respectively.

3.3.1. Calculation of factor α

In the control experiments with a non-irradiated bilirubin (100 mg/l) in human serum albumin (20 g/l), each sample was analysed by HPLC, and the very large peak (94.4%) of (ZZ)-bilirubin IXα was separated in addition to the small (ZZ)-bilirubin IIIα (2.8%) and (ZZ)-bilirubin XIIIα (2.9%) peaks. Analysis by HPLC of the (ZZ)-bilirubin solution irradiated by blue light for 30 s under anaerobic conditions revealed that the peak of (ZZ)-bilirubin decreased, and the main significant peak of (ZE)-bilirubin appeared (Fig. 4). The sum of the integrated peak areas of (ZE)-bilirubin corrected by an unknown factor α and the integrated peak area of unchanged (ZZ)-bilirubin [(ZZ)-B] after 30 s photoirradiation was assumed to be equal to the integrated peak area of the initial (ZZ)-bilirubin [(ZZ)-Bi] before photoirradiation.

In the following experiment, we measured the

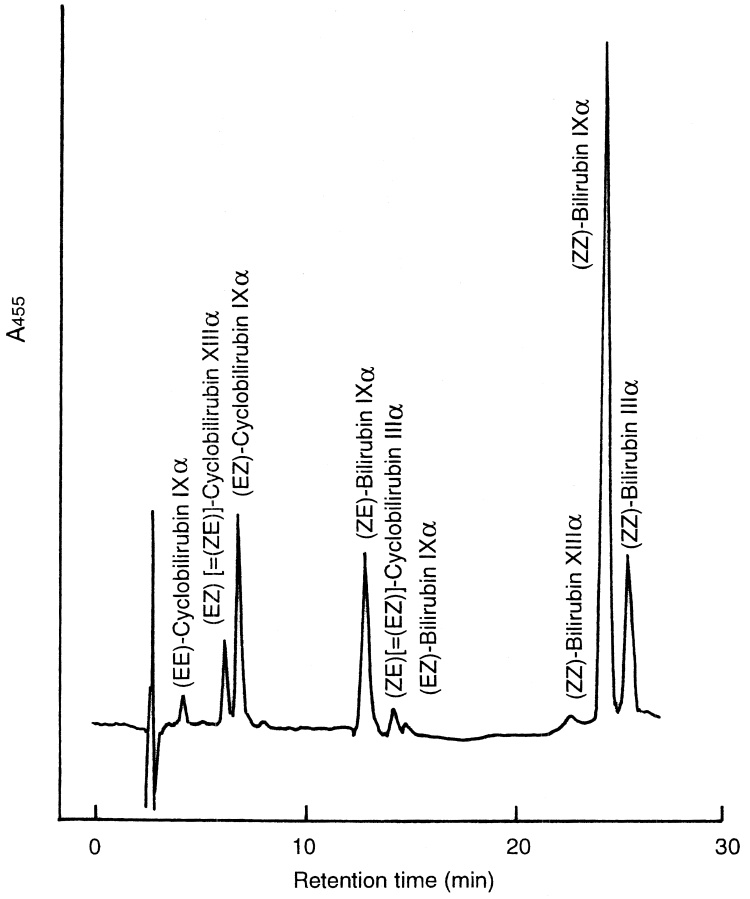

Fig. 2. HPLC scans of (ZZ)-bilirubin human serum albumin solution after photoirradiation by blue light for 10 min under anaerobic conditions. Analysis by HPLC of the (ZZ)-bilirubin containing rich -XIIIα (10%) and -IIIα (11%) irradiated by blue light for 10 min under anaerobic conditions showed that the peaks of (ZZ)-bilirubin IIIα, IXα, XIIIα and its photoisomers were well separated.

mean area from three HPLC analyses at 455 nm. Each RSD from the respective three integrated areas of (ZZ)-bilirubin and its photoisomers was less than 5%.

$$[(ZZ)\text{-Bi}] = \frac{[(ZE)\text{-B}]}{\alpha} + [(ZZ)\text{-B}]$$

mean [(ZZ)-Bi]=325 652, mean [(ZE)-B]=68 859, mean [(ZZ)-B]=240 910. Hence $\alpha = 0.81$.

3.3.2. Calculation of factor β

In the control experiments with a non-irradiated (ZZ)-bilirubin dimethylsulfoxide solution (100 mg/l), each sample was measured by HPLC, and the very large peak (94.1%) of (ZZ)-bilirubin IXα was separated in addition to small (ZZ)-bilirubin IIIα (3.0%) and (ZZ)-bilirubin XIIIα (2.9%) peaks. β was calculated by the same procedure used to estimate factor α. On photoirradiation of the (ZZ)-bilirubin dimethylsulfoxide solution, the peak of (ZZ)-bilirubin decreased and only two significant peaks of (ZE)-bilirubins and (EZ)-bilirubin appeared. The sum of both integrated peak areas of (ZE)- and (EZ)-bilirubin corrected by the factor α and unknown factor β, and the integrated peak area of unchanged (ZZ)-bilirubin [(ZZ)-B] after 30 s photoirradiation, should be equal to the integrated

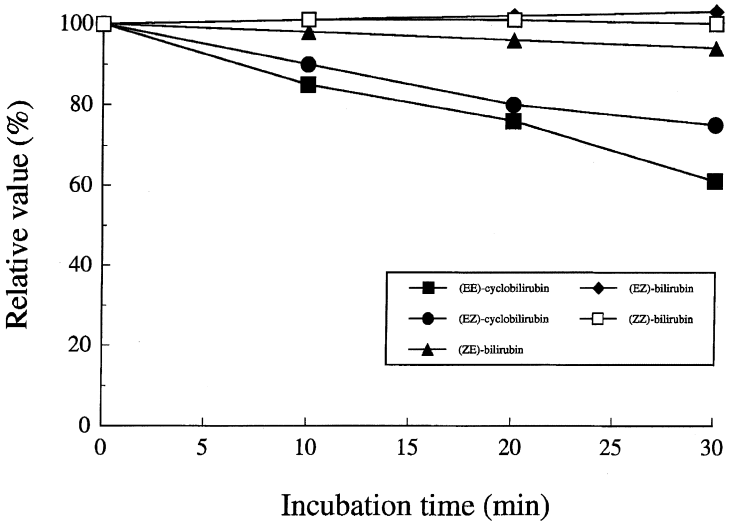

Fig. 3. Stability of (ZZ)-bilirubin and the photoisomers in HPLC eluents.

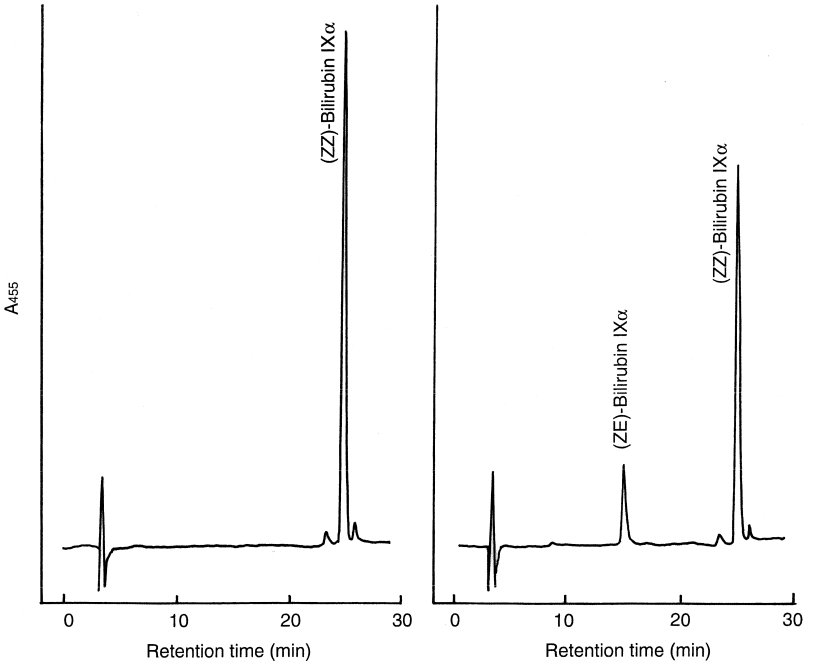

Fig. 4. HPLC scans of (ZZ)-bilirubin human serum albumin solution before and after photoirradiation by blue light for 30 s under anaerobic conditions. In the control experiments with a non-irradiated bilirubin in human serum albumin, the large peak of (ZZ)-bilirubin IXα was separated. Analysis by HPLC of the (ZZ)-bilirubin solution irradiated by blue light for 30 s under anaerobic conditions showed that the peak of (ZZ)-bilirubin IXα decreased, and one significant peak of (ZE)-bilirubin IXα and a very small peak of (EZ)-cyclobilirubin appeared.

peak area of the initial (ZZ)-bilirubin [(ZZ)-Bi] before photoirradiation

$$[(ZZ)\text{-Bi}] = \frac{[(ZE)\text{-B}]}{\alpha} + \frac{[(EZ)\text{-B}]}{\beta} + [(ZZ)\text{-Bi}]$$

mean [(ZZ)-Bi]=1 072 764, mean [(ZZ)-B]= 897 279, mean [(ZE)-B]=77 348 mean [(EZ)-B]= 42 600. Hence, $\alpha=0.81$ and $\beta=0.54$.

3.3.3. Calculation of factors δ/γ ratio

After the peak of (EZ)-cyclobilirubin was collected, the sample (50 μl) was injected into the HPLC, and only the peak of (EZ)-cyclobilirubin was separated. The (EZ)-cyclobilirubin in the HPLC eluent by blue light for 10 s under anaerobic conditions showed that the peak decreased abruptly and the (EE)-cyclobilirubin peak appeared at the same time (Fig. 5).

The sum of the integrated peak areas of (EZ)-cyclobilirubin and (EE)-cyclobilirubin, corrected by unknown factors γ and δ, respectively, after 10 s photoirradiation, should be equal to the integrated peak area of the initial (EZ)-cyclobilirubin [(EZ)-CBi] corrected by unknown factor γ before photoirradiation:

$$\frac{[(EZ)\text{-CBi}]}{\gamma} = \frac{[(EZ)\text{-CB}]}{\gamma} + \frac{[(EE)\text{-CB}]}{\delta}$$

mean [(EZ)-CBi]=106 817, mean [(EZ)-CB]= 65 060, mean [(EE)-CB]=34 969. Hence, $\delta/\gamma=$ 0.84.

3.3.4. Calculation of factors δ and ε

The experimental calculating factors δ and ε were performed using the same solution as factor α. Green light was used for this cyclization experiment, because in the photochemical reaction by green light, the cyclization is the most predominant reaction [7,15]. When photoirradiation by green light was applied for 15 min, the peak of the (ZZ)-bilirubin decreased, and the peaks of (ZE)-bilirubin, (EZ)-cyclobilirubin and (EE)-cyclobilirubin appeared (Fig. 6). In the same manner as before, the following equation was formulated:

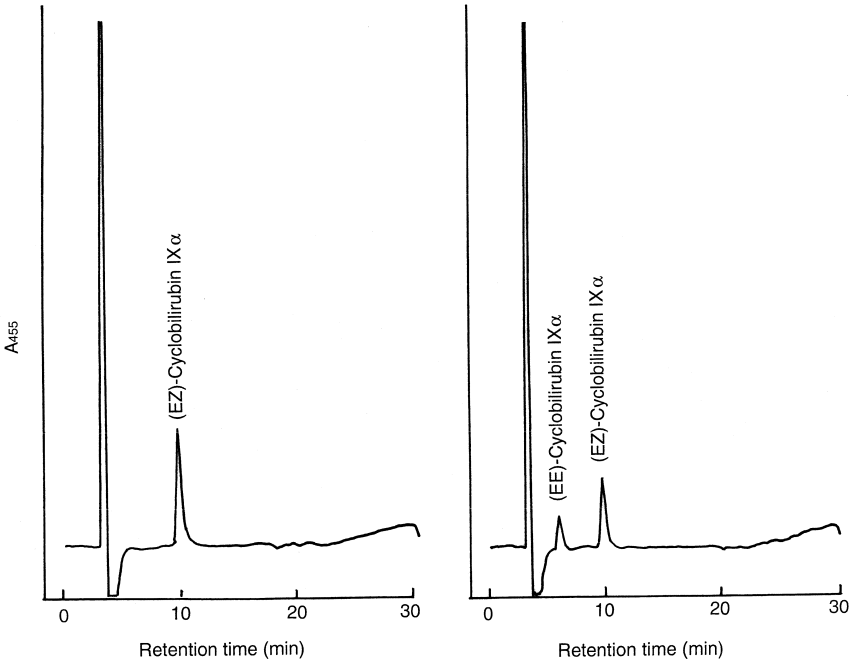

Fig. 5. HPLC scans of (EZ)-cyclobilirubin IXα solution before and after photoirradiation by blue light for 10 s under anaerobic conditions. In the control experiments with an (EZ)-cyclobilirubin IXα solution, only the peak of (EZ)-cyclobilirubin was separated. Analysis by HPLC of the (EZ)-cyclobilirubin IXα solution irradiated by blue light for 10 s under anaerobic conditions showed that the peak decreased abruptly and a peak of (EE)-cyclobilirubin IXα appeared at the same time.

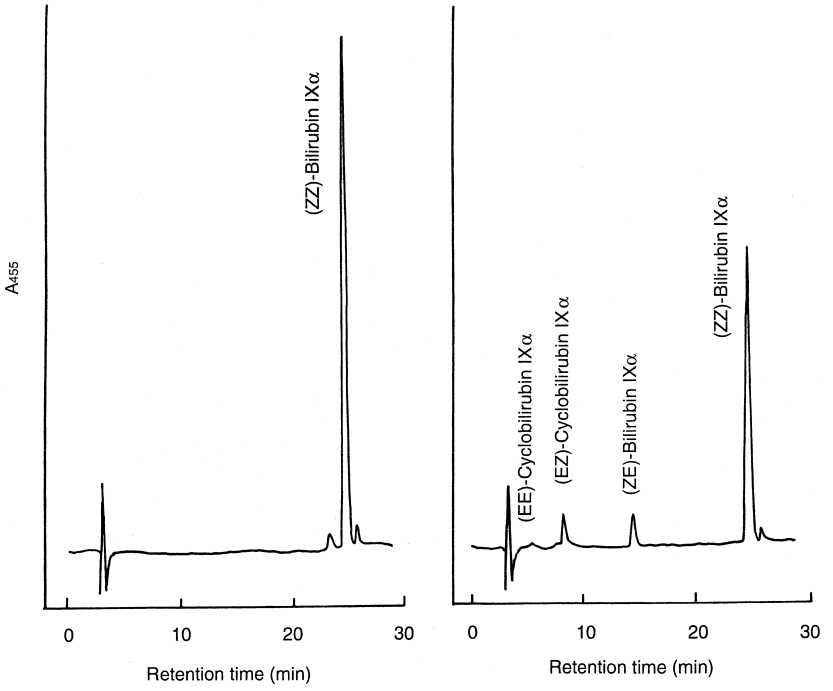

Fig. 6. HPLC scans of (ZZ)-bilirubin human serum albumin solution before and after photoirradiation by green light for 15 min under anaerobic conditions. In the control experiment with (ZZ)-bilirubin in human serum albumin solution, the large peak of (ZZ)-bilirubin IXα was separated. When photoirradiation by green light was applied for 15 min, the peak of the (ZZ)-bilirubin IXα decreased and the peaks of (ZE)-bilirubin IXα, (EZ)-cyclobilirubin IXα and (EE)-cyclobilirubin IXα appeared.

$$\text{mean} = \frac{[34\ 833]}{\alpha} + \frac{[30\ 262]}{\gamma} + \frac{[4626]}{\delta} + [238\ 932]$$

$$\alpha = 0.81, \frac{\delta}{\gamma} = 0.84; \text{ hence, } \gamma = 0.46 \text{ and } \delta = 0.39.$$

4. Conclusions

The factors used to correct the integrated peak areas on the chromatograms were calculated from the decrease in the relative molar absorptivity values at 455 nm for each photoisomer produced by photoirradiation compared with that for (ZZ)-bilirubin. As a result, the total integrated peak area estimated by HPLC at 455 nm decreased due to a decrease in the relative molar absorptivity values of geometrical and structural photoisomers which appeared during photoirradiation. Then, the relative 455 nm molar absorptivity values for (ZZ)-bilirubin: (ZE)-bilirubin: (EZ)-bilirubin: (EZ)-cyclobilirubin: (EE)-cyclobilirubin = 1.00: 0.81: 0.54: 0.46: 0.39. The changes in the relative molar absorptivity values at 455 nm for Δ^4 geometrical photoisomerization by (ZE)-bilirubin formation from (ZZ)-bilirubin and by (EE)-cyclobilirubin formation from (EZ)-cyclobilirubin were theoretically expected to be 81% (0.81/1.00) and 83% (0.39/0.46), respectively. Thus, similar values were obtained.

However, the concentrations of (EZ)-cyclobilirubin and (EE)-cyclobilirubin were 6–7% lower than their actual concentrations of those because the structural photoisomers were unstable in the HPLC eluents.

Now the maximum sensitivity and selectivity of the photoisomers may be achieved by modern photodiode array detector or computer-controlled variable-wavelength detector.

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