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THE SEPARATION OF CONFIGURATIONAL ISOMERS OF BILIRUBIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND THE MECHANISM OF JAUNDICE PHOTOTHERAPY

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SUMMARY When deoxygenated chloroform solution of bilirubin IXα (ZZ) was irradiated by blue light, ion-pair reversed-phase high pressure liquid chromatography technique revealed that the pigment was converted to a mixture containing IIIα, IXα, XIIIα (ZZ-configuration), and more polar geometric isomers (E-configuration). All azodipyrroles derived from each peak of Z- or E-configuration resulted in one of the exo- or endo-vinyl isomers, indicating that the bilirubin molecule is not affected by any of the phenomena except for geometric isomerization under this photochemical condition.

INTRODUCTION

The study of bilirubin metabolism in neonatal jaundice during phototherapy has chiefly focused on two main subjects (1,2): one is the dipyrrole pathway which mainly provides excretion into urine via photo-oxygenation of bilirubin (3); the other is the tetrapyrrole pathway by which there is excretion into bile (4,5). However, it is puzzling that during phototherapy considerable amounts of unconjugated bilirubin are excreted into bile, and it has never been shown how phototherapy enables bilirubin to slip through the liver and into the bile without conjugation. Bonnett et al. (6) discovered by X-ray analysis that the bilirubin molecule normally has a Z-configuration about the exocyclic eneamide carbon-carbon double bonds and forms an intramolecular hydrogen bond resulting in a compact and stable three-dimensional

*Abbreviations: HPLC = high pressure liquid chromatography, DEA = diazotized ethyl anthranilate

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structure (ZZ-configuration). Based on these findings, it has been suggested that conformational or configurational modification of the pigment by photoenergy is probably responsible (7,8,9).

Recently, McDonagh, Lightner and Wooldridge (10,11,12) reported that bilirubin IXα dimethyl ester undergoes rapid and reversible photo-isomerization on irradiation with visible light giving photobilirubin (ZE, EZ, and EE configuration). Chromatography and purification of geometric isomer of naturally occurring bilirubin IXα were frustrated by its tendency to revert to bilirubin IXα (ZZ). For the first time, the present authors were able to resolve it completely into its components and they also demonstrated by phototherapy, that the increase of IIIα (ZZ) and XIIIα (ZZ) isomer and photobilirubin in neonatal icteric serum occurred. A preliminary report of these results has been published earlier (13). The present paper presents the conditions for the complete separation of bilirubin IXα, IIIα, XIIIα (ZZ configuration) and these photobilirubin by HPLC.

MATERIALS AND METHODS

Purification of bilirubin (Tokyo Kasei, Tokyo) and confirmation of its purity were performed as previously described by McDonagh and Assisi (14). Photoproduct formation was followed by high pressure liquid chromatography on Shimadzu PCH column (25 cm x 4.6 mm I.D.). HPLC analyses were run on a Shimadzu LC-2 liquid chromatograph equipped with a model SPD-2 variable wavelength detector. Detectors were set at 455 nm. Photochemical experiments were carried out using chloroform solution of bilirubin in a stoppered 25 ml flask.

The light source was four 20-watt Matsushita blue-white fluorescent tubes. In all experiments the solutions were deoxygenated with pure (99.999 %) argon. Solvents for HPLC were used as supplied. Reagent grade solvents were purified and redistilled. Nonphotochemical manipulations were carried out under subdued light. HPLC condition for separation of azopigments: Separation was best achieved by using a linear gradient of acetonitrile in 0.1 M acetate buffer pH 4.0 (20 % to 60 % v/v in 80 minutes). HPLC condition for separation of bilirubin and photobilirubin: Separation was best achieved by using a linear gradient of acetonitrile in 0.01 M phosphate buffer pH 8.0 containing 0.1 % tetra-n-butylammonium hydroxide (Wako, Osaka) as counter ion (10 % to 40 % v/v in 120 minutes).

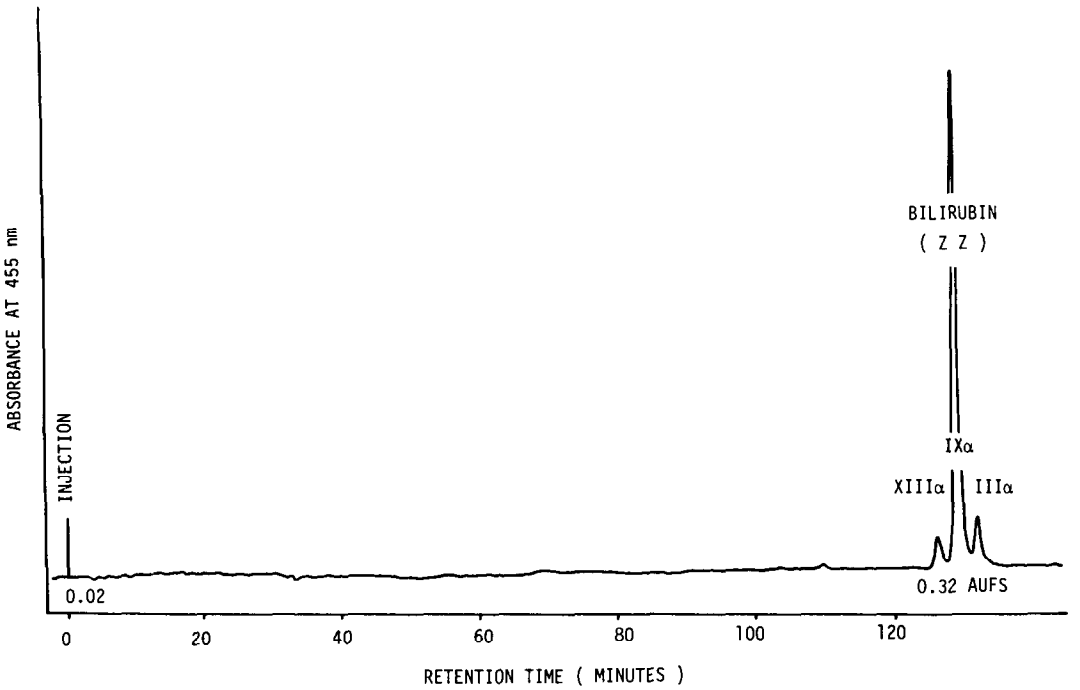


Figure 1. HPLC scan of non-irradiated bilirubin. The detector was set at 455 nm and the solvent flow rate was 1.0 ml/min. The retention times (min) of isomers are: XIIIα ZZ(126), IXα ZZ (130) and IIIα ZZ9133).

RESULTS AND DISCUSSION

The residue obtained after evaporation of an irradiated (30 min.) solution of bilirubin IXα (ZZ) in deoxygenated chloroform was dissolved in the primary eluent. Ion-pair reversed-phase HPLC was then introduced to analyze the eluent. As shown in figure 1, in the control experiment with a nonirradiated chloroform solution, a large peak of bilirubin IXα (ZZ) which has retention time at 130 min. and two small peaks of bilirubin IIIα (ZZ) and XIIIα (ZZ) were clearly separated. When photo-irradiation was applied, decreased bilirubin IXα peak and increased isomer peaks of bilirubin IIIα and XIIIα were observed (Figure 2). Many peaks of more polar photoproducts which have a shorter retention time were also noted. Then each peak was fractionated and conducted into dipyrrolic azopigments by

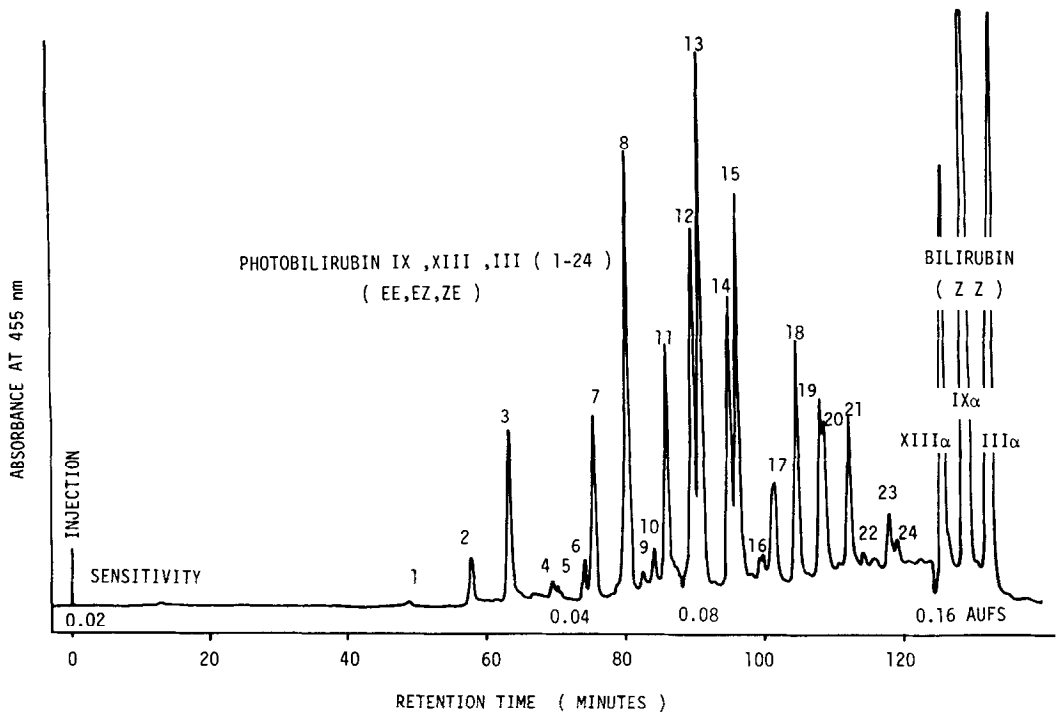
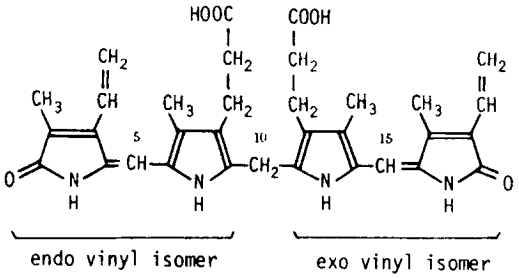
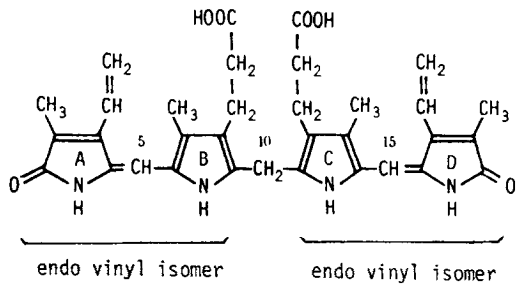


Figure 2. HPLC scan of photoproducts derived from irradiated bilirubin in deoxygenated chloroform solution. The detector was set at 455 nm and the solvent flow rate was 1.0 ml/min. The retention times (min) of the isomers are: photobilirubin (48-120), XIIIα ZZ(126), IXα ZZ(130) and IIIα ZZ(133). Main peaks of photobilirubin XIIIα: Peak No. 2, 7, 11, 18. Main peaks of photobilirubin IXα: Peak No. 9, 12, 14, 17, 19, 20, 23. Main peaks of photobilirubin IIIα: Peak No. 3, 8, 10, 13, 15, 21.

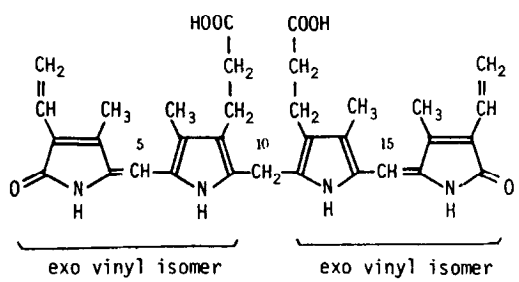
diazotized ethyl anthranilate after addition of ethanol. In order to analyze the composition of exo- or endo-vinyl isomers in each bilirubin peak, HPLC was introduced. It was then confirmed that azodipyrroles derived from bilirubin IXα consisted of equal amounts of endo- and exo-vinyl isomers, and those of bilirubin IIIα and XIIIα consisted of a single isomer of exo- and endo-vinyl respectively (Figure 3). Azopigments derived from peaks which are more polar than ZZ-configuration were also confirmed to have resulted in completely the same substances. This phenomenon may be recognized by the facts that E-configuration, one of the geometric isomers (EZ, ZE, and EE configuration)



Bilirubin IXα



Bilirubin XIIIα



Bilirubin IIIα

Figure 3. The structural relationship between exo vinyl and endo vinyl isomer in bilirubin IXα, IIIα and XIIIα structures.

reverted to Z configuration by the diazo reaction in the presence of ethanol and intramolecular hydrogen bonding of varying degrees are all cut off (Figure 4).

Using the same technique and analysis condition, we also observed the appearance of more polar bilirubin IXα in the serum of hyperbilirubinemic neonate during phototherapy (Figure 5).

BR IXα + DEA ⟶ Azodipyrrole (endo vinyl + exo vinyl)
(ZZ,EZ,ZE,EE)

BR XIIIα + DEA ⟶ Azodipyrrole (endo vinyl + endo vinyl)
(ZZ,EZ=ZE,EE)

BR IIIα + DEA ⟶ Azodipyrrole (exo vinyl + exo vinyl)
(ZZ,EZ=ZE,EE)

DEA : diazotized ethyl anthranilate

Figure 4. The structural relationships between endo vinyl or exo vinyl isomer of azodipyrroles derived from Z- or E-configuration of bilirubin IXα, IIIα, or XIIIα and the structure of bilirubin IXα, XIIIα or IIIα.

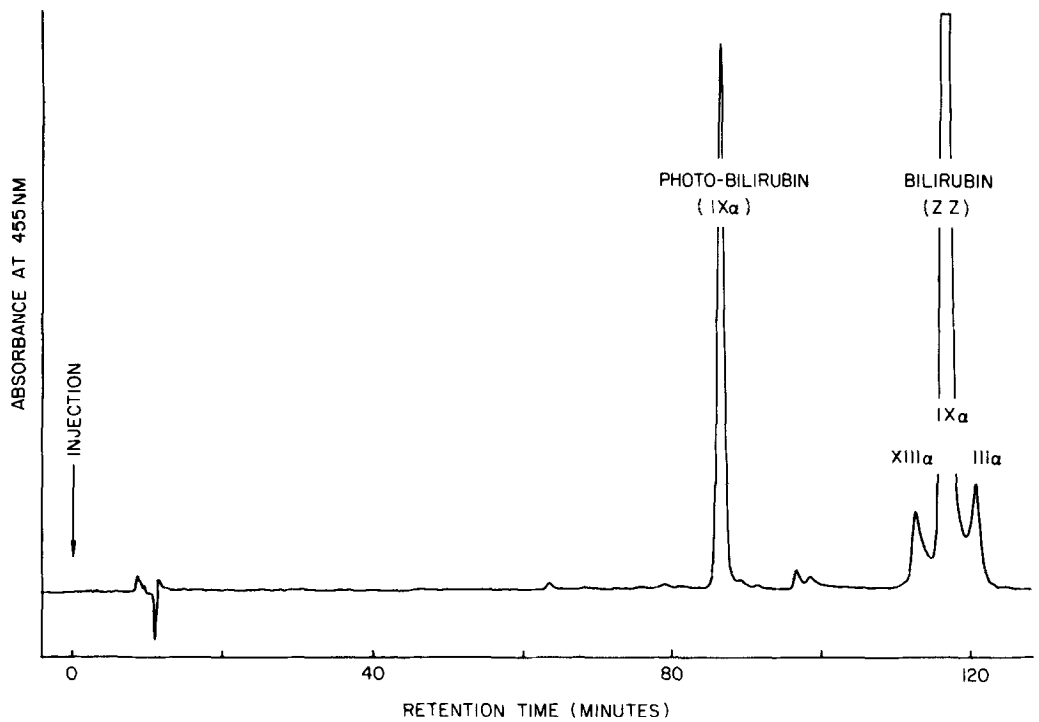


Figure 5. Using the same technique and analysis condition, the remarkable increment of one peak of more polar bilirubin IXα (photobilirubin IXα) was observed in the serum of hyperbilirubinemic neonate during phototherapy.

The following characteristics support the hypothesis that all peaks of bilirubin obtained on HPLC are geometric isomers.

1. These peaks disappear and reconvert to ZZ-configuration by

trifluoroacetic acid reaction (10). 2. By leaving them for a short period at room temperature, photobilirubin peaks rather markedly decreased or disappeared. These photobilirubin peaks would correspond to bilirubin IXα (EE), IXα (EZ) and IXα (ZE) described by McDonagh, Lightner, and Wooldridge (10,11,12). 3. These peaks disappear and reconvert to ZZ-configuration by incubation for 4 hours at 50°C (11). The confirmation of geometric isomers of bilirubin IIIα and XIIIα was performed by conduction into azodipyrrole and also by analysis of the retention time of the irradiated chloroform solution of bilirubin IIIα and XIIIα.

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