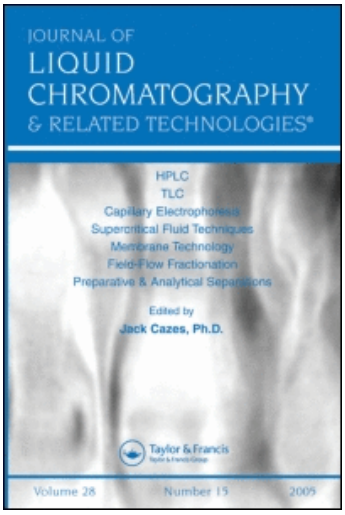

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SEPARATION OF THE III-α, IX-α, AND XIII-α ISOMERS OF BILIRUBIN AND BILIRUBIN DIMETHYL ESTER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

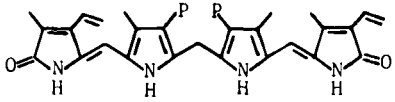
Conditions are described for the rapid separation of the III-α, IX-α, and XIII-α isomers of both free bilirubin (BR) and its dimethyl ester (BRDME) by normal-phase HPLC on silica. The HPLC method for BR is an alternative for the tedious TLC method for determination of the isomeric purity of commercial and other BR samples.

INTRODUCTION

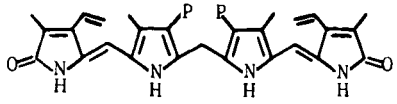
The chemistry, photochemistry and clinical analysis of the important bile pigment, bilirubin IX-α (BR IX-α) has required increasingly sophisticated methods of detection and quantitative determination. This is especially true for investigations of the phototherapy for newborn infants with unconjugated hyperbilirubinemia (1).

Commercial samples of BR IX-α contain variable amounts of the symmetrical III-α and XIII-α isomers of BR (2) which probably arise in the isolation-purification procedures. Larger amounts of the BR III-α and XIII-α isomers can be intentionally generated

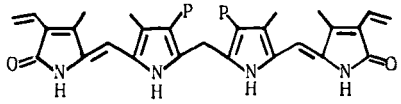
from BR IX-α by treatment with conc. HCl in dimethylsulfoxide (3). Analysis of BR samples for isomer content or separation of pure samples of each isomer has previously been accomplished by analytical TLC on polyamide (4) or silica (4) and preparative TLC on silica (3). These slower procedures can be replaced by HPLC on silica using recently developed conditions described herein. Conditions have also been worked out for the separation of the III-α, IX-α, and XIII-α isomers of bilirubin dimethyl ester (BRDME).



BR IX-α



BR XIII-α



BR III-α

P = CH_2CH_2COOH

EQUIPMENT AND MATERIALS

A Perkin-Elmer Series 3 liquid chromatograph equipped with a Rheodyne 7105 injector and an LC-65 detector-over unit was used for all HPLC work. The column used was a Dupont Zorbax-SIL (25 cm x 4.6 mm I.D.). Separations were achieved at 25°. The variable wavelength detector was set at 450 nm for work with BR and at 410 nm for work with BRDME.

The $CHCl_3$ used contained 0.75% ethanol stabilizer (Fisher HPLC grade). The toluene (Fisher reagent grade) was washed with water, dried (K_2CO_3) and distilled. The toluene with 5% (v/v) of 95% ethanol was filtered through filter paper to remove excess suspended water while leaving the solution nearly water-saturated.

The BR (MCB) was purified by dissolving in $CHCl_3$, followed by 5% aq. $NaHCO_3$ extraction (3X) and crystallization from $CHCl_3$-CH_3OH. The BRDME was prepared by diazomethane (5) or 1-methyl-3-*p*-tolytriazene (6) methylation of BR IX-α. Separation of the BRDME from over-methylated products was achieved by column chromatography on neutral alumina, activity II-III (E. Merk, Darmstadt), eluted with 1:1 $CHCl_3$-60^0 petroleum ether, $CHCl_3$, and then 20:1 $CHCl_3$-CH_3OH in that order. Final purification was achieved by thick-layer chromatography on M. Woelm, Eschewege silica gel F using benzene with 10% (v/v) of 95% ethanol. Two different mixtures of BR III-α, IX-α, and XIII-α were generated by treatment of BR IX-α with conc. HCl in dimethylsulfoxide for one minute and 60 minutes exactly as described by the published procedure (3).

METHODS AND RESULTS

Separation of the BR isomers was best achieved by using a linear gradient of glacial acetic acid in $CHCl_3$ [0.2% to 0.999% (7) (v/v) in 20 minutes, Figure 1]. Although gradient elutions are not always repeated on silica, the column used re-equilibrated rapidly with this solvent system and gave reproducible retention times. As shown in Figure 1a, the commercial (MCB) BR sample contains only small amounts of the III-α and XIII-α isomers while the sample treated for one minute with acid (Figure 1b) contains the isomers in a ratio of approximately 1:2:1. The sample treated for 60 minutes shows (Figure 1c) mostly BR XIII-α and IX-α with little III-α. Peak assignments were based on previous work (2,3).

Separation of the BRDME isomers was best achieved by an isocratic elution with toluene with 5% (v/v) of 95% ethanol. The sample of BRDME prepared from BR IX-α shows (Figure 2) somewhat increased amounts of III-α and XIII-α which probably result from the preliminary isolation-purification procedure. Peak assignments were confirmed by comparison with the BRDME esters derived from acid treatment of BR IX-α (above), which produces a nearly 1:2:1 ratio of the III-α, IX-α and XIII-α isomers (3).

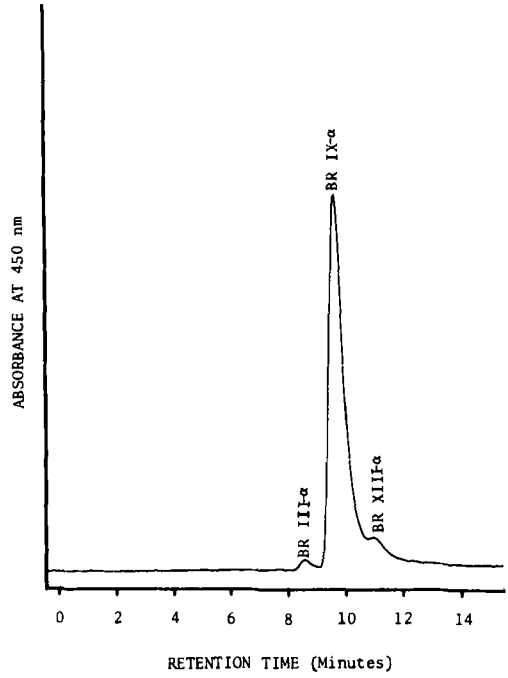


FIGURE 1a. Commercial BR IX α, zorbax-sil, acetic acid in $CHCl_3$ -0.75% ethanol 0.2% to 0.99% linear gradient in 20 min, 0.5ml/min, 450 nm.

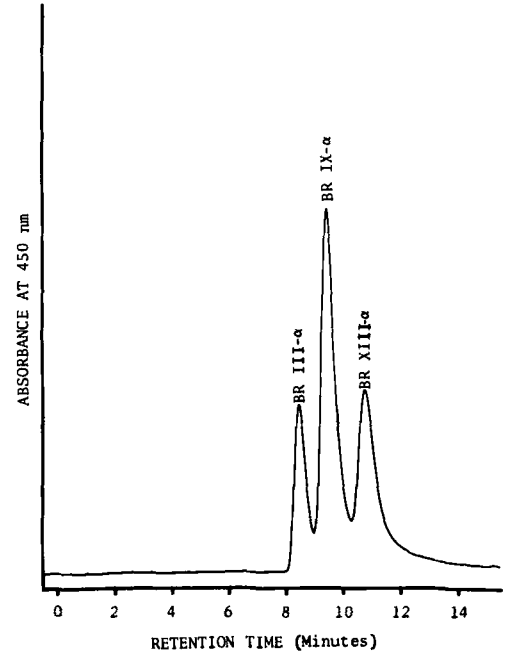


FIGURE 1b. Acid treated BR IX-α (1 min), zorbax-sil, acetic acid in $CHCl_3$ -0.75% ethanol, 0.2% to 0,99% linear gradient in 20 min, 0.5ml/min, 450 nm.

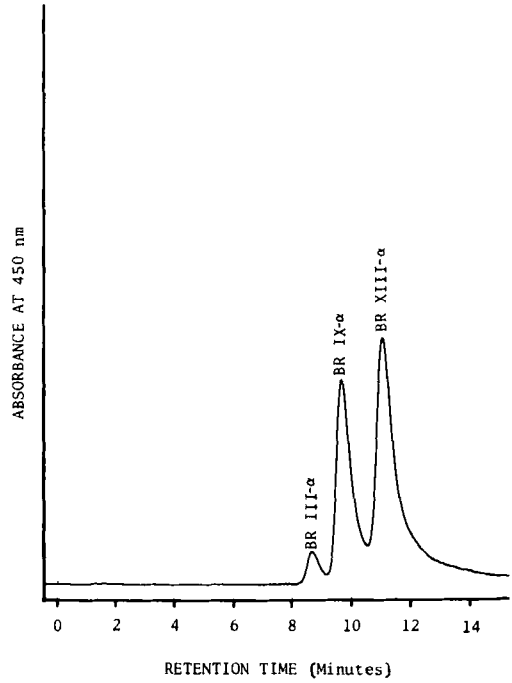


FIGURE 1c. Acid-treated BR IX-α (60 min), zorbax-sil, acetic acid in $CHCl_3$ -0.75% ethanol, 0.2% to 0.99% linear gradient in 20 min, 0.5ml/min, 450 nm.

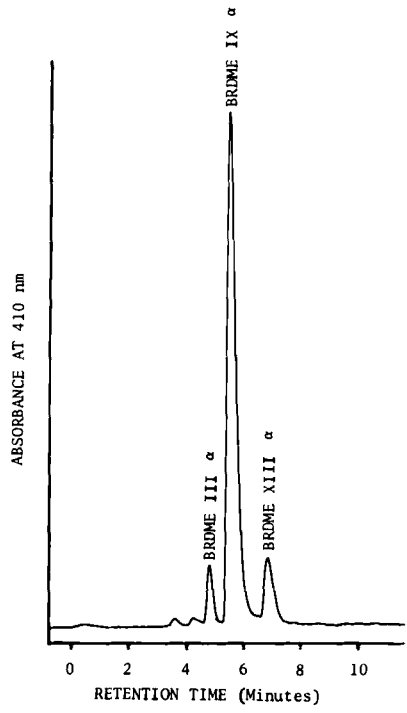


FIGURE 2. BRDME from BR IX-α, zorbax-sil, toluene-5% 95% ethanol isocratic, 1.0 ml/min, 410 nm.

Small samples of the BR or BRDME isomers sufficient, for example, for UV-Visible spectrophotometric analysis can easily be collected by making several 10µl injections of approximately 10^{-4} M solutions.

ACKNOWLEDGEMENTS

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REFERENCES

(1) For leading references see: Bilirubin Metabolism in the Newborn (II), Bergsma, D. and Blondheim, S. H., eds, American Elsevier, New York, 1976.

(2) McDonagh, A. F. and Assisi, F., Commercial Bilirubin: A Trinity of Isomers, FEBS Letters, 18, 315, 1971.

(3) McDonagh, A. F. and Assisi, F., Direct Evidence for the Acid-Catalysed Scrambling of Bilirubin-IXα, Chem. Commun., 117, 1972.

(4) McDonagh, A. F., Bilatrienes and 5·15 Bilatrienes in The Porphyrins, Vol. VI, Part A, Dolphin, D., ed, Academic Press, New York, 1978.

(5) Kuenzle, C. C., Weibel, M. H. and Pelloni, R., The Reaction of Bilirubin with Diazomethane, Biochem. J., 133, 357, 1973.

(6) Hutchinson, D. W., Johnson, B. and Knell, A. J., The Synthesis of Esters of Bilirubin, Biochem. J., 133, 493, 1973.

(7) The 0.999% value reflects only a setting on the solvent programming equipment used. The actual value was 1.0%.