Journal of Chromatography, 182 (1980) 105—109
Biomedical Applications

CHROMBIO. 486

Note

Accurate and sensitive analysis of ethyl anthranilate azopigments from bile by reversed-phase high-performance liquid chromatography

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(First received June 29th, 1979; revised manuscript received November 9th, 1979)

Van Roy and Heirwegh [1] developed a very elegant thin-layer chromatographic (TLC) method by which it was elucidated that the conjugates of bilirubin are composed of glucoside and xyloside in addition to glucuronide [2]. This finding reflects the considerable progress being made in research techniques in the field of conjugated bilirubin structure. However, Bonnett and Stewart pointed out that photodecomposed substances of bilirubin were incompletely resolved on TLC [3]. In order to overcome these difficulties, we introduced the new high-performance liquid chromatographic (HPLC) method in 1973 and reported our results at the Second International Symposium on Bilirubin Metabolism in the Newborn, held in Jerusalem in 1974 [4]. Lately, Billing has pointed out that the most promising approach would appear to be that of HPLC [5]. We concluded that HPLC is a useful method for separation of azopigments, conjugated bilirubin [6] or geometric isomers of bilirubin in biological fluids, and have published a preliminary report of these results [7, 8]. The present paper describes the conditions for the complete separation of azopigments of dog bile by HPLC.

MATERIALS AND METHODS

High-performance liquid chromatography

A Shimadzu LC-2 liquid chromatograph with an SPD-1 detector and Chromatopac C-R1A was used for all HPLC procedures. The column used was a Shimadzu PCH-05/S2504 (25 cm × 4.6 mm) packed with a reversed-phase packing material based on silica gel (5-μm particles). Separations were achieved at room temperature. The variable-wavelength detector was set at 530 nm. The mobile phase was a mixture of acetonitrile, distilled water and sodium acetate.

Separation was best achieved by using a linear gradient of acetonitrile [20% to 60% (v/v) in 80 min] in 0.1 *M* acetate buffer (pH 4.0). Peak assignments were conducted using two methods; one was the TLC method described below, the other by comparison with the retention time of reference samples on HPLC. The ethyl anthranilate azopigments derived from bilirubin conjugates which were obtained from incubating bilirubin with UDP-glucuronic acid (or either UDP-glucose or UDP-xylose) and adult human liver homogenate, yielding bilirubin glucuronides (or either glucosides or xylosides), respectively, were used as a reference substance. Fourier transform proton NMR spectrometry was also performed for the identification of azodipyrrole (*exo*-vinyl and *endo*-vinyl isomer).

Formation of ethyl anthranilate azo derivatives

A 1% (v/v) suspension of ethyl anthranilate in 0.15 *M* HCl was prepared, and 0.6 ml was diluted with 9.4 ml of water. To this was added 0.6 ml of $NaNO_2$ solution (5 mg/ml) and, 5 min later, 0.2 ml of ammonium sulphamate solution (10 mg/ml). The diazo reaction was carried out by mixing 1 vol. of bile with 1 vol. of citrate—phosphate buffer (pH 6.0), 0.5 vol. of formamide—ethanol (1:1, v/v) and 1 vol. of the diazo reagent. After 45 min at 0°, 1 vol. of ascorbic acid solution (10 mg/ml) in glycine—HCl buffer (pH 2.0) was added to destroy the excess diazo reagent and to lower the pH for extraction. The azopigments were extracted without delay by vigorous shaking with 2 vols. of pentan-2-one [9]. The organic phase was dried at room temperature in a rotary evaporator connected to an oil vacuum pump. The residue was dissolved in the primary eluent of HPLC before analysis.

Analysis of azopigments by TLC

Portions of the extracted azopigments with pentan-2-one were applied to precoated silica gel plates (DC-kieselgel, E. Merck, Darmstadt, G.F.R.) and developed at room temperature in the dark. Separation was best obtained by developing the plates first with chloroform—methanol (17:3, v/v) for 3 cm, followed by chloroform (containing 0.6—1% ethanol) for 18 cm, and then chloroform—methanol—water (65:25:3, v/v) for 15 cm [10]. The azopigments were eluted from the thin-layer plates with ethanol.

Pigments and reagents

Bilirubin (Merck) and ethyl anthranilate (Kodak, London, Great Britain) were used without further purification. Pentan-2-one (Tokyo Kasei, Tokyo, Japan) was distilled before use. UDP-glucuronic acid, UDP-glucose and UDP-xylose were purchased from Sigma (St. Louis, Mo, U.S.A.). Acetonitrile was of analytical grade.

RESULTS AND DISCUSSION

The azopigments were obtained from dog bile treated with diazotized ethyl anthranilate [9]. This was followed by confirmation of the separation of azodipyrrole, its xyloside, glucoside and glucuronide at bands α_0, α_2, α_3 and δ (Fig. 1), respectively, by TLC, as demonstrated by Heirwegh et al. [2]. Then, using

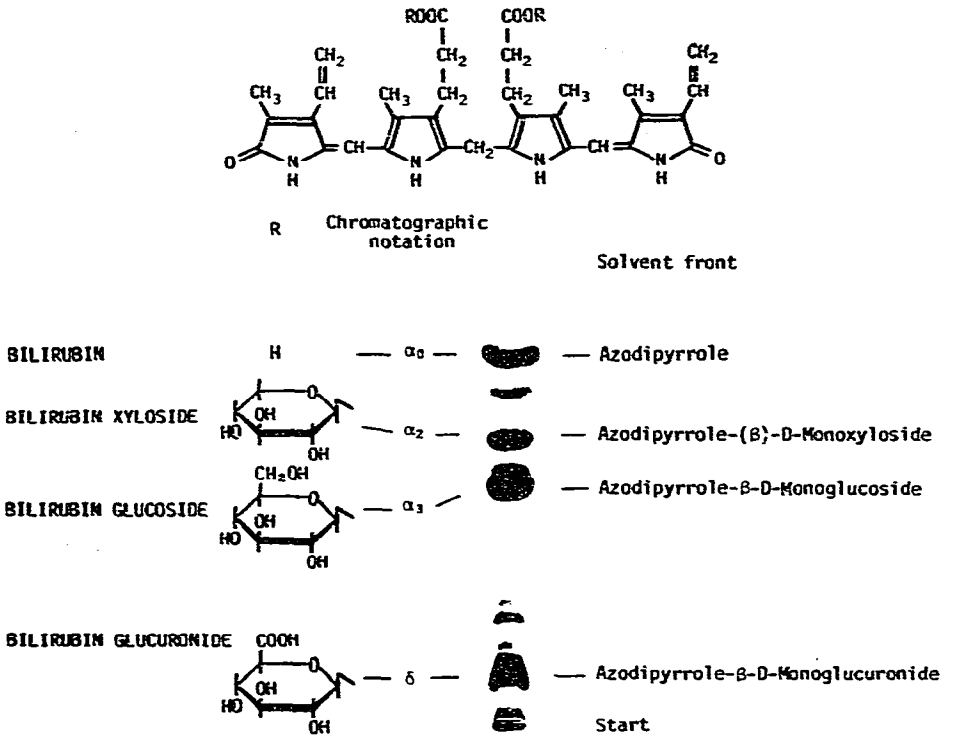

Fig. 1. Schematic diagram of TLC of ethyl anthranilate azopigments (α_0, α_2, α_3 and δ) derived from diazo coupling of bilirubin and its conjugates in dog bile.

the HPLC method, it was observed that each band (α_0, α_2, α_3 and δ) had a pair of peaks (Fig. 2). Moreover, by analyzing each α_0 peak using Fourier transform proton NMR spectrometry [11], it was confirmed that the former peak was *endo*-vinyl isomer and the latter *exo*-vinyl isomer (Fig. 3). Accordingly, it was demonstrated that the mixture of the *endo*- and *exo*-vinyl isomers of azopigment which gave one band on the TLC plate obtained by several developments could be clearly separated by only one HPLC run, and that the *exo*-vinyl peak was usually smaller than that of *endo*-vinyl and easily degraded despite careful attention to light, oxygen and related factors.

The calibration curve of peak area, obtained from the electronically integrated peak area calculated by Chromatopac C-R1A, versus amount of bilirubin loaded, is linear up to 1–3 μg of bilirubin. If the concentration is above this range, the value can be precisely estimated either by decreasing the amount of sample injected into the chromatograph or by diluting the sample with the primary eluent.

ACKNOWLEDGEMENTS

We thank Dr. K.P.M. Heirwegh for valuable advice and comments on this work. This research was supported by Research Grant No. 487097 from the Ministry of Education of Japan. We thank Professors J. Ogawa and Y. Wada for encouragement.

JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 8 1980

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
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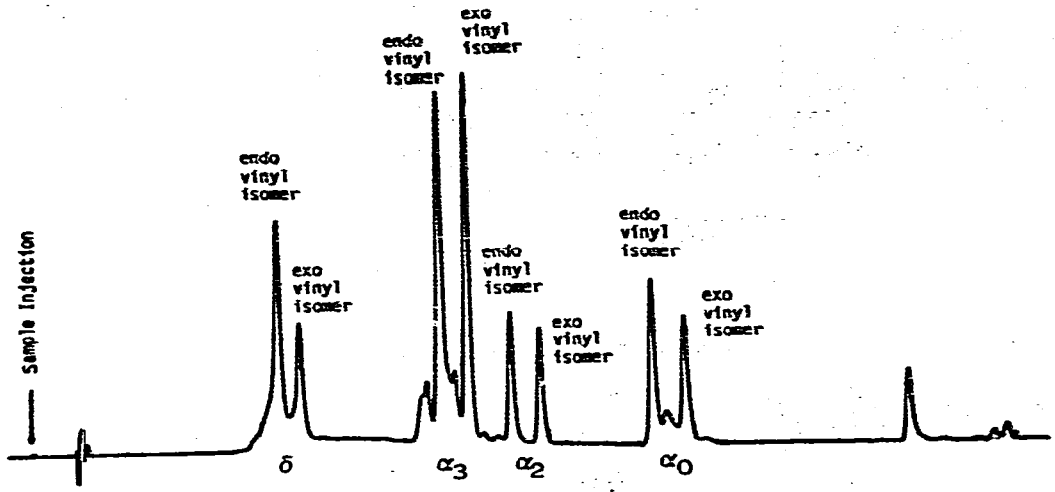

Fig. 2. Separation of ethyl anthranilate azopigments (α_0, α_2, α_3 and δ) derived from dog bile. Conditions: column, Shimadzu PCH-05/S2504; mobile phase, a linear gradient of acetonitrile (20% to 60% in 80 min); flow-rate, 1 ml/min; detection, 530 nm, 0.02 a.u.f.s.

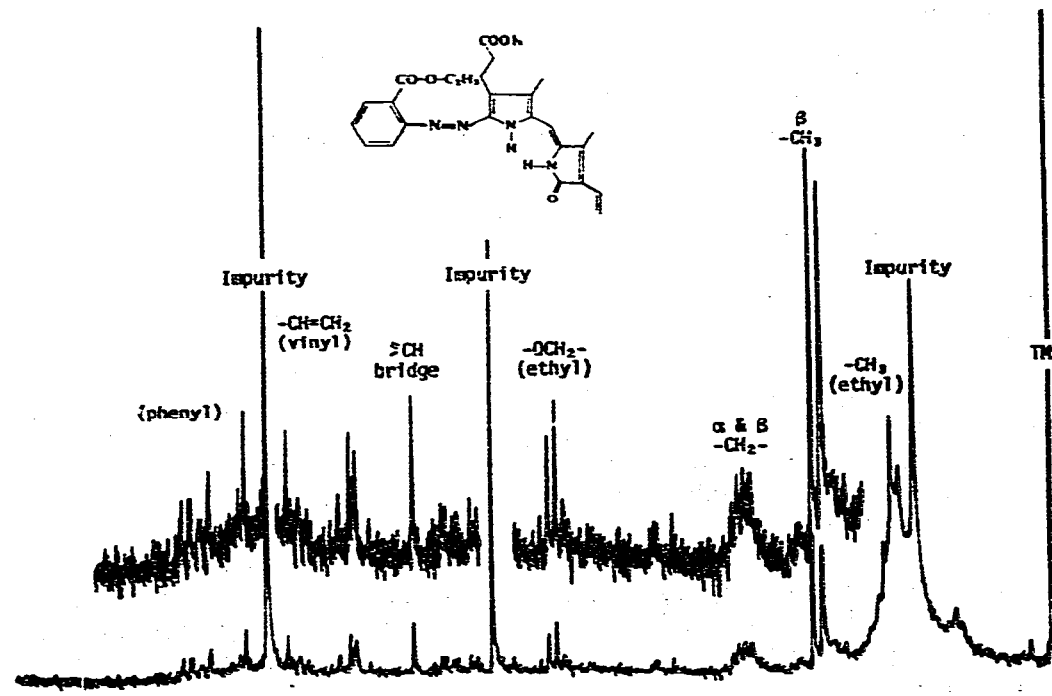

Fig. 3. Fourier transform proton NMR spectrum of *exo*-vinyl isomer of azodipyrrole (α_0) run on a JMN-PFT-100 type NMR (JEOL) instrument with tetramethylsilane as an internal standard.