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Note

Separation of bilirubin azopigments from bile by high-performance liquid chromatography

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Recent reports from several laboratories have revealed that bilirubin is excreted in bile as a heterogenous group of relatively polar derivatives which arise by hepatic conjugation of bilirubin with a variety of carbohydrates and possibly other compounds as well [1—7]. Factors which influence the formation of bilirubin conjugates are of clinical interest with regard to a number of conditions which result in jaundice. Investigations of the nature of such factors should be materially aided by the ability to rapidly and specifically determine the various bilirubin conjugates in body fluids.

I have developed a high-performance liquid chromatography (HPLC) method for the resolution of *p*-iodophenylazo derivatives of bilirubin and its conjugates from dog bile. The method employs ion-pair chromatography on a reversed-phase column and is performed directly on the derivatized samples without intervening extractions or other preliminary purification steps.

EXPERIMENTAL

Chemicals

Reagent grade solvents were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and were filtered through an 0.5-μm Fluoropore filter (Millipore) before use. β-Glucuronidase (type B-10 from bovine liver) and saccharic acid 1—3 lactone were obtained from Sigma (St. Louis, Mo., U.S.A.). Bilirubin standards were from American Monitor (Indianapolis, Ind., U.S.A.). All other chemicals were the best grade commercially obtainable and were used without further purification.

Apparatus

All experiments described herein employed an ALC 200 Series liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model U6K injector and a Model 440 absorbance detector operating at a fixed wavelength

of 546 nm. The reversed-phase column (30 cm × 4 mm I.D.) was obtained prepacked with μBondapak C_{18} (10 μm) from Waters Assoc.

Preparation of samples

Bile was collected from the gall bladders of anesthetized dogs via a hypodermic needle and immediately expelled into a foil-covered, screw-capped tube and frozen. The frozen bile was stored at -20°C until used. Preparation of *p*-iodophenylazo derivatives of bilirubin was accomplished by mixing 100 μl of bile with 100 μl of acetonitrile followed by 200 μl of methanol—ethyl acetate (1:1) and 50 μl of diazo reagent (diazotized *p*-iodoaniline). After allowing the reaction to proceed on ice for 30 min, 25 μl of ascorbic acid (100 mg/ml) were added to destroy excess diazo reagent. The samples were centrifuged for 5 min in an Eppendorf micro centrifuge to remove suspended matter, and aliquots of the supernatant were chromatographed. The diazo reagent was prepared as follows [8]: 75 μl of sodium nitrite (100 mg/ml) was mixed with 100 μl of 2 *M* *p*-toluenesulfonic acid, and 0.5 ml of *p*-iodoaniline (21 mg/ml in glacial acetic acid) was added. This mixture was allowed to stand for 2 min then diluted with 2.5 ml of water followed by 50 μl of 1.5 *M* ammonium sulfamate. The reagent was used after being allowed to stand on ice for 5 min.

β-Glucuronidase hydrolysis

In order to identify which components were susceptible to hydrolysis by β-glucuronidase, bile samples were incubated with the enzyme before derivatization. The reaction mixtures consisted of 100 μl of β-glucuronidase (2 mg/ml), 100 μl of 0.2 *M* citrate—phosphate buffer (pH 5.6) and 75 μl of bile. Samples were incubated for varying periods of time at 30°, then aliquots of the incubation mixtures were derivatized and chromatographed as described above. To demonstrate inhibition by saccharic acid 1—3 lactone, a buffered 1 m*M* solution of the inhibitor was substituted for the buffer in the incubation mixtures. Each sample incubated with β-glucuronidase was accompanied by a control incubated without enzyme.

HPLC operating conditions

Separations were performed at ambient temperature with a flow-rate of 2.0 ml/min (column inlet pressure 1500—3000 p.s.i.). The solvent was a solution of acetonitrile—ethyl acetate—methanol—water (1.0:1.1:1.5:3.5) containing 1.4 ml of tetrabutyl ammonium phosphate solution (Waters Assoc. PIC-A) per 100 ml of solvent. The volume of sample injected was generally 5 μl.

RESULTS

HPLC of azopigments from dog bile yielded 9 major peaks (Fig. 1A). Fig. 1B is a chromatogram of a commercial bilirubin standard treated in the same manner as the bile sample. Bilirubin yielded two azopigment peaks whose retention times were the same as peaks 4 and 5 from bile.

As a first step toward establishment of the identities of the other components separated, their susceptibility to hydrolysis by β-glucuronidase was investigated.

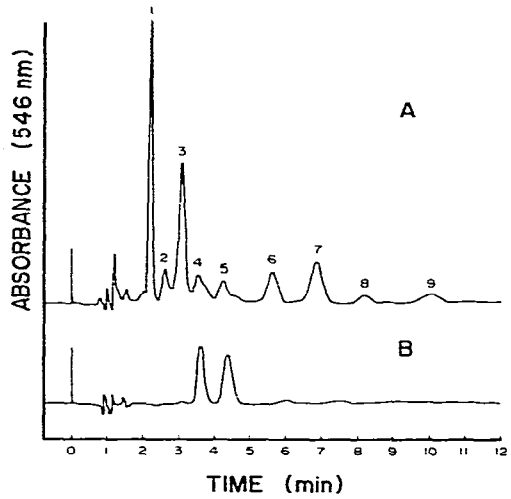
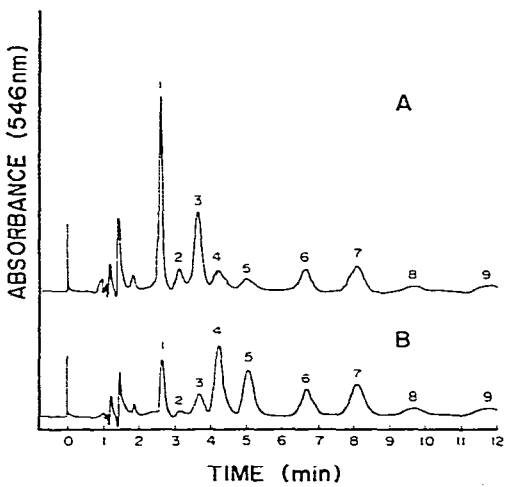


Fig. 1. (A) Separation of bilirubin azopigments from dog bile. (B) Bilirubin standard (20 mg/dl) treated in same manner as bile. Column packing μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s.; eluent, acetonitrile—ethyl acetate—methanol—water (1.0:1.1:1.5:3.5) with 1.4 ml of tetrabutyl ammonium phosphate (Waters PIC-A) per 100 ml; flow-rate, 2.0 ml/min.

Figs. 2 and 3 show the results of these investigations. The sample depicted in Fig. 2A was a control incubated for 2 h without enzyme and Fig. 2B shows the results obtained from bile incubated for 30 min in the presence of β-glucuronidase. It is apparent that the quantity of azopigments represented by peaks 1, 2,



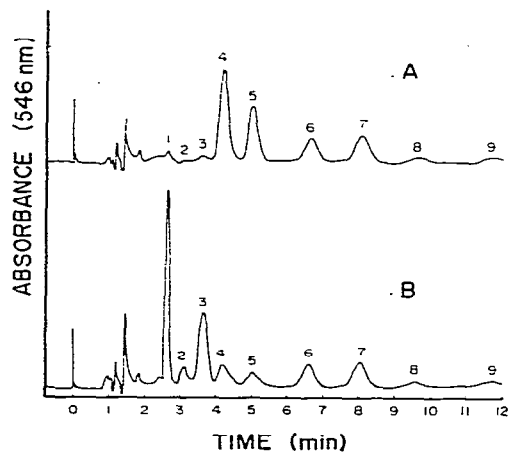


Fig. 2. (A) Control bile sample incubated at 30° for 2 h without enzyme. (B) Bile sample incubated with β-glucuronidase for 30 min at 30°. Other conditions same as in Fig. 1.

Fig. 3. (A) Bile sample incubated with β-glucuronidase for 2 h at 30°. (B) Bile sample incubated with β-glucuronidase plus saccharic acid 1—3 lactone for 2 h at 30°. Other conditions same as in Fig. 1.

and 3 was diminished while components 4 and 5 were increased. Incubation with β-glucuronidase for 2 h resulted in the virtual disappearance of peaks 1, 2, and 3 with a further increase in the magnitude of peaks 4 and 5 (Fig. 3A). When bile was incubated for 2 h with β-glucuronidase plus saccharic acid 1—3 lactone (Fig. 3B) no changes were observed relative to the control.

DISCUSSION

It is customary in investigations of bilirubin conjugates to couple the bile pigments with diazonium salts to produce azopigments of greatly enhanced stability. While this approach has the disadvantage of being unable to distinguish monoconjugated from diconjugated bilirubin, it does permit identification of the various moieties with which bilirubin is conjugated [9]. Previous investigators have employed solvent extraction combined with column chromatography and thin-layer chromatography to separate the various azopigments [2, 3]. Such multistep approaches inevitably result in incomplete yields making quantitation unreliable, and increase the risk of introducing artifacts. By means of the HPLC method reported here, the azopigments are separated in a single step requiring no preliminary fractionation.

In the coupling of bilirubin with diazonium salts the molecule is cleaved into two isomeric dipyrroles [9]. The present system is capable of resolving these isomers as shown in Fig. 1B. Peaks 6—7 and 8—9 probably represent isomeric conjugated azo dipyrroles. The nature of the conjugating moieties is presently unknown. The findings of Fevery et al. [3] suggest that they are probably glucose and xylose.

The predominant form in which bilirubin is excreted in bile is the diglucuronide, with lesser amounts of bilirubin monoglucuronide being present [4, 10]. Components 1, 2, and 3 in our system were susceptible to hydrolysis by β-glucuronidase. This does not unequivocally establish their identity as simple glucuronides. However, since peaks 1 and 3 are quantitatively the major constituents they probably represent the vinyl and isovinyl isomers of azodipyrrole glucuronic acid [1, 4—6]. Peak 2 is quantitatively minor. Since it is susceptible to the action of β-glucuronidase, constituent 2 may be the same as pigment B6 reported by Kuenzle [2], a pseudoaldobiouronide. Experiments designed to unequivocally identify all of the components separated are currently in progress.

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