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Purification of Conjugated Bilirubin: A New Approach Utilizing Albumin-Agarose Gel Affinity Chromatography (39318)

ALLAN W. WOLKOFF, BRUCE F. SCHARSCHMIDT, PAUL H. PLOTZ, AND PAUL D. BERK

Section on Diseases of the Liver, Digestive Diseases Branch, and the Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Bilirubin, an organic anion and breakdown product of heme, is excreted chiefly by the liver by a process involving uptake by the hepatocyte and subsequent conjugation to form a more polar compound (1). The availability of purified preparations of bilirubin, especially of radiolabeled bilirubin, has greatly facilitated studies of its metabolism (1-5). Study of the transport of bilirubin from blood to bile, however, must also include consideration of the mechanisms for excretion of bilirubin conjugates. Such study has been limited because of difficulty in obtaining conjugated bilirubin free of significant quantities of contaminants (6-19).

Recent studies of albumin-agarose gel affinity chromatography have revealed that the gels have a high affinity for bilirubin and a relatively low affinity and capacity for tri- and dihydroxy bile acids (20, 21), suggesting that this method might effectively separate bilirubin conjugates from other components in bile. We describe here, a rapid and relatively simple method for purification of conjugated bilirubin from bile.

Materials and methods. Albumin-agarose gel was prepared by a modification of the cyanogen bromide method (20, 22), and packed in small columns of 0.9-cm i.d. Bile acids were quantitated in bile and column eluates by gas-liquid chromatography (23), cholesterol by the method of Zak *et al.* (24), and phospholipids by a modification of the method of Chen *et al.* (25, 26).

Absorption spectra were obtained with a Beckman Acta III spectrophotometer, and fluorimetric analyses with a Farrand Mark I spectrofluorimeter.

Tritium was quantitated by adding up to 0.5 ml of sample to 15 ml of Aquasol (New England Nuclear) and by counting in a liquid scintillation counter (Nuclear Chicago). All samples were recounted after addition of [^{3}H]toluene (New England Nuclear) and results were corrected for background and quenching.

Bilirubin conjugates were quantitated after diazotization with ethyl anthranilate according to the method of Van Roy and Heirwegh (27). The resultant azopigments were separated by thin-layer chromatography (28) and bilirubin conjugates were identified (28, 29). Multiple determinations of the proportions of monoglucuronide and diglucuronide in a single bile specimen agreed within 1% of the mean. Presence of conjugated bilirubin was confirmed by Weber-Schalm fractionation (30) and by thin-layer chromatography performed on pigment applied directly to a silica gel plate and developed with chloroform (31).

Glucuronic acid content of conjugated bilirubin preparations was determined by a modification of the carbazole reaction (32) after hydrolysis of the ester linkage by incubation with 0.2 *N* NaOH.

Bile collection. Biliary cannulae were inserted into male Wistar rats under light ether anesthesia. The rats were kept in a restraining cage within an incubator; body temperature was maintained at 37°. Starting 2 hr after insertion of the cannulae, when a constant rate of bile flow had been established, bile was collected in the dark under ice and frozen until used. Bile containing radiolabeled conjugated bilirubin was collected in a similar fashion. A jugular cannula was inserted simultaneously with the construction of the bile fistula, and 100 μCi of 2,3-^{3}H delta-aminolevulinic acid (33,000 mCi/mmole) (33) was injected via the cannulated jugular vein 2 hr after insertion of the cannulae.

Preparation of purified bilirubin conju-

gates. Rat bile, 8 to 10 ml, was applied to an 8-ml albumin-agarose column equilibrated with 0.02 *M* phosphate buffered saline (PBS) at pH 7.0. The column was washed with 150 ml of PBS at pH 7.0 at a rate of 3 ml/min. Elution in 2- to 4-ml aliquots was performed by distilled water followed by 50% (v/v) ethanol in water. The water and ethanol eluates were lyophilized, dissolved in 1 ml of 0.02 *M* PBS at pH 5.8, and extracted twice with 2 vol of chloroform to remove unconjugated bilirubin. Protein, present as a trace contaminant, was removed by applying the resulting PBS solution to Sephadex G-25 (2 × 20 cm) which had been equilibrated with PBS at pH 7.0. Most of the yellow pigment, representing protein-free conjugated bilirubin, remained bound to the column, whereas protein and protein-bound pigment eluted with 150 ml of PBS at pH 7.0. Residual pigment on the column was rapidly eluted with a small volume of distilled water. The aqueous solution was lyophilized, dissolved in 1 ml of 0.02 *M* PBS at pH 7.0, applied to the Sephadex G-25 column, washed with 150 ml of 0.02 *M* PBS at pH 7.0, eluted with approx 10 ml of distilled water, and lyophilized. Further purification of these preparations to remove trace phospholipid contamination was performed by extraction of the aqueous solution of conjugated bilirubin three times with 25 vol total of chloroform-methanol (2:1).

Results. Figure 1 illustrates the elution pattern of radiolabeled bile from an albumin-agarose affinity column. Approximately 20–30% of labeled material did not bind and washed through with PBS. Distilled water eluted a fraction of pigment; 50% ethanol eluted a second fraction. The elution pattern of unlabeled bile was identical to that of radiolabeled bile.

Thin-layer chromatography of pigment from both the water and ethanol eluates (31) revealed that only conjugated bilirubin was present in each. Following diazotization, thin-layer chromatography of the ethyl anthranilate azopigments of conjugated bilirubin in bile and in each of the eluates was performed (Fig. 1). In the Wistar rat, 70–80% of total bilirubin conjugates in bile are glucuronide conjugates; bilirubin monoglucuronide represents 80% of glucuronide conjugates, and bilirubin diglucuronide the remainder. Proportions of bilirubin conjugates in the PBS wash and in the ethanol eluate were similar to that in bile. However, 80% of glucuronide conjugates in the distilled water eluate was bilirubin diglucuronide. The Wistar-R rat, unlike other strains of Wistar rats (28), has less than 10% nonglucuronide conjugates of bilirubin; of the glucuronide conjugates, 50–60% is bilirubin diglucuronide. Chromatography of bile

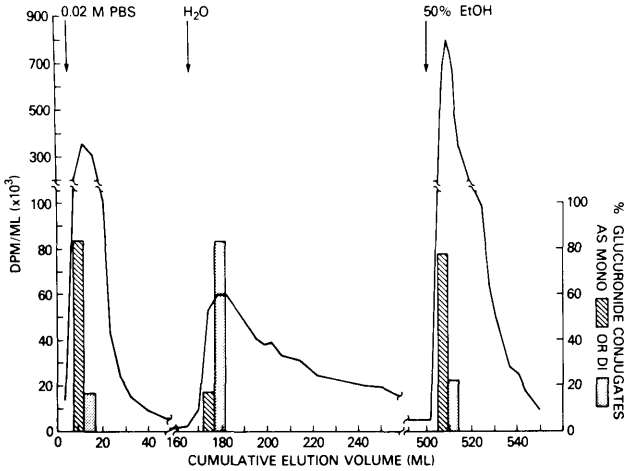

FIG. 1. Elution of radiolabeled bile after application to an albumin-agarose gel affinity chromatography column. After rat bile was applied, unbound biliary constituents were washed from the column with 0.02 *M* phosphate buffered saline at pH 7.0 (0.02 *M* PBS). Distilled water (H_2O) eluted a fraction of pigment; 50% ethanol (50% EtOH) eluted a second fraction. The relative proportions of bilirubin mono- and diglucuronide in the unbound material and the two eluates are indicated by the bars.

from a Wistar-R rat on albumin–agarose gel revealed almost exclusively bilirubin diglucuronide in the water eluate.

When tritiated bilirubin conjugates from either the water or ethanol eluates were diazotized and applied to a thin-layer plate, more than 90% of the radioactivity was recovered in the visible azo bands. Weber-Schalm fractionation (30) of these eluates, before or after lyophilization, revealed that 90% or more of the radioactivity and pigment distributed into the upper, polar layer.

The absorption spectrum of the ethanol eluate after lyophilization and subsequent solution in distilled water demonstrated a peak at 450 nm. However, there was a significant shoulder at 280 nm and marked fluorescence upon excitation at this wavelength. Because the absorption and fluorescence patterns were of the sort expected of protein and suggested contamination with albumin either from the affinity column or from the bile itself, an additional purification step was undertaken. As described above, the sample was extracted with 2 vol of chloroform to remove any possible traces of unconjugated bilirubin and subjected to gel filtration through Sephadex G-25. The spectrum of the resulting preparation demonstrated removal of the material with peak absorbance at 280 nm. This preparation did not fluoresce upon excitation at 285 nm and thin-layer chromatography with chloroform (31) again confirmed the absence of a band corresponding to unconjugated bilirubin. Ethyl anthranilate diazotization and thin-layer chromatography of this protein-free preparation revealed it to contain bilirubin diglucuronide almost exclusively, suggesting preferential binding of bilirubin diglucuronide, as compared with monoglucuronide, to the Sephadex G-25.

Glucuronic acid content was determined in two protein-free preparations of bilirubin diglucuronide as determined by thin-layer chromatography of the azo pigments. The molar ratio of glucuronic acid to bilirubin was 1.8:1 and 2.3:1, respectively.

Analysis of bile and column eluates for bilirubin and bile acids (Table I) demonstrated a marked decrease in the molar ratio of bile acids to bilirubin most evident in the ethanol and Sephadex eluates in which this ratio was 0.05:1 and <0.01:1, respectively, as compared to 11:1 in the starting bile. Simultaneous cholesterol analysis demonstrated it to be absent from all eluates; phospholipid content was 14 μmole in bile, 0.013 μmole in the water eluate, 1.7 μmole in the ethanol eluate, and 0.2 μmole in the Sephadex eluate. All phospholipid contamination was removed after extraction with chloroform–methanol; such extraction removed 10% or less of conjugated bilirubin.

When stored in a standard freezer for 2 weeks at −15°, these lyophilized preparations showed no change in Weber-Schalm fractionation. When diazotized after 2 weeks and subjected to thin-layer chromatography, less than 10% of radioactivity was lost from the visible bands. The ratio of glucuronide conjugates to other conjugates remained constant, but there was a 30 to 40% decrease in bilirubin diglucuronide content with a corresponding increase in bilirubin monoglucuronide.

Discussion. Previous attempts to purify conjugated bilirubin have resulted in preparations containing significant amounts of bile acids and other contaminants (6–19). The present method, utilizing affinity chromatography of rat bile, is relatively rapid and simple to perform, and produces a preparation of conjugated bilirubin virtually free of the major biliary contaminants. A degree of separation of monoglucuronide from diglucuronide may be accomplished by differential elution with distilled water followed by 50% ethanol. If bile from a Wistar-R rat is used, a preparation of conjugated bilirubin which is more than 90% bilirubin diglucuronide can be obtained from the water

TABLE I. PURIFICATION OF CONJUGATED BILIRUBIN FROM RAT BILE.

	Bilirubin[a]	Total bile acids[a]	Cholesterol[a]	Phospholipids[a]
Bile	2.8	31	18	14
Water eluate	0.2	0.1	0	0
Ethanol eluate	1.1	0.06	0	0
Sephadex eluate	0.2	<0.002	0	0

[a] μmole.

eluate. Bilirubin diglucuronide may also be obtained by subjecting the ethanol eluate to further chromatography on Sephadex G-25. These preparations of conjugated bilirubin are relatively stable when lyophilized and stored at −15°. Their long-term stability has yet to be established.

Previous studies of hepatic organic anion transport have been unable to directly examine the metabolism of conjugated bilirubin, in part due to difficulty in its purification. The method described here may facilitate investigation into this question as well as investigation into the physical characteristics and composition of the conjugates of bilirubin.

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