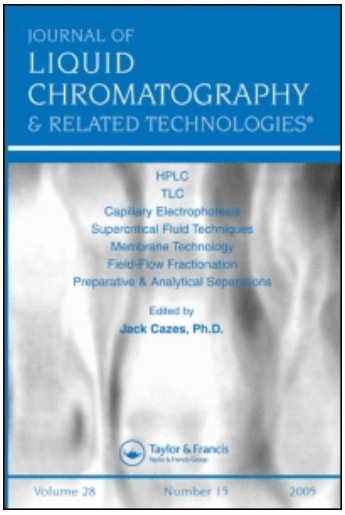

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THE SEPARATION OF CONJUGATED AND UNCONJUGATED BILIRUBIN IN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A fast and simple method was developed for the separation of unconjugated bilirubin and its mono- and di-glucuronide conjugates from bile by high-performance liquid chromatography (HPLC). Unconjugated bilirubin was separated on a reversed-phase column using acetonitrile-water (70:30 v/v) as the mobile phase, while the conjugates were separated on a μ-Bondapak-carbohydrate column employing acetonitrile-water (90:10 v/v) as the eluent. The application of this method was demonstrated by the analysis of the bile pigments in rat bile.

INTRODUCTION

The major conjugates of bilirubin in normal bile are the mono- and di-glucuronides (Figure 1) with small amount of other conjugates (1, 2). The compounds are both photosensitive and easily oxidised, making their separation and analysis difficult. They are therefore usually isolated and identified as their more stable diazo derivatives by coupling with a diazonium salt; this cleaves the tetrapyrroles forming dipyrrolic azopigments which are then characterised by thin layer chromatography (2). Bilirubin di-glucuronide yields two molecules of a slowly

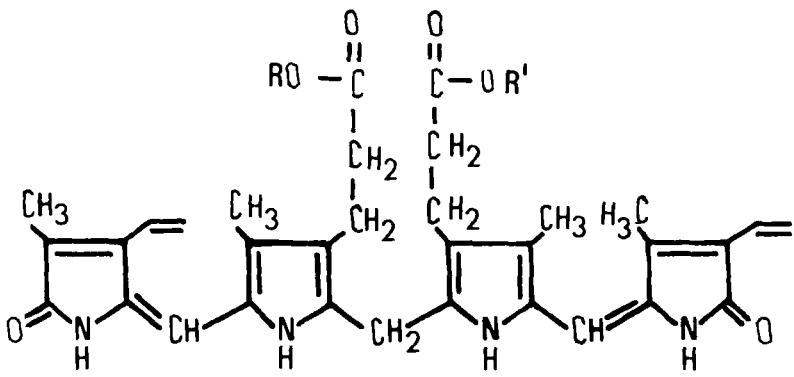

FIGURE 1 Structures of bilirubin and its conjugates
Bilirubin : R = R^1 = H
Bilirubin monoglucuronide : R = H, R^1 = glucuronide
or R = glucuronide, R^1 = H
Bilirubin diglucuronide : R = R^1 = glucuronide

migrating pigment (δ-azopyrrole) while the monoglucuronide gives a molecule of a faster moving pigment (α-azopyrrole) and one molecule of δ-azopyrrole. Ignoring isomerism due to arrangement of the β-side chain, δ-azopyrrole is the mono-glucuronide of α-azopyrrole.

This paper describes a fast and simple method for the separation of bilirubin and its conjugates in bile. Bile was collected from rats by biliary cannulation and analysed directly. No chemical manupulation and derivatisation was required. The conjugates were separated on a μ-Bondapak-carbohydrate column; unconjugated bilirubin was not retained on this column but was separated on a reversed-phase (octadecylsilane) column.

EXPERIMENTAL

Chemicals

Acetonitrile was HPLC grade from Rathburn Chemicals (Walkerburn) Ltd., Walkerburn, Peebleshire, Scotland. Bilirubin was from Sigma Chemical Company, St. Louis, Mo., U.S.A. A solution was made up by dissolving bilirubin in chloroform.

Apparatus

A Waters Associate's Model 6000A pump and U6K injector (Waters Associate, Milford, Ma., U.S.A.) was linked to a Cecil CE 212 (Cecil Instruments Ltd., Cambridge, U.K.) variable wavelength UV monitor set at 450 nm. Two columns were used: a reversed-phase column (Partisil-10-ODS, 25 cm x 4.6 mm I.D., Whatman Lab Sales Ltd., Maidstone, U.K.) for the separation of unconjugated bilirubin and a μ-Bondapak-carbohydrate column (30 cm x 3.9 mm I.D., Waters Associates) for the separation of the glucuronides.

Methods

Bile was collected in ascorbic acid solution from Sprague-Dawley rats by biliary cannulation under halothane-oxygen anaesthesia and with diazepam as a post-operation tranquilliser. The bile was protected from oxidation and photodegradation by covering the cannula and the specimen with black paper. For the separation of unconjugated bilirubin, freshly collected bile (20 μl) was injected directly into the reversed-phase column; the mobile phase was acetonitrile-water (70:30 v/v) and the flow rate was 1 ml/min (Figure 2). For bilirubin glucuronides, bile (50 μl) was injected directly into the μ-Bondapak-carbohydrate column with acetonitrile-water (90:10 v/v) as the mobile solvent at a flow rate of 1 ml/min. The eluates of the two major peaks M and D

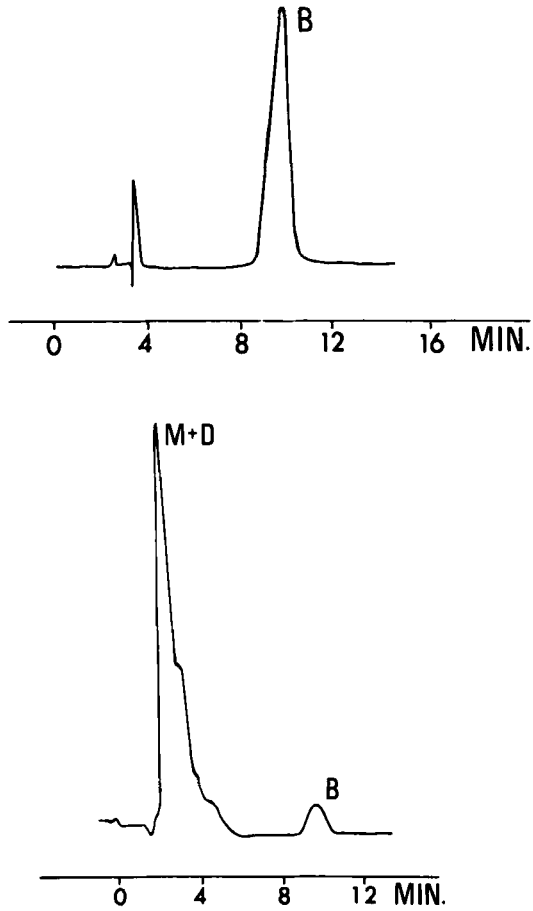

FIGURE 2 Separation of unconjugated bilirubin (a) standard (b) bile
B = bilirubin; M + D = bilirubin mono- and di-glucuronide.
Condition : column, Partisil-10 ODS; mobile phase, acetonitrile-water (70:30 v/v); flow rate, 1.0 ml/min; detection, u.v. 450 nm, 0.02 A.U.F.S.

(Figure 3) were combined to give two M and D fractions which were immediately freeze-dried in the dark. The residues were converted into their corresponding ethyl anthranilate azopyrroles and were

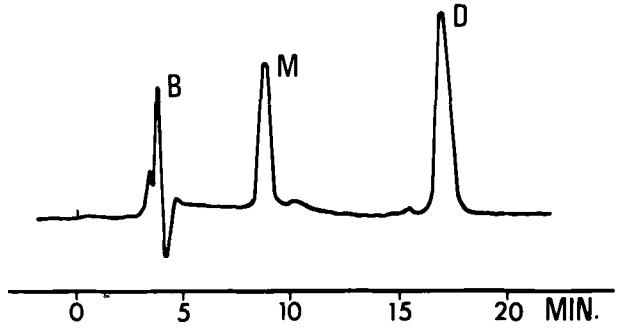

FIGURE 3 Separation of bilirubin mono- (M) and di-glucuronides (D) in bile. Conditions : column, μ-Bondapak-carbohydrate; mobile phase, acetonitrile-water (90:10 v/v); flow rate, 1.0 ml/min; detection, u.v. 450 nm, 0.02 A.U.F.S.

separated by t.l.c. (2). To reduce loss by oxidation the HPLC solvent systems were thoroughly degassed and saturated with nitrogen before use.

RESULTS AND DISCUSSION

The separation of unconjugated bilirubin by reversed-phase chromatography is shown in Figure 2. It is generally believed that little unconjugated bilirubin is present in rat bile (3, 4). The relatively small peak (B) observed supported this view (Figure 2b).

The peak M (Figure 3) obtained from the μ-Bondapak-carbohydrate column was proved to be mono-glucuronide of bilirubin by the preparation of the two different ethyl anthranilate azopyrroles (α and δ). The peak D contained bilirubin di-glucuronide as indicated by the marked predominance of δ-azopyrrole.

The μ-Bondapak-carbohydrate column, designed for the analysis of sugars and carbohydrates, is ideal for the separation of the mono- and di-glucuronides of bilirubin especially because the number of glucuronide groups is different in the two compounds. The pigment without a glucuronide group, i.e. unconjugated bilirubin, is not retained.

Conjugated bilirubin has never been isolated and characterised as a chemically pure compound (5-7) mainly because of contamination of the pigments with bile acid. As the great majority of bile acids do not possess a sugar group they will not be retained by the carbohydrate analysis column. HPLC is therefore an effective way of removing bile acids from bile pigments; it offers a closed system and provided the pigments collected from the effluents are well protected from light, photodegradation can be minimised.

CONCLUSION

Bilirubin glucuronides can be separated by HPLC using a carbohydrate analysis column. This technique is suitable for the isolation of large amounts of pure bilirubin glucuronides from bile. The speed and simplicity of the separation and purification procedure suggest that the method could easily be modified to measure conjugates in human plasma and facilitate the detailed study of hepatic secretion of bilirubin and its kinetics.

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