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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CONJUGATED AND UNCONJUGATED BILIRUBINS IN BODY FLUIDS

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SUMMARY

A novel high-performance liquid chromatography (HPLC) system is described for the separation of bilirubin and its conjugates in body fluids. Biliary bilirubin mono- and diglucuronide can be analysed directly on a C_{18} reversed-phase column with acetonitrile–dimethyl sulphoxide–0.1 *M* ammonium acetate (pH 5.16) (50:50:85, v/v/v) as mobile phase. However, the simultaneous determination of conjugated and unconjugated bilirubins in plasma required conversion of the conjugates into their methyl esters by alkaline methanolysis before HPLC separation of the C_{18} column eluted with acetonitrile–dimethyl sulphoxide–0.50 *M* ammonium acetate (pH 4.6) (50:50:40, v/v/v). The method is superior to, and more flexible than, previously described reversed-phase systems by allowing precise control of retention times by adjustment of pH, buffer concentration and the relative proportion of organic modifier in the mobile phase.

INTRODUCTION

Bilirubin in normal plasma is almost entirely unconjugated as it is transported from the reticuloendothelial system to the liver, where conjugation of one or both propionic acid side-chains to glycones occurs. In humans, conjugation with glucuronic acid (Fig. 1) predoninates, although small amounts of glucoside and xyloside conjugates are also present[1,2]. The conjugated bilirubins are then excreted in the bile.

The determination of conjugated and unconjugated bilirubins is important for studying bilirubin metabolism and for the differential diagnosis of various patholog-

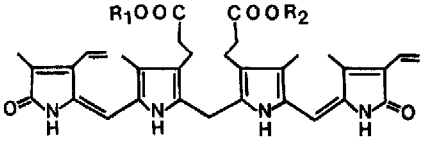

Fig. 1. Structures of bilirubin and bilirubin mono- and diglucuronides. Bilirubin: $R_1 = R_2 = H$; bilirubin C-8 monoglucuronide: R_1 = glucuronic acid, $R_2 = H$; bilirubin C-12 monoglucuronide: $R_1 = H$, R_2 = glucuroric acid; bilirubin diglucuronide: $R_1 = R_2$ = glucuronic acid.

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ical conditions in which bilirubin, either conjugated or unconjugated or both, accumulates in the plasma. The pigments are usually estimated by the direct and indirect diazo reaction under specific conditions[3,4], by solvent partition[5,6], or by direct spectrophotometry[7]. These methods lack specificity, sensitivity and/or accuracy. There is, therefore, a need for a specific and reliable method for separating and assaying these clinically important compounds. High-performance liquid chromatography (HPLC) is an obvious technique for achieving this purpose and reversed-phase ion-pair chromatography has been reported for the separation of conjugated and unconjugated bilirubins in bile[8,9]. These methods, however, are not applicable to the analysis of conjugated bilirubins in plasma due to strong binding of the pigments to proteins, which are difficult to release by solvent extraction. Blanckaert[10] solved this problem by treating conjugated bilirubins in plasma with 2% potassium hydroxide in methanol. The resulting mono- and dimethyl esters, together with the unreacted bilirubin, were then separated by adsorption chromatography[11]. This paper describes a novel reversed-phase system for the direct and effective separation of conjugated and unconjugated bilirubins in bile and, after transesterification, in plasma. The retention behaviour of these pigments was studied in order to develop an optimum system for a particular application.

EXPERIMENTAL

Materials and reagents

Bilirubin-IX α, the natural pigment, was from Sigma (Poole, U.K.). The IIIα and XIIIα isomers were prepared by acid-catalysed rearrangement of bilirubin-IXα and isolated by thin-layer chromatography[12]. Bilirubin mono- and dimethyl esters were synthesised according to the method of Blanckaert[10]. Ascorbic acid, EDTA (disodium salt), glycine, hydrochloric acid, potassium hydroxide, chloroform, methanol, ammonium acetate, glacial acetic acid and dimethyl sulphoxide (DMSO) were AnalaR grade from BDH (Poole, U.K.). Acetonitrile was HPLC grade from Rathburn Chemicals (Walkerburn, U.K.).

Bile samples

Human bile was obtained by duodenal aspiration and was injected directly onto the HPLC column. Bilirubin mono- and diglucuronide-enriched bile was obtained from rats infused intravenously with bilirubin[10]. This bile was used as reference for bilirubin mon- and diglucuronide.

Plasma samples

Alkaline methanolysis[10] was used. The whole procedure was carried out in dim light. Ascorbic acid (30 mg), EDTA (1.5 mg) and methanol (2 ml) were mixed with 250 μl of plasma. A 3-ml aliquot of 2% (w/v) potassium hydroxide in methanol was added and the mixture was immediately vortex-mixed for 60–90 s. Chloroform (4 ml) and 5 ml of glycine–hydrochloric acid buffer (0.4 mol/l hydrochloric acid adjusted to pH 2.7 with solid glycine) were added sequentially. The mixture was shaken vigorously and then centrifuged for 2 min at 2000 *g*. The chloroform layer was transferred to a dry tube and evaporated to dryness under nitrogen at 30°C. The residue was redissolved in DMSO (250 μl) for HPLC analysis.

High-performance liquid chromatography

A Pye Unicam (Cambridge, U.K.) PU4010 liquid chromatograph with a variable-wavelength UV–VIS detector set at 450 nm was used. Sample injection was by Rheodyne injector, fitted with a 100-μl loop. The separation was carried out on a 25 cm × 5 mm I.D. ODS-Hypersil column (Sandon Southern, Runcorn, U.K.). The mobile phases contained various proportions of acetonitrile–DMSO–ammonium acetate buffer, typically 50:50:90 or 50:50:40 (v/v/v), at different pH levels (4.6–6.8) and buffer concentrations (0.1–0.5 *M*).

RESULTS AND DISCUSSION

HPLC of bilirubin mono- and diglucuronide

Fig. 2 shows the separation of bilirubin mono- and diglucuronides, isolated from rat bile, by thin-layer chromatography[13] on an ODS-Hypersil column eluted with acetonitrile–DMSO–0.1 *M* ammonium acetate (pH 5.16) (50:50:85, v/v/v). This system compares favourably with reversed-phase ion-pair chromatography[8,9] in terms of speed and resolution, as shown by the rapid and effective separation of the C-8 and C-12 monoglucuronide isomers. It is also well suited to the preparative isolation of the bile pigments where ion-pair chromatography is not applicable.

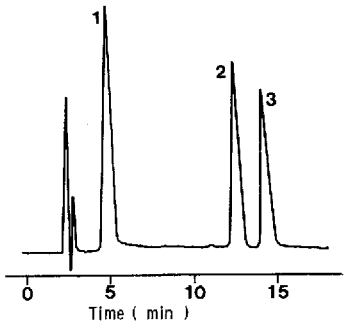

Fig. 2. Separation of a standard mixture of bilirubin mono- and diglucuronide. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.1 *M* ammonium acetate (pH 5.16) (50:50:85, v/v/v); flow-rate, 1 ml/min; detector, 450 nm. Peaks: 1 = bilirubin diglucuronide; 2 = bilirubin C-8 monoglucuronide; 3 = bilirubin C-12 monoglucuronide.

The inclusion of DMSO, a solvent widely used for bilirubin, is intended to improve the solubility of the bile pigments. This is particularly important when preparative HPLC is performed. Acetonitrile is added to reduce the relatively high column back-pressure generated by mixing DMSO and ammonium acetate, and to provide a better and more flexible system afforded by the ternary mobile-phase system. Solubility considerations also led to the choice of a C_{18} column rather than one with a shorter bonded-phase chain-length, as higher proportions of organic modifier can be used for elution.

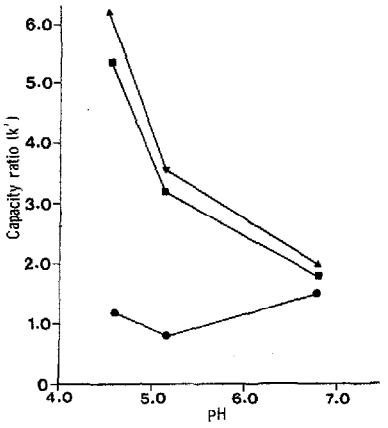

Fig. 3. Effect of pH on the retention of bilirubin mono- and diglucuronide. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.25 *M* ammonium acetate at various pH values (50:50:90, v/v/v). ● = Diglucuronide; ■ = C-8 monoglucuronide; ▲ = C-12 monoglucuronide.

Effects of pH and buffer concentration on retention and resolution of bilirubin mono- and diglucuronides

The retention and resolution of bilirubin mono- and diglucuronides can be easily controlled by altering the pH and/or buffer concentration of the mobile phase. Increasing the pH significantly decreased the retention of the monoglucuronides (Fig. 3). This indicated that retention control by pH adjustment is mainly mediated by the ionization state of the propionic acid moiety. Complete ionization of this group in the monoglucuronides at higher pH would obviously lead to rapid elution.

The molar concentration of ammonium acetate between 0.1 and 0.25 *M* at pH 5.16 had little effect on the retention of bilirubin mono- and diglucuronides (Fig. 4). At higher buffer concentration (0.5 *M*), however, retention increased, the monoglucuronides being more significantly affected than the diglucuronide.

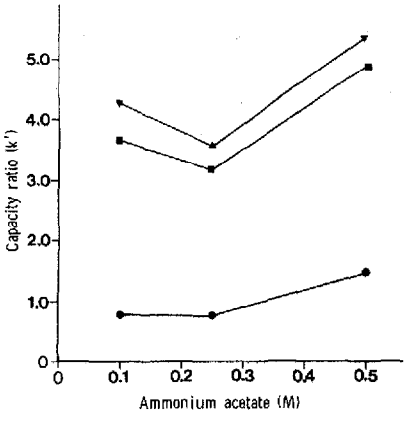

Fig. 4. Effect of ammonium acetate concentration on the retention of bilirubin mono- and diglucuronide. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–ammonium acetate (pH 5.16) at various concentrations (50:50:90, v/v/v). ● = Diglucuronide; ■ = C-8 monoglucuronide; ▲ = C-12 monoglucuronide.

HPLC of conjugated and unconjugated bilirubin in bile

Fig. 5 shows the separation of conjugated and unconjugated bilirubins in human bile by using the above reversed-phase system. Bilirubin diglucuronide was the main component, with smaller amounts of both C-8 and C-12 monoglucuronides. A trace of bilirubin (unconjugated) was also detected. This strongly-retained compound required acetonitrile–DMSO–ammonium acetate (50:50:40, v/v/v) for elution. A number of unidentified minor peaks on the chromatogram were probably due to the glucoside and xyloside conjugates of bilirubin.

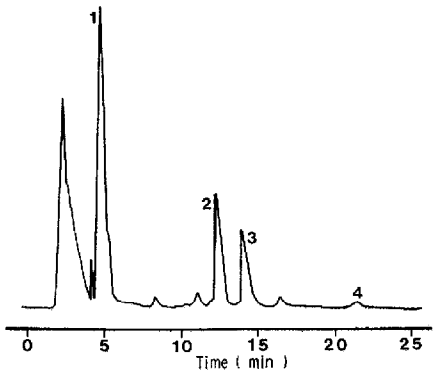

Fig. 5. Separation of conjugated and unconjugated bilirubin in human bile. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.1 *M* ammonium acetate (pH 5.16) (50:50:85, v/v/v) for 15 min, then 50:50:40 (v/v/v) for 10 min; flow-rate, 1 ml/min; detector, 450 nm. Peaks: 1 = bilirubin diglucuronide; 2 = bilirubin C-8 monoglucuronide; 3 = bilirubin C-12 monoglucuronide; 4 = bilirubin.

HPLC of bilirubin, bilirubin mono- and dimethyl esters

The separation of these compounds is essential, since the simultaneous determination of conjugated and unconjugated bilirubin in plasma is based on the conversion of bilirubin conjugates to their methyl esters by alkaline methanolysis[10]. Fig. 6 shows the separation of a standard mixture containing the IIIα, IXα and XIIIα isomers of bilirubin, bilirubin monomethyl ester and bilirubin dimethyl ester on ODS-Hypersil. These pigments are much more hydrophobic than the glucuronide conjugates, and acetonitrile–DMSO–0.5 *M* ammonium acetate (pH 4.6) (50:50:40, v/v/v) was necessary for elution.

The efficiency of the system is clearly demonstrated by the ease with which the isomers can be resolved. The ability to separate the IIIα, IXα and XIIIα isomers is important because commercially available bilirubin-IXα (the natural isomer) invariably contains the IIIα and XIIIα isomers as impurities, and purification may be necessary for certain biological applications. For preparative purification of bilirubin isomers, a mobile phase of acetonitrile–DMSO–0.1 *M* ammonium acetate (pH 4.6) (40:60:50, v/v/v), is recommended. Fig. 7 shows the degree of separation that can be achieved with this system. This is far superior to the previously described reversed-phase method for separating bilirubin isomers[14], where the only parameter for controlling resolution was the content of organic modifier in the eluent. The presence of a buffer in the mobile phase allows precise control of retention and resolution by

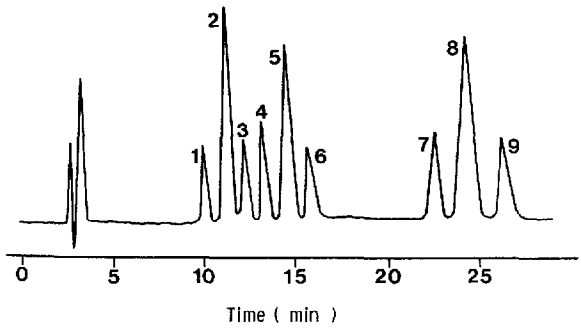

Fig. 6. Separation of a standard mixture of bilirubin, bilirubin monomethyl and dimethyl esters and their isomers. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.5 *M* ammonium acetate (pH 4.6); flow-rate, 1 ml/min; detector, 450 nm. Peaks: 1 = bilirubin-XIIIα monomethyl ester; 2 = bilirubin-IXα monomethyl ester; 3 = bilirubin-IIIα monomethyl ester; 4 = bilirubin-XIIIα; 5 = bilirubin-IXα; 6 = bilirubin-IIIα; 7 = bilirubin-XIIIα dimethyl ester; 8 = bilirubin-IXα dimethyl ester; 9 = bilirubin-IIIα dimethyl ester.

manipulation of pH, buffer concentration and organic modifier content. The buffer pH provides one of the best parameters for retention control. The effect is shown in Fig. 8. Increasing the pH decreased the retention of bilirubin and bilirubin monomethyl ester, with the former being affected more than the latter. The elution order was reversed when the pH was changed from 4.6 to > 5.0. This behaviour is consistent with the fact that as the pH is raised, bilirubin, having two ionizable propionic acid groups, becomes progressively less hydrophobic than bilirubin monomethyl ester, which has one propionic acid group. The opposite trend was observed for bilirubin dimethyl ester, *i.e.*, retention increased with increasing pH. Without an acid group, the ionization state of the pyrrole nitrogens becomes a factor in controlling the retention by pH variation. Increasing the pH suppressed nitrogen ionization and, consequently, increased the hydrophobicity and, therefore, the retention.

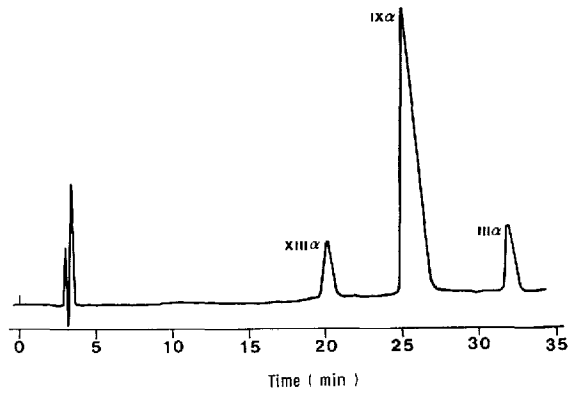

Fig. 7. Separation of bilirubin-XIIIα, IXα and IIIα isomers. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.1 *M* ammonium acetate (pH 4.6) (40:60:60, v/v/v); flow-rate, 1 ml/min; detector, 450 nm.

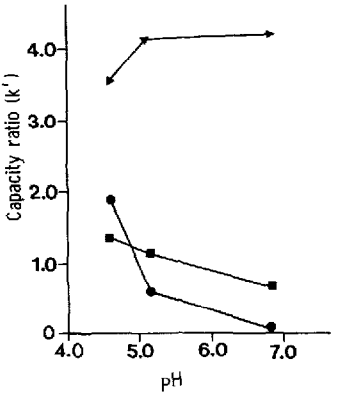

Fig. 8. Effect of pH on the retention of bilirubin and bilirubin monomethyl and dimethyl esters. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.1 *M* ammonium acetate at various pH values (50:50:40, v/v/v). ● = Bilirubin-IXα; ■ = bilirubin-IXα monomethyl ester; ▲ = bilirubin-IXα dimethyl ester.

HPLC of conjugated and unconjugated bilirubin in plasma

The application of the system to the analysis of plasma bilirubin is shown in Fig. 9. In normal plasma (Fig. 9a), only bilirubin-IXα was detected. In the plasma of a patient with conjugated hyperbilirubinaemia caused by posthepatic (obstructive) jaundice (Fig. 9b), both mono- and diconjugated bilirubins were markedly elevated. The method is particularly useful for the differential diagnosis of neonatal jaundice and for monitoring the effectiveness of phototherapy.

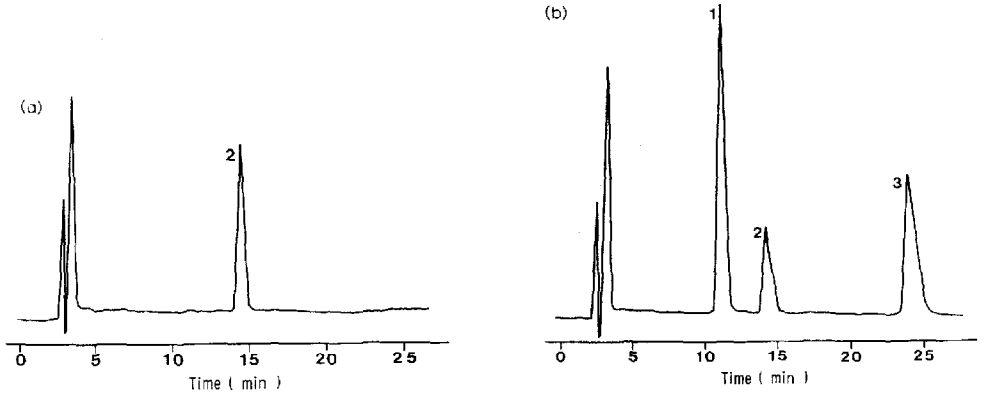

Fig. 9. Separation of conjugated and unconjugated bilirubins in plasma after alkaline methanolysis. (a) Normal subject and (b) patient with hyperbilirubinaemia. Column, ODS-Hypersil; eluent, acetonitrile–DMSO–0.5 *M* ammonium acetate (pH 4.6); flow-rate, 1 ml/min; detector, 450 nm. Peaks: 1 = bilirubin monomethyl ester; 2 = bilirubin; 3 = bilirubin dimethyl ester.

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