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High-performance liquid chromatographic separation of bilirubin conjugates: the effects of change in molarity and pH

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SUMMARY

A fast, sensitive high-performance liquid chromatographic method has been developed for the separation and quantitation of biliary bile pigments; this utilizes a C_{18} reversed-phase column with two solvents, a buffer and an organic solvent, which were changed in a linear gradient from a polar to a less polar combination. Nine glycosidic conjugates of bilirubin as well as unconjugated bilirubin and a suitable internal standard, unconjugated mesobilirubin IXα, were all separated to baseline by gradient elution; the species eluted in a polar to less polar fashion. Increasing the molarity of the solvent decreased the binding of non-glucuronide pigments to the column, with a decrease in their retention times, whereas for bilirubin monoglucuronide they increased. Decrease in pH, similarly, preferentially increased bilirubin monoglucuronide retention times.

INTRODUCTION

Since 1975 high-performance liquid chromatography (HPLC) has been utilized as a separation tool to resolve the constituents of mixtures of bile pigments through their differential migration [1]. This allows the separation and quantitation of both the polar bilirubin conjugates and the non-polar bilirubin. In this approach, the conjugates of bilirubin separate, as a result of their phys-

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ical and chemical properties, in an increasing buffer solvent gradient and the non-polar bilirubin is bound to the hydrophobic column and removed with an organic solvent. For the various systems developed, reproducibility and completeness of separation of the multiplicity of species have been difficult to assess because the conjugates of bilirubin are not commercially available and biological sources are uncharacterized mixtures of the compounds. The matter becomes even more complex at an analytical level because, after their biological formation, the conjugates of bilirubin are easily destroyed by processes involving photochemical and oxidative degradation or modified by molecular rearrangement and hydrolysis, and substantial precautions are needed to avoid these processes. Since either formation of dipyrrolic diazo derivatives or alkaline methanolysis of conjugates provide for the formation of stable compounds [2,3], methods utilizing these have been used to approach the analysis of mixtures of conjugates. With each of these approaches, however, the identification and quantitation of the original native tetrapyrroles present in biological fluids become difficult or impossible to carry out, when any substantial number of the conjugates is present.

In the present work, an HPLC method was developed, with a reversed-phase column, for the analysis of the native bilirubin tetrapyrroles. Bilirubin and its conjugates are bound more or less strongly to the column, in a dynamic equilibrium, and are progressively eluted with variation in the mobile phase to modulate the association of the pigments with the column. Nine conjugates of bilirubin in dog gallbladder bile were separated to baseline by this approach, and their quantification was facilitated by the addition of commercially available mesobilirubin IXα as an internal standard. The occurrence of the scrambling phenomenon [4] in vitro in the laboratory (the conversion of the physiologically characteristic IXα compounds by dipyrrolic schism and recombination to a reconstituted mixture of the symmetric bilirubin XIIIα, asymmetric IXα and symmetric IIIα forms, in 1:2·1 proportions) has also made it necessary to ensure that the analytical methodology developed separates not only the parent IXα conjugates but also the XIIIα and IIIα isomers. We also explore the effects of change in molarity and pH, and define the systematic changes in the elution patterns of bilirubin and its conjugates which are associated with their variation. The resulting generalities provide a key to the adaptation of the approach in various other methodological environments.

Dog bile was chosen as the suitable substrate for these explorations because its diazo derivatives [5–7] had been shown to contain all three of the glycosidic moieties (glucuronic acid, glucose and xylose) which are conjugated with bilirubin in various species. It is readily available and can serve as a reference material.

EXPERIMENTAL

Materials

HPLC-grade dimethylsulfoxide, chloroform, methanol, 1-butanol and acetonitrile were obtained from Caledon Labs. (Georgetown, Canada); 1-pentanesulfonic acid (sodium salt), tetraheptylammonium chloride and ethyl anthranilate were obtained from Eastman Kodak (Rochester, NY, U.S.A.). Pentan-2-one was obtained from BDH (Montreal, Canada), Whatman PLK5 thin-layer chromatographic (TLC) plates were obtained from Chromatography Specialities (Montreal, Canada) and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), uridine-5′-diphosphoglucuronic acid, uridine-5′-diphosphoglucose and uridine-5′-diphosphoxylose were obtained from Sigma (St. Louis, MO, U.S.A.). Bilirubin and mesobilirubin IXα were obtained from Porphyrin Products (Logan, UT, U.S.A.). Analysis of the commercial bilirubin used in these studies by HPLC indicated that it contained the following isomeric forms by weight: 9% XIIIα, 80% IXα and 11% IIIα. High-purity nitrogen was used. All other chemicals were of reagent grade.

High-performance liquid chromatographic equipment

A Hewlett-Packard 1084A high-performance liquid chromatograph with two pumps, a fraction collector and a variable-wavelength detector set at a wavelength of 440 nm were used in this study. Solvent and column temperatures were maintained at 40°C. For the analytical separations, a 5-μm fully capped Spherisorb C_{18} column (25 cm×0.40 cm I.D.) (Chromatography Sciences, Montreal, Canada) or, alternatively, a Hewlett-Packard (Kirkland, Canada) reversed-phase C_{18} column (20 cm×0.46 cm I.D.) were used.

Extraction of dog gallbladder bile

Bilirubin and bilirubin conjugates were extracted from dog bile by the addition of 300 mg of ammonium sulfate and 1 ml of chloroform–ethanol (50:50, v/v) to 1 ml of diluted gallbladder bile, containing 5–40 μg of bilirubin equivalents. Samples were then vigorously mixed, centrifuged at 100 *g* for 10 min at 2°C, and the bottom layer was removed and dried down under nitrogen.

Preparation of bilirubin conjugates

Bilirubin diglucoside, bilirubin dixyloside, bilirubin monoglucoside and bilirubin monoxyloside were formed by use of the UDP-bilirubin transferase (EC 2.4.1.17) [8] in dog microsomal preparations [9] in the presence of the cosubstrates UDP-glucose or UDP-xylose. Microsomes were prepared from perfused dog liver by differential centrifugation [10] and activated with CHAPS (10 m*M* in a 10 mg/ml suspension of microsomal protein). Each ml of assay contained 5 mg protein, 10.5 μmol magnesium chloride, 20 nmol bilirubin and either 7.3 μmol UDP-glucose or 7.7 μmol UDP-xylose in 0.05 *M* triethanolamine buffer, pH 7.8. The assay was incubated at 37°C in the dark for 30 min

and the reaction was stopped by immersion in dry ice and acetone. The samples were then diluted by a factor of 1.5 and each ml was extracted with 300 mg ammonium sulfate and 1 ml of chloroform–ethanol (50:50, v/v) as outlined above.

Separation of bilirubin and its conjugates by high-performance liquid chromatography

The previously described extracts were resuspended in 1 ml dimethylsulfoxide, passed through a 0.45-μm filter, and aliquots of 10 to 50 μl were injected into the liquid chromatographic system. With the first column, two solvent systems (buffers A and B) were utilized to separate the compounds: buffer A contained distilled water–acetonitrile–dimethylsulfoxide (58:16:26, v/v) and buffer B contained sodium acetate (0.1 *M*) with 1-pentanesulfonic acid (5 m*M*) at a pH of 4.0–acetonitrile–dimethylsulfoxide (15:56:34, v/v). The flow-rate for the chromatographic assay system was set at 0.7 ml/min and the initial solvent mixture was set at 85% A and 15% B. Over the first 18 min the proportion of solvent B was increased linearly to 55% and, over the following 7 min, to 85% and maintained there for the rest of the run (40 min). An equilibration delay of 10 min, at initial composition, was necessary before the next run.

With the second column, two different solvent systems were used: buffer A consisted of 0.1 *M* sodium acetate buffer at pH 4.0 with 5 m*M* 1-pentanesulfonic acid and buffer B consisted of acetonitrile–dimethylsulfoxide–buffer A (56:34:15, v/v). The flow-rate was set at 0.9 ml/min and the initial solvent mixture at 53% A and 47% B. Over the first 23 min the proportion of B was increased linearly to 75%, then to 88% at 28 min and maintained at this level until 41 min. An equilibrium delay of 10 min at initial composition was again necessary before the next run. Both approaches yielded essentially identical patterns.

Identification of the compounds

The compounds were identified by two methods: the microsomal enzymatic formation of the compound with appropriate cosubstrates and the subsequent identification of its elution pattern both alone and on addition of the compound to extracts of bile; and collection of the peaks eluting from the HPLC instrument and then formation and identification of the dipyrrolic ethyl anthranilate derivatives of the compound [2]. In the second procedure, the peaks were collected and the acetonitrile evaporated under nitrogen, after which an ammonium sulfate and chloroform–ethanol (50:50, v/v) extraction was performed as described above for bile. The dried down extract was then resuspended in dimethylsulfoxide and the diazo derivatives formed with ethyl anthranilate. Prior to diazotization, an aliquot of the resuspended sample was

reinjected into the liquid chromatograph to assure the identity and purity of the collected compound.

Thin-layer chromatography for the preparation of reference bile pigments

Tetrapyrroles. Dried down extracts of dog gallbladder bile were resuspended in chloroform containing 10 m*M* tetrapheptylammonium chloride and spotted on preparative TLC plates. The plates were developed in a solvent system consisting of chloroform–methanol–water (60:30:6, v/v) [7]. The resulting bile pigment bands were then scraped, extracted with dimethylsulfoxide, and the extract was filtered through a 0.45-μm filter. An aliquot was injected onto the HPLC system and a second aliquot was taken to prepare diazo derivatives.

Ethyl anthranilate diazo derivatives. The prepared dipyrrolic diazo derivatives were spotted on TLC plates and developed in a solvent system consisting of chloroform–methanol–water (65:25:3, v/v) [2]. The separated dipyrrolic azo derivatives corresponding to unconjugated species (α_0), and the glucuronic (δ), glucose (α_3) and xylose (α_2) conjugates were then collected and quantitated.

Quantification of bile pigments

The conjugates of bilirubin are not available commercially. We were able to prepare each conjugate (nine in total) and develop the standard curves for each, but due to the relative instability of the conjugates and the extensive preparation time involved, this approach did not prove to be useful. We therefore looked for a commercially available tetrapyrrole related to bilirubin, one with a similar spectrum, and developed a procedure for quantifying the bilirubin conjugates with reference to this, utilized as an internal standard. We found mesobilirubin IXα to be such an appropriate reference compound. It provides the key to extending the methodology for assaying bilirubin conjugates to the whole spectrum of biological fluids in which they are encountered.

Preparation of bilirubin and mesobilirubin IXα standard curves

Stock solutions of both compounds were prepared as follows: 10 mg of dry bilirubin and 10 mg of mesobilirubin IXα were weighed out and each was dissolved in 20 ml of dimethylsulfoxide. Further dilutions were made from the stock solution and aliquots were taken, filtered through a 0.45 μm filter and injected into the HPLC system. The peak areas for various concentrations of bilirubin IXα and mesobilirubin IXα (concentration range 5–200 μM) were identical. Thus the mesobilirubin IXα provides an ideal reference compound.

RESULTS

Separation and identification of the bilirubin conjugates in dog bile

HPLC analysis of dog bile, carried out with the first approach, revealed the following compounds eluting in sequence, as indicated in Fig. 1: 1 = bilirubin

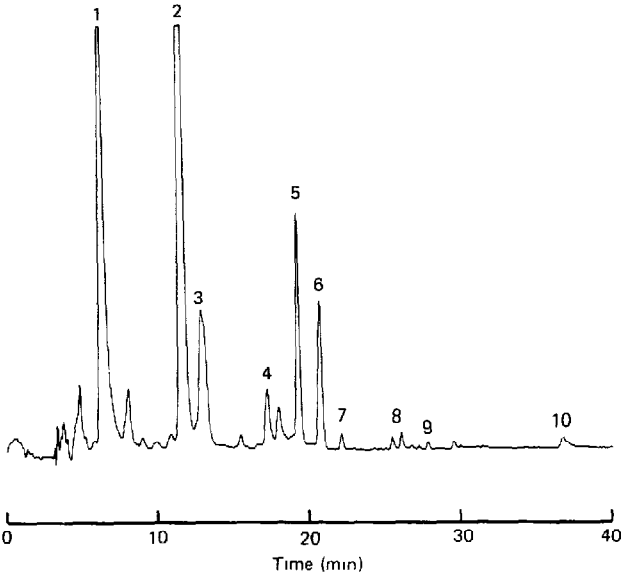


Fig. 1. Chromatograms of dog gallbladder bile. Peaks: 1 = bilirubin diglucuronide; 2 = bilirubin monoglucuronide monoglucoside; 3 = bilirubin monoglucuronide monoxyloside; 4 = the bilirubin monoglucuronide isomer pair (C_8, C_{12}); 5 = bilirubin diglucoside; 6 = bilirubin monoglucoside monoxyloside; 7 = bilirubin dixyloside; 8 = bilirubin monoglucoside isomer pair (C_8, C_{12}); 9 = bilirubin monoxyloside; 10 = bilirubin IXα.

diglucuronide; 2 = bilirubin monoglucuronide monoglucoside; 3 = bilirubin monoglucuronide monoxyloside; 4 = the bilirubin monoglucuronide isomer pair (C_8, C_{12}); 5 = bilirubin diglucoside; 6 = bilirubin monoglucoside monoxyloside; 7 = bilirubin dixyloside; 8 = the bilirubin monoglucoside isomer pair (C_8, C_{12}); 9 = bilirubin monoxyloside; 10 = bilirubin IXα. With the addition of known amounts of commercial bilirubin IXα and its isomers (XIIIα and IIIα), and of mesobilirubin IXα to dog gallbladder bile, the elution profile of the chromatogram remained the same except that now bilirubin IXα (peak 10) was increased by the amount added, together with its earlier XIIIα and later IIIα symmetrical isomers, and the peak for mesobilirubin (peak 11) then followed (Fig. 2).

Bilirubin diglucuronide and bilirubin monoglucuronide monoglucoside were isolated from extracted dog bile by use of TLC. The extracted bands were each divided into two aliquots: one aliquot was injected directly into the HPLC column, both alone and mixed with an extract of the bile sample; the other was utilized to form ethyl anthranilate derivatives, which were then identified by use of a diazo TLC system [2]. Bilirubin diglucuronide (peak 1), with a retention time of 6.6 min on the liquid chromatographic column, was identified by the formation of the single ethyl anthranilate derivative δ. Bilirubin monoglucuronide monoglucoside (peak 2), with a retention time of 11.7 min, was

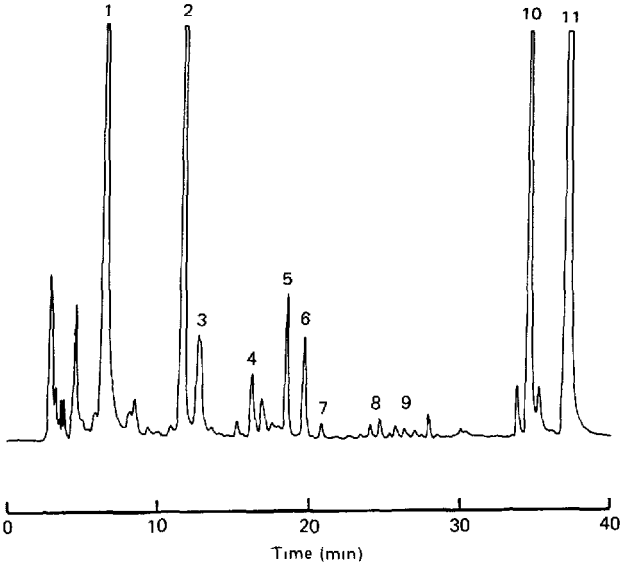


Fig. 2. Chromatograms of dog gallbladder bile with added bilirubin (and its isomers) and mesobilirubin. Peaks 1–9 as listed in Fig. 1; peak 10 = bilirubin IXα with its preceding bilirubin XIIIα and following bilirubin IIIα isomers; peak 11 = mesobilirubin IXα.

identified by the formation of equal amounts of the ethyl anthranilate derivatives δ and α_3.

The remaining compounds were not adequately separated by TLC and it was necessary to identify them by other means. As an alternative, compounds were formed by use of the enzyme UDP-bilirubin transferase in hepatic microsomal preparations, with UDP-glucose and UDP-xylose as cosubstrates. An aliquot of the extracted medium of the biosynthetic system was chromatographed, and a second aliquot was then also added to the extract from dog bile and the combination was injected into the high performance liquid chromatograph (Fig. 3). A remaining aliquot was diazotized with ethyl anthranilate and analyzed. The retention times of the tetrapyrrole conjugates formed with UDP-glucose were 18.4 min for a single peak and 23.8 and 24.5 min for a later doublet. The first compound (peak 5), on recollection, formed a single α_3 derivative with ethyl anthranilate, indicating that its parent compound was bilirubin diglucoside. The two elements of the second set of twin peaks (the peaks 8) formed α_0 and α_3 as derivatives, each in equal amounts, indicating that this twin peak contained the two isomers (C_8, C_{12}) of bilirubin monoglucoside. Addition of the isolated bilirubin conjugates to the extract from dog bile resulted in increases in the corresponding peak areas, indicating an identity of the corresponding compounds in the extracts.

Utilizing UDP-xylose as the cosubstrate, we found the peak retention times of the products were, on the HPLC system (Fig. 4), 21.5 min for the first

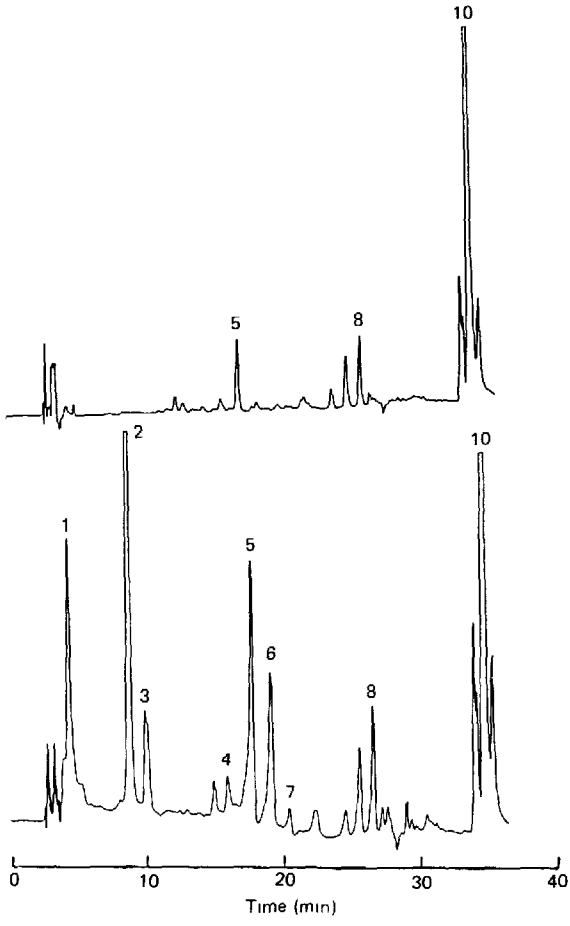


Fig. 3. (Upper) Chromatogram of compounds formed by the microsomal UDP-bilirubin transferase with UDP-glucose as cosubstrate. Peak 5 = bilirubin diglucoside; peak 8 = the two isomers (C_8 and C_{12}) of bilirubin monoglucoside. (Lower) Chromatogram of dog gallbladder bile with the addition of compounds shown in the upper panel. An increase in peak areas 5 and 8, that is, in the bilirubin diglucoside and the two monoglucoside isomer peaks, occurred, equivalent to the amount added.

compound (peak 7) and 26.9 min for the second compound (peak 9). The ethyl anthranilate derivative formed from the first compound was α_2 alone, indicating that it was bilirubin dixyloside, whereas both α_0 and α_2 were formed in equal amounts from the second compound, indicating that it was a bilirubin monoxyloside. The pattern obtained on mixture with an extract of dog bile again demonstrated identity with the corresponding compounds in the extract from dog bile.

In another approach, the compounds with a retention time of 12.3 min (peak 3) and 19.9 min (peak 6) were collected from the HPLC effluent, and the

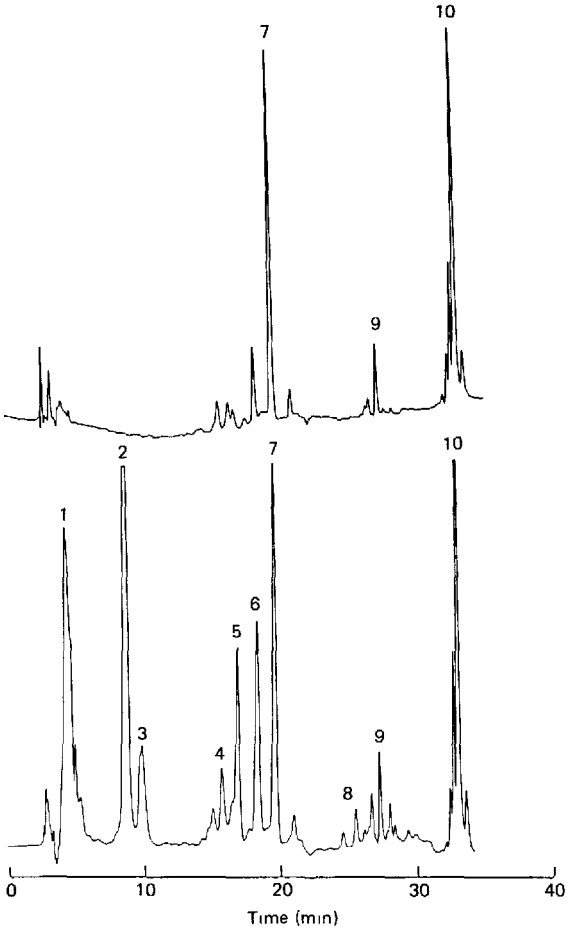


Fig. 4. (Upper) Chromatogram of the compounds formed by microsomal UDP-bilirubin transferase with UDP-xylose as the cosubstrate. Peak 7 = bilirubin dixyloside; peak 9 = bilirubin monoxyloside. Note the XIIIα and IIIα isomers of peak 7, the bilirubin dixyloside. (Lower) Chromatogram of dog gall bladder bile with the addition of the compounds shown in the upper panel. An increase in the peak areas of bilirubin dixyloside and bilirubin monoxyloside, that is, peaks 7 and 9, occurred equivalent to the amount added.

fractions from several runs were pooled. The pooled eluates were extracted with ammonium sulfate and chloroform–ethanol, and the ethyl anthranilate derivatives were produced. In the case of peak 3, the diazo derivatives formed were δ and α_2 in equal amounts, indicating that the compound was bilirubin monoglucuronide monoxyloside. Peak 6 formed the diazo derivatives α_2 and α_3 in equal amounts, demonstrating that this compound was bilirubin monoglucoside monoxyloside.

The bilirubin monoglucuronide isomer pair (that is, the C_8 and C_{12} forms)

were isolated by TLC from pig bile, in which these form the dominant conjugate. It was added to dog gallbladder bile and the mixture pattern showed an identity with the two peaks 4. The retention times of these were 16.3 and 16.9 min, respectively (Fig. 5). From the remainder of the extract, the ethyl anthranilate diazo dipyrrolic derivatives were formed and identified. These were found, for each of the peaks 4, to consist of δ and α_0 in equal amounts, indi-

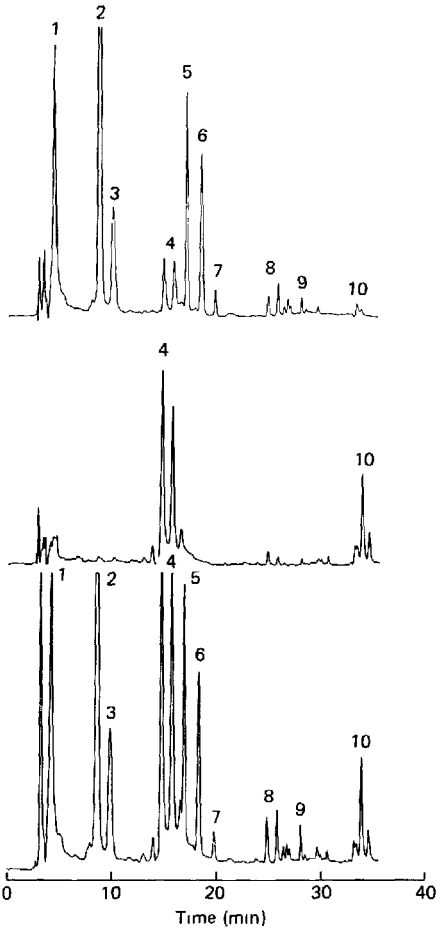


Fig. 5. (Upper) Chromatogram of the dog gallbladder bile used for the following mixture experiment. (Middle) Chromatogram of bilirubin monoglucuronide (C_8 and C_{12} isomers) isolated from pig bile, predominantly the peaks 4. Four isomers of bilirubin monoglucuronide are present (the XIIIα, IX-α C-8, IXα C-12 and IIIα isomers), the XIIIα and IIIα species being present in much lower concentration. (Lower) Chromatogram of gallbladder bile with the addition of the pig bilirubin monoglucuronide characterized in the middle panel. An increase in the bilirubin monoglucuronide (peaks 4) occurred, equivalent to the amount added. The bilirubin IIIα monoglucuronide isomer overlaps the front edge of peak 5, the bilirubin diglucoside.

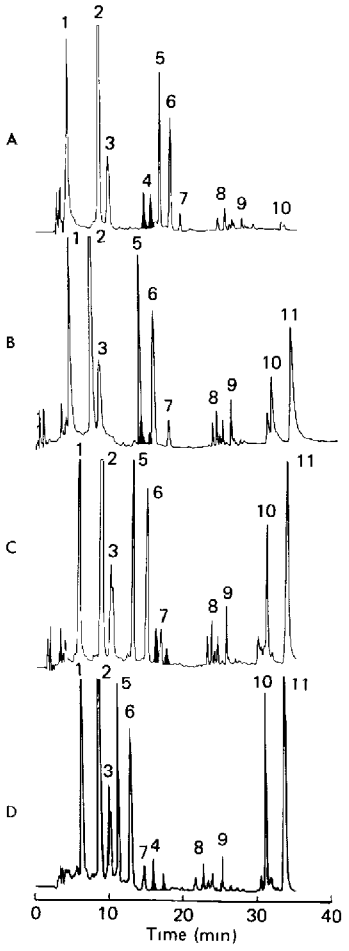
cating that the compound consisted of the two (C_8, C_{12}) bilirubin monoglucuronides.

Influence of changes in molarity on the separation patterns for various conjugates

The quite good separation of bilirubin, mesobilirubin and the nine conjugates of bilirubin, illustrated above, was achieved as the result of a previous general exploration of the effect of changing the molarities of solvents A and B and of changing the pH values of these solvents. The phenomena encountered are of interest. To display the effects of changes in molarity, we utilized the first column and an entirely different set of buffers for the two solvent systems. The flow-rate in the HPLC system was set at 0.78 ml/min. The solvent systems utilized were sodium acetate (0.0065 *M*) and 1-pentanesulfonic acid (0.3125 m*M*) at pH 4.0 in buffer A, and acetonitrile and dimethylsulfoxide (60:40, v/v) in buffer B. The solvent gradient was developed in the following fashion: the initial solvent mixture was set at 53% A and 47% B; the proportion of B was linearly increased to 52% at 15 min and to 80% at 25 min. Separation of the conjugates of bilirubin was excellent. However, as the gradient was developed and the proportion of buffer B was increased to levels of 80%, although unconjugated bilirubin was eluted from the column, the separation of its isomers did not occur in any clear-cut fashion (Fig. 6A). The molarity of the system was therefore increased in buffer A to 0.0125 *M* sodium acetate and 0.625 m*M* 1-pentanesulfonic acid while the composition of buffer B was allowed to remain the same and the gradient was developed in similar fashion. As illustrated in Fig. 6B, the pattern of separation of the conjugates changed. The retention times decreased for all of the compounds except the bilirubin diglucuronide and the bilirubin monoglucuronide peaks; the retention times of the latter increased slightly. As a consequence, the latter were now partially masked by the bilirubin diglucoside and bilirubin monoglucoside monoxyloside peaks. By increasing the molarity of buffer A to 0.0625 *M* sodium acetate and 3.125 m*M* 1-pentanesulfonic acid, while leaving that of B the same, retention times were decreased further for the bilirubin monoglucoside monoxyloside and bilirubin dixyloside (Fig. 6C). At these molarity values the bilirubin monoglucuronide peaks did not yet, however, clearly separate from the bilirubin dixyloside peak. The molarity in buffer A was therefore increased to 0.25 *M* sodium acetate and 12.5 m*M* 1-pentanesulfonic acid, while the composition of buffer B remained the same. Separation of the conjugates was now excellent and the two bilirubin IXα monoglucuronides shifted further to the right and appeared distinctly later than the bilirubin dixyloside (Fig. 6D). Bilirubin IXα and mesobilirubin IXα were added to the bile sample prior to the determinations illustrated in the lower three panels of Fig. 6. Their recoveries, which were decreased in the middle panels, became complete in the lower panel.

The changes illustrated above have been construed to be due to changes in

molarity. Alternatively, one could ask whether the increased molarity simply provides sufficient capacity in the initial mobile phase to bring all species that are injected into the column to the same initial pH sooner after injection. The pH profiles for various mixtures of dog bile extracted in dimethylsulfoxide and the solvent mixtures at initial proportions were examined (Fig. 7). The injection rate utilized was expected to result in initial extract/buffer ratio (v/v) values of approximately 1:1, with lower values occurring as the result of further mixing. Nevertheless, pH profiles as a function of molarity were explored for extract alone, extract/buffer proportions (v/v) of 2:1, and 1:2, and buffer alone. The pH dropped progressively from extract alone to buffer alone. For given proportions of each mixture, over a 100-fold range of molarities (0.0065–0.65 *M*), the pH showed essentially no change, especially over the more important extract/buffer ratios (v/v) of 1:1 and 1:2. However, in the transition



to 0.25 *M*, the pH of the mixture did decrease by 0.3 units at the lowest molarity and 0.7 units at the highest molarity. The alterations found with molarity for the retention times of the conjugates of bilirubin can then be explained by changes in the molarities of the solvents except at 0.25 *M* where a slight pH change may also contribute to the differential binding of species to the column.

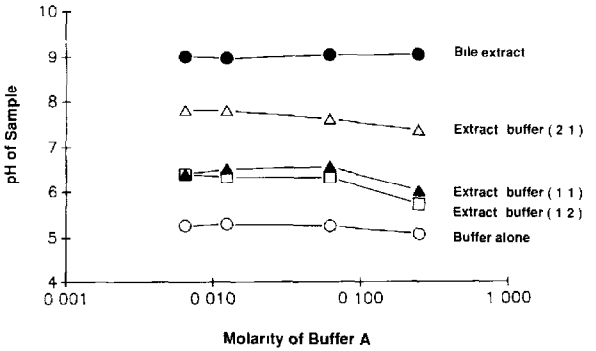


Fig. 7. Effect of change in the molarity of solvent A on the pH of a mixture of the bile extract and the initial solvent mixture. The pH profiles are shown for various proportions of dog bile, extracted with dimethylsulfoxide, and solvent mixtures A and B at initial composition. Values are given for extract alone, extract/buffer ratios (v/v) of 2.1, 1 1 and 1 2, and buffer alone.

Fig. 6. Effect of increasing the molarity of the buffer. (A) Chromatogram of the bile pigments in dog gallbladder bile developed in a solvent gradient system of sodium acetate (0.0065 *M*) with 1-pentanesulfonic acid (0.3125 m*M*) pH 4.0 in buffer A, and acetonitrile and dimethylsulfoxide (60:40, v/v) in buffer B. Excellent compound separation occurred except for the XIIIα and IIIα isomers of bilirubin, which was poor. Separation of the latter is needed especially when the possibility of randomization is being appraised, and particularly when commercial bilirubin, containing measurable proportions of these, is being utilized. The bilirubin monoglucuronide peaks are darkened here and in the following panels. (B) Chromatogram of dog gallbladder bile pigments with bilirubin IXα and mesobilirubin IXα added in a solvent gradient system of sodium acetate (0.0125 *M*) with 1-pentanesulfonic acid (0 625 m*M*) at pH 4.0 in buffer A and acetonitrile and dimethylsulfoxide (60:40, v/v) in buffer B. The retention times were generally less, except for the bilirubin monoglucuronide (C_8, C_{12}) species, which was displaced to the right in the sequence of the chromatogram and is now partially masked by the bilirubin diglucoside and bilirubin monoglucoside monoxyloside peaks. (C) Chromatogram of dog gallbladder bile pigments in a solvent gradient system of sodium acetate (0.0625 *M*) with 1-pentanesulfonic acid (3.125 m*M*) at pH 4.0 in buffer A and acetonitrile and dimethylsulfoxide (60.40, v/v) in buffer B. The retention times of the bilirubin diglucoside, bilirubin monoglucoside monoxyloside and bilirubin dixyloside decreased further and the bilirubin monoglucuronide C_8 and C_{12} peaks are now detected on either side of the bilirubin dixyloside, or peak 7. The bilirubin diglucuronide has also been displaced to the right, but no overlapping has occurred. (D) Chromatogram of dog gallbladder bile pigments developed in a solvent gradient system consisting of sodium acetate (0.25 *M*) with 1-pentanesulfonic acid (12.5 m*M*) pH 4.0 in buffer A and acetonitrile and dimethylsulfoxide (60 40, v/v) in buffer B. The retention times of all of the compounds except the bilirubin diglucuronide and the two bilirubin monoglucuronides decrease with the change. This change now clearly separates the bilirubin monoglucuronides from the bilirubin dixyloside.

Effect of varying the pH

The pH of the buffer was then varied from 4.0 to 4.8. Several chromatograms were run and it was found that increase of the pH from 4.0 to 4.8 resulted in a minor decrease in the retention times for the conjugates, apart from the monoglucuronide, for which there was a major decrease in the retention time, so that its doublet peaks eluted earlier, in a relative sense, within the chromatogram (Fig. 8). At the higher pH, the C_8 (exovinyl) isomer of the bilirubin IXα monoglucuronide fell between bilirubin monoglucoside monoxyloside and bilirubin diglucoside and the C_{12} exovinyl isomer was found under bilirubin diglucoside.

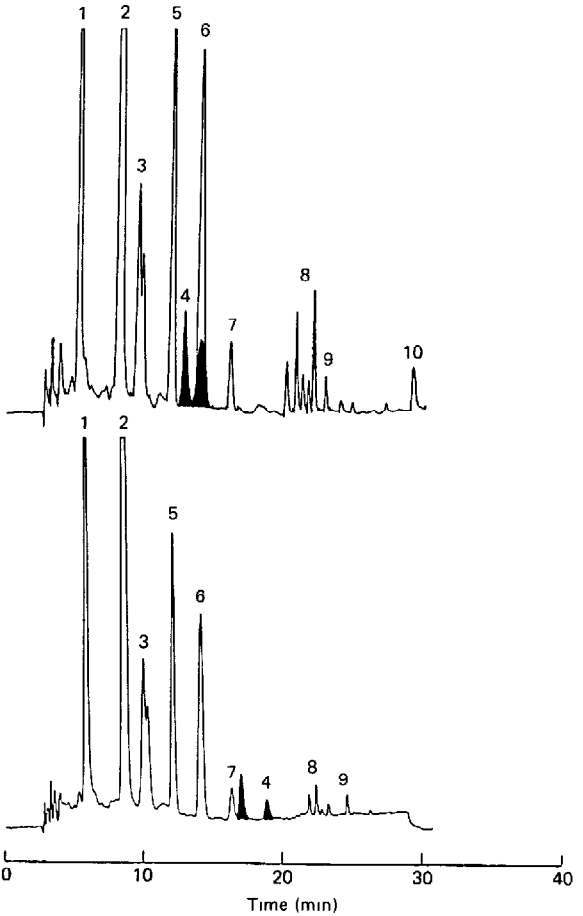


Fig. 8. Effect of increasing the pH of the buffer in solvent A from 4.0 (lower panel) to 4.8 (upper panel). The retention times of all conjugates decreased, with the most marked change being found in the retention of the two bilirubin monoglucuronides (which are again shaded black in this illustration), which therefore advance within the sequence.

The changes resemble, in part, those previously illustrated in Fig. 6 and indicate that increase in the pH results in an alteration in the elution profile somewhat analogous to that produced by a decrease in the molarity and ionic strength of buffer A.

Choice of an assay system

There was excellent separation and quantitation of the conjugates of bilirubin with sodium acetate at 6.5 m*M* at a pH of 4.0, but the system did not separate the isomers of unconjugated bilirubin. While the assay system would be fine for characterizing the conjugates from biological samples, where the species are virtually all of the bilirubin IXα or non-randomized variety, it does not lend itself to in vitro studies of the microsomal conjugation mechanism, in which commercial bilirubin, containing measurable proportions of the randomized symmetrical bilirubin XIIIα and IIIα isomers, must necessarily be utilized, and in which potential further randomization must be separated from enzymic effects, to avoid inappropriate conclusions. We therefore changed to the systems outlined in the Experimental section.

Analysis of varying known amounts of bilirubin IXα and its isomers and of mesobilirubin IXα, with this system, resulted in a linear correlation between amounts injected (in nmol) and the areas obtained on the chromatogram; the mesobilirubin IXα and bilirubin IXα values superimposed on the same curve.

TABLE I

COMPARISON OF ANALYSES FOR CONJUGATES OF BILIRUBIN IN BILE AFTER EXTRACTION AND DIRECT INJECTION

All samples from dog gallbladder bile are analyzed in two ways: (1) chloroform–ethanol extraction, filtered and analyzed by HPLC; (2) dissolved in dimethylsulfoxide, filtered and analyzed by HPLC. The values derived in each way were virtually identical.

Bile pigments in dog gallbladder bile	Percentage of total conjugates of bilirubin					
	Chloroform–ethanol extraction			Sample of bile dissolved in dimethylsulfoxide		
	A	B	Average	C	D	Average
Diglucuronide	29.8	33.4	31.6	30 7	31.0	30.9
Glucuronide glucoside	39.3	38.0	38.7	40.6	39.5	40.1
Glucuronide xyloside	11.3	10.5	10.9	9 2	10.1	9.7
Monoglucuronide (exovinyl and endovinyl)	3.6	3.5	3.6	1.7	3.5	2.6
Diglucoside	7.8	7.0	7.4	9.2	8.1	8.7
Glucoside xyloside	6.8	6.3	6.6	8.0	6.5	7.3
Dixyloside	0.7	0.5	0.6	0.7	0.7	0.7
Monoglucoside	0.8	0 7	0.8	0.7	0.7	0.7
Bilirubin	< 01	<.01	<.01	< 01	<.01	<.01

Therefore, to quantitate the various bilirubin conjugates in bile, mesobilirubin IXα was added as an internal standard and the amount of each conjugate was, in this system, calculated in equivalents of the amount of the mesobilirubin IXα. The methodology clearly can be extended to other biological fluids.

The quantitation of the amounts of conjugates of bilirubin found in bile, either extracted with ammonium sulfate and chloroform–ethanol (50:50, v/v), supernatant-dried and resuspended in dimethylsulfoxide, or bile diluted in the dimethylsulfoxide and injected into the high-performance liquid chromatograph is presented in Table I. Both methods of extraction yielded essentially identical values for the amounts of conjugates of bilirubin in bile, with mesobilirubin IXα as the internal standard.

DISCUSSION

The present work provides both a new methodology for the resolution of the bilirubin conjugates in biological samples and a description of how this can be modified by changes in molarity and pH, to provide a framework for the transfer of the methodology to other chromatographic systems.

The nine conjugates of bilirubin from dog gallbladder bile and bilirubin IXα have been separated to baseline by HPLC and their peak areas have been quantified by use of mesobilirubin IXα as an internal standard. The solvent systems also separate the randomization-created XIIIα and IIIα isomers of these compounds. There are substantial species-specific differences in bilirubin conjugation [11]. Not all mammals excrete the number of conjugates of bilirubin found in dog gallbladder bile: the rat secretes mainly bilirubin diglucuronide and bilirubin monoglucuronide (C_8, C_{12}) [12]; the guinea pig, hamster, deer mouse, prairie dog and pig excrete mainly bilirubin monoglucuronide (C_8, C_{12}) [13–16], and human bile has been found to contain bilirubin diglucuronide and small amounts of bilirubin monoglucuronide monoglucoside and the bilirubin monoglucuronides (C_8, C_{12}) [17–19]. All of these may also potentially excrete some of the other glucose and xylose conjugates presently identified in dog bile, since the methods previously utilized for their resolution were poor. Only an exceedingly small proportion of the bilirubin in bile is found to be unconjugated [17–19].

Several HPLC methods have been employed to separate bilirubin and its conjugates, either by use of ion-pair reagents or by manipulating hydrophobic interactions, or a combination of both approaches [20]. Uesugi et al. [21] described a sensitive method which utilized a citrate buffer containing methanol and acetonitrile to separate the three isomers of unconjugated bilirubin (XIIIα, IXα, and IIIα), four isomers of bilirubin monoglucuronide [XIIIα, endovinyl IXα (C_8), exovinyl IXα (C_{12}) and IIIα], three isomers of bilirubin diglucuronide (XIIIα, IXα, and IIIα) and bilirubin mono- and diglucosides and mono- and dixylosides. The separation procedure was quite long; it took

approximately 70 min. Onishi et al. [22] also completely separated the native tetrapyrroles and their isomers by using a linear gradient of acetonitrile in 0.1 *M* acetate buffer, pH 4.0, containing 5 m*M* sodium pentanesulphonate as a counter-ion. This separation took approximately 100 min, which makes it difficult to use for routine assay and it does not resolve all of the species separated by the present method. Roy Chowdhury et al. [17] also employed a reversed-phase column, an ion pair with an acetate buffer (pH 4.0) and a concave methanol gradient (50–100%). After 70 min, resolution of di- and monoconjugates but not the endovinyl IXα and exovinyl IXα of bilirubin monoglucuronide was achieved. Spivak and Carey [16] improved their original separation method by using a 3-μm C_{18} column and a methanol–sodium acetate–aqueous ammonium acetate buffer. Three isomers of bilirubin and of bilirubin diglucuronide, two isomers of bilirubin monoglucuronide, three isomers of unconjugated bilirubin and minor conjugates containing glucose and xylose were separated within 12 min. However, the elution times of some of the conjugates overlap and quantitation of the compounds becomes difficult [18].

Two isocratic solvent systems have been described by Lim [23] and Jansen [24]. Each separated the mono- and diconjugates and unconjugated bilirubin, and in the Jansen method the various sugar conjugates were also isolated.

In our previous chromatographic approach [19], the diglucuronide and monoglucuronide and their isomers, the monoglucuronide monoglucoside and the diglucoside, were well separated with an ion pair in an acetate buffer (pH 4.8) and a stepped acetonitrile gradient and flow program. To provide for a better separation and for the resolution of all of the mixed sugar diconjugates found in bile, the present system was developed, which utilizes an ion pair and a hydrophobic column together with a change in molarity of a pH 4.0 acetate buffer. With this, the reversed-phase column gives a much enhanced separation of the bile pigments found in dog gallbladder bile. In this column the stationary phase is an alkylsilyl compound with a non-polar hydrophobic surface. The solute retention time is mainly due to the hydrophobic interaction between the solutes and the hydrocarbaceous stationary surface. Solutes are eluted in order of decreasing polarity (increasing hydrophobicity) [1,20,25,26]. As illustrated in our results, the hydrophobicity can be precisely adjusted by altering the molarity and pH of the buffers, the effect being largest where the pK_a is near the pH of the mobile phase (Figs. 6 and 8). Thus the choice of the buffer and its molarity are important because they will significantly affect the selectivity, efficiency and resolving power of the column [27].

This was shown by changing the concentration of the sodium acetate buffer from 6.5 m*M* to 0.25 *M*, which altered the retention times especially of the bilirubin monoglucuronides (C_8 and C_{12}). As the molarity was increased, retention times of the non-polar conjugates decreased whereas those of the glucuronides, and especially of the bilirubin monoglucuronide, increased. This indicates that, with the increase in the molarity of the buffer, the bilirubin

monoglucuronide has become more hydrophobic. These observations were made at a buffer A pH of 4.0. By increasing the pH to 4.8, with the buffer at 0.2 *M*, the retention times of the bilirubin monoglucuronide peaks were found to decrease, in relation to those of the other conjugates (Fig. 8). The change in the elution time indicates a likely more complete ionization of the bilirubin monoglucuronide. Thus, by utilizing the physical and chemical properties of these bile pigments and a reversed-phase column, the conjugated bile pigments can be manipulated by use of classic chemical methods so that they can be separated and quantitated by HPLC [25–27]. The technique thus has now been developed to an extent which will permit the evaluation of disturbances in bile pigment metabolism in animals and man.

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