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High-performance liquid chromatographic analysis of bile pigments as their native tetrapyrroles and as their dipyrrolic azosulfanilate derivatives

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) analysis of bile pigments is described that provides baseline separation of the major bilirubin conjugates found in bile. The advantage of the technique is that the bile pigments can be analyzed directly as their native tetrapyrroles without prior solvent extractions or derivatization. The use of ammonium acetate in place of sodium salts permits preparative isolation and lyophilization of the pigments for mass spectroscopy. The derivatization of the pigments as their dipyrrolic azosulfanilates with subsequent HPLC analysis demonstrates baseline separation of the endo- and exovinyl azodipyrroles and allows identification of that half of the tetrapyrrole which contains the conjugate in the instances of monoglycosides.

INTRODUCTION

The precise analysis of bile pigments is hampered because of their susceptibility to photo-oxidation, disproportionation from the naturally occurring IXα isomer to formation of the XIIIα and IIIα isomers [1], acylmigration of the glucuronide conjugates to non-C-1-0 glycosides [2] and transesterification to methyl esters when exposed to methanol during purification [3] (Fig. 1). Transesterification of bilirubin glucuronides also occurs in vitro in the presence of albumin [4,5] whereby the bilirubin becomes covalently bound to albumin with release of the glucuronic acid. Considerable species variation of
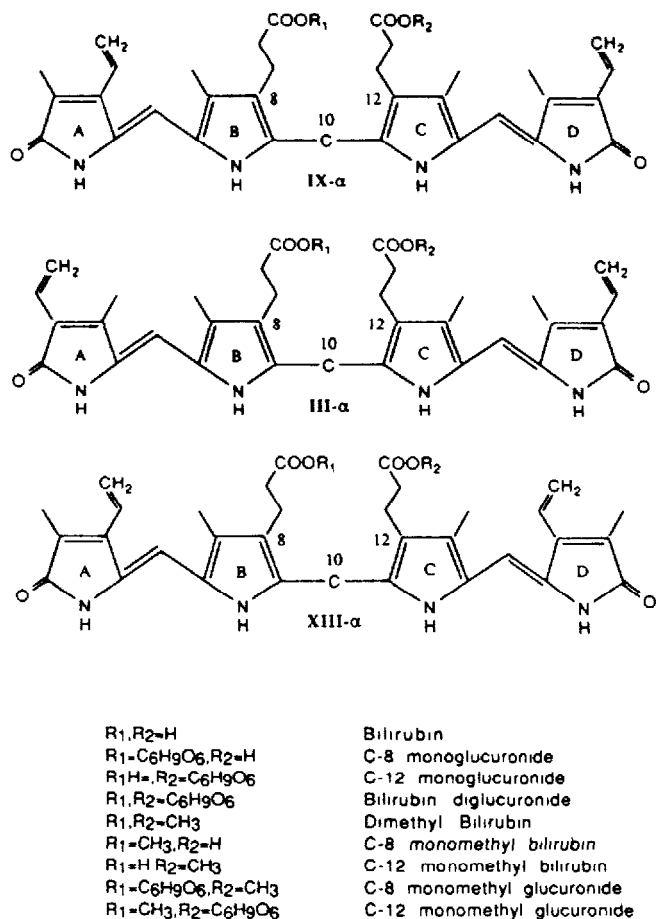
R1,R2=H   Bilirubin

$R_1,R_2=H$	Bilirubin
$R_1=C_6H_9O_6,R_2=H$	C-8 monoglucuronide
$R_1H=,R_2=C_6H_9O_6$	C-12 monoglucuronide
$R_1,R_2=C_6H_9O_6$	Bilirubin diglucuronide
$R_1,R_2=CH_3$	Dimethyl Bilirubin
$R_1=CH_3,R_2=H$	C-8 monomethyl bilirubin
$R_1=H\ R_2=CH_3$	C-12 monomethyl bilirubin
$R_1=C_6H_9O_6,R_2=CH_3$	C-8 monomethyl glucuronide
$R_1=CH_3,R_2=C_6H_9O_6$	C-12 monomethyl glucuronide

Fig. 1. Structures of the bile pigments.

bile pigment conjugates has also been found [6–8] that requires precise analytic separation for subsequent independent chemical characterization for proper identification. In view of the above cited in vitro reactivities and species variation of conjugation in vivo, analysis of bile pigments which minimizes their in vitro storage and handling, prior to analysis, is preferable [9–12].

In the present study we report an improved chromatographic separation and quantitation of bile pigments as their tetrapyrroles, without any prior treatment. Further analysis by high-performance liquid chromatography (HPLC) of the tetrapyrroles as their dipyrrolic azosulfanilate derivatives demonstrates, in the instance of monoconjugates, which half of the tetrapyrrole contains the conjugate.

EXPERIMENTAL

Chemicals

Acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.) and ammonium acetate (Mallinckrodt, Paris, KY, U.S.A.) were of spectrophotometric grade. Bilirubin was from Sigma (St. Louis, MO, U.S.A.) and all other reagents were of C.P. standards.

Methods

All bile pigment analyses were performed on either a Waters reversed-phase C_{18} μBondapak analytical (30 cm × 0.39 cm I.D.) or preparative (30 cm × 0.78 cm I.D.) column. The columns were equipped with a Waters Guard-Pak module containing RCSS C_{18} Guard-Pak disposable precolumn inserts. All samples were delivered by a Perkin-Elmer ISS-100 automatic sample injector equipped with either a 0.2- or a 1.0-ml sample loop. All elution solvents were pre-filtered and delivered by a Perkin-Elmer Series 4 liquid chromatograph microprocessor deaerated with helium. Column eluates were monitored with a Hewlett-Packard 1040A diode-array detector controlled by an HP85B personal computer, with an on-line HP3392A integrator and an HP7470A graphic plotter. The diode-array detector data was stored on a Bering Series 3000 Subsystem data storage terminal. The bile pigments were monitored as their tetrapyrroles at 450 nm (bandwidth 4 nm) against a reference at 590 nm (bandwidth 10 nm),

TABLE I

SOLVENT ELUTION COMPOSITION FOR HPLC SEPARATION OF BILE PIGMENTS

For the analytical column, flow-rates were 1.0 and 4.0 ml/min with the preparative column. Equilibration time of 10 min was used for both elution programs.

Time (min)	Solvent A (0.1 M ammonium acetate, pH 4.85) (%)	Solvent B (acetonitrile) (%)	Gradient slope
Native tetrapyrroles, monitored at 450 nm			
0	70	30	
15	60	40	1
25	60	40	—
26	20	80	1
36	20	80	—
41	70	30	1
Derivatized dipyrrolic azosulfanilates, monitored at 540 nm			
0	80	20	
10	80	20	—
30	40	60	1
35	80	20	1

and the derivatized dipyrrolic azosulfanilates at 540 nm (bandwidth 4 nm) against a reference of 590 nm (bandwidth 10 nm).

The solvents and gradients for elution of the tetrapyrroles and dipyrrolic azosulfanilates are given in Table I. The 0.1 M ammonium acetate was titrated to pH 4.85 with glacial acetic acid.

Bile pigment collections

Five heterozygous (Jj) and five homozygous (JJ) non-jaundiced rats from our Gunn rat colony [13] were surgically equipped with bile duct and jugular vein catheters (PE-10) after pentobarbital anesthesia (35 mg/kg intraperitoneally). After a basal bile collection, the animals were infused over 20 min with 5 mg of dimethylbilirubin (DMB) dissolved in 0.35 ml of dimethylsulfoxide (DMSO). The bile was subsequently collected for four 30-min intervals on ice in dim light (Safe Lamps). Fluid replacement of the animals was given as isotonic saline containing 5 g/dl glucose by a Harvard Apparatus infusion pump. The animal's temperature was maintained at 37.5°C by a 40-W incandescent bulb reflected onto the animal from a goose-necked lamp. The animals were euthanized with pentobarbital intravenously at the end of the collection period.

The bile samples (10–800 μl) were injected directly without any form of modification for HPLC analysis.

Preparation of bile pigments and their derivatives

The IXα DMB was prepared as previously reported [12]. The purified XIIIα DMB used for infusions was prepared by acid scrambling of bilirubin IXα [1] and isolated by thin-layer chromatography (TLC) on silica gel plates developed in chloroform–acetic acid (95:5, v/v). The isolated bilirubin XIIIα and an authentic sample of bilirubin XIIIα (kindly provided by Professor D. Lightner [14]) were converted to XIIIα DMB with etheral diazomethane [12].

Preparations of standards of bilirubin and DMB conjugates were synthesized by in vitro incubation of freshly isolated liver microsomes [15] in which the donor glycoside was UDP glucuronic acid (5 mM), UDP glucose (20 mM) or UDP xylose (100 mM). After 30 min of incubation the incubates were centrifuged at 8800 g for 5 min, and an aliquot of supernatant (5–15 μl) was directly injected. For preparative elutions the microsomal supernatant was mixed with an equal volume of absolute ethanol to deproteinize and precipitate unconjugated bilirubin and/or DMB.

Preparation of the dipyrrolic azosulfanilates

The di- and monoconjugates of bilirubin and DMB were collected directly into iced glass tubes in dim light after their flow through the detector. The eluate volumes were measured and the absorbance of an aliquot determined at 450 nm in a 1-cm cuvette in a Varian spectrophotometer. Another aliquot (0.4

ml) was converted to its dipyrrolic azosulfanilate by addition of 0.1 ml of freshly prepared diazotized sulfanilic acid, 21.0 mM in 0.68 M hydrochloric acid. The reaction mixture was diluted with an equal volume of methanol to 1 ml and its absorbance was determined at 540 nm after 30 min against 50% aqueous methanol. The dipyrrolic azosulfanilate derivatives were then analyzed by HPLC using the solvent elution program of Table I.

Quantitation of the pigments from the integrated area under the peaks of the HPLC elution profile was standardized by injection of known amounts of crystalline bilirubin solution in chloroform with millimolar absorptivity of 60 at 450 nm, and its dipyrrolic azosulfanilate derivative in 50% methanol with a millimolar absorptivity of 60 at 540 nm.

Hydrolysis of bile pigment conjugates

Hydrolysis of the bile pigment conjugates was performed by exposure of 100 μl of freshly collected bile samples to 10 000 U of β-glucuronidase (Sigma) in 0.1 M phosphate buffer (pH 6.8) at 37°C for 30 min, in a final volume of 0.2 ml. After incubation and centrifugation, a 50-μl aliquot of the supernatant was analyzed by HPLC. A similar volume of bile incubated at pH 6.8 in the phosphate buffer without addition of β-glucuronidase served as a control.

RESULTS

The elution of the bile pigment conjugates associated with the infusion of IXα DMB is illustrated in Fig. 2. The incubation of the bile with β-glucuronidase indicates that the eluted conjugates (retention time, t_R, 9–27 min) were all β-D-C–1-0 acylglycosides. The monomethyl esters eluting at 33–35 min were derived from the monomethyl glucuronides and the unconjugated bilirubin isomers (37–41 min) were derived from both the bilirubin di- and monoglucuronides. As previously described [12], for purposes of continuity the C-8 monoglucuronide C-12 monomethyl IXα bilirubin diester is denoted as C-8 IXα monomethyl glucuronide and the C-12 monoglucuronide C-8 monomethyl IXα bilirubin diester as C-12 IXα monomethyl glucuronide. To be noted is that after β-glucuronidase hydrolysis, the previous C-8 and C-12 diesters become C-12 and C-8 monomethyl bilirubin, respectively, with the C-8 monomethyl ester eluting prior to the C-12 monomethyl ester. This accounts for the greater peak area of the C-8 monomethyl bilirubin at 33.8 min than that of the C-12 at 34.1 min, since the former was derived from the C-12 IXα monomethyl glucuronide.

Confirmation of the isomer assignments of eluted pigments is illustrated in Fig. 3 which contrasts the elution profiles of the bilirubin conjugates after the sequential infusion of XIIIα DMB (50% offset) and IXα DMB (10% offset) in the same animal. As shown, the XIIIα diglucuronide at 9.8 min and the XIIIα monomethyl monoglucuronide at 23.1 min are exaggerated compared to
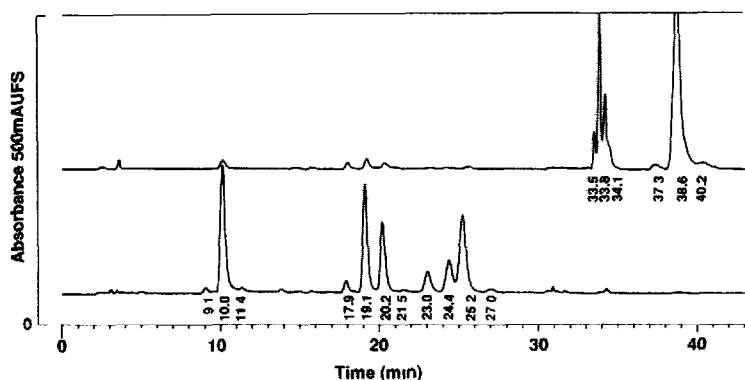
Fig. 2. Elution profile of bile pigments as their tetrapyrroles, monitored at 450 nm, collected from a non-jaundiced heterozygous (Jj) Gunn rat after infusion with dimethyl-bilirubin IXα. The tracings are of the bile pigments before (10% offset) and after (50% offset) incubation with β-glucuronidase. The elutions of the lower tracing between 9.1 and 11.4 min are XIIIα, IXα and IIIα bilirubin diglucuronides, respectively; those between 17.9 and 21.5 min are XIIIα, C-8 and C-12 IXα and IIIα monoglucuronides; those between 23.0 and 27.0 are XIIIα, C-8 and C-12 IXα and IIIα monomethyl glucuronide diesters of bilirubin. The elutions of the upper tracing labeled between 33.5 and 34.1 min are the corresponding monomethyl esters of bilirubin. The shoulder on the descent of the C-12 monomethyl ester peak at 34.1 min represents the IIIα derivative. The elutions between 37.3 and 40.2 min are the XIIIα, IXα and IIIα unconjugated bilirubin.
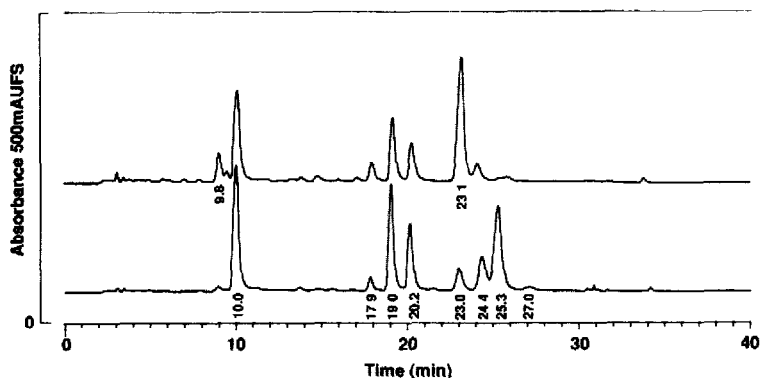

Fig. 3. Elution profile of bile pigments as their tetrapyrroles, monitored at 450 nm, collected from a non-jaundiced homozygous (JJ) Gunn rat after sequential infusion of XIIIα dimethylbilirubin (50% offset) and IXα dimethylbilirubin (10% offset). Identification of the eluted pigments is as described in Fig. 2. To be noted are the prominent elutions at 9.8 and 23.1 min in the upper tracing representing the XIIIα bilirubin diglucuronide and the XIIIα monomethyl glucuronide diester during the infusion of XIIIα dimethylbilirubin.

the excretion following the IXα DMB infusion. Further confirmation (data not shown) was obtained by collection of the C-8 IXα monoglucuronide and C-12 IXα monomethyl glucuronide and allowing them to disproportionate by

storage in the elution solutions. Subsequent chromotography revealed the presence of XIIIα diglucuronide and IIIα bilirubin from the C-8 IXα monoglucuronide, and IIIα diglucuronide and XIIIα DMB from the C-12 IXα monomethyl glucuronide, respectively. Such isomeric elutions could only have occurred by disproportionation and recombination of the dipyrrolic halves of the IXα isomers [1].

The elution profiles of the supernatants from individual in vitro microsomal incubations of bilirubin with the donor UDP glycosides of xylose, glucose and glucuronic acid are illustrated in Fig. 4. Each elution profile demonstrates the expected three diglycosides preceding the four monoglycosides, with the glucuronides (C) eluting earlier than the respective glucosides (B) and xylosides (A). As illustrated, the IXα dixyloside (t_R 17.4 min, A) overlaps that of the C-8 IXα monoglucuronide (t_R 17.6 min, C) and the C-8 IXα monoxyloside coelutes with the IIIα monoglucoside (t_R 26.4 min, A and B, respectively).

Such coelutions of the tetrapyrroles could lead to misinterpretation of bile composition. However, by further HPLC analysis of the bile pigment tetrapyrroles as their dipyrrolic azosulfanilates, such misinterpretations can be avoided. The monoglycoside conjugates of Fig. 4 were converted to their dipyrrolic azosulfanilates. These derivatives were subsequently chromatographed and eluted by the protocol of Table I. After the elution times were recorded, a pool of the three conjugated dipyrrolic azosulfanilates was mixed with diazotized bilirubin and diazotized DMB and rechromatographed. The results are illustrated in Fig. 5. As dipyrrolic azosulfanilates, the conjugated and non-conjugated dipyrroles elute in pairs, as their endo- and exovinyl derivatives separated by approximately 0.8 min. As illustrated, the endo- and
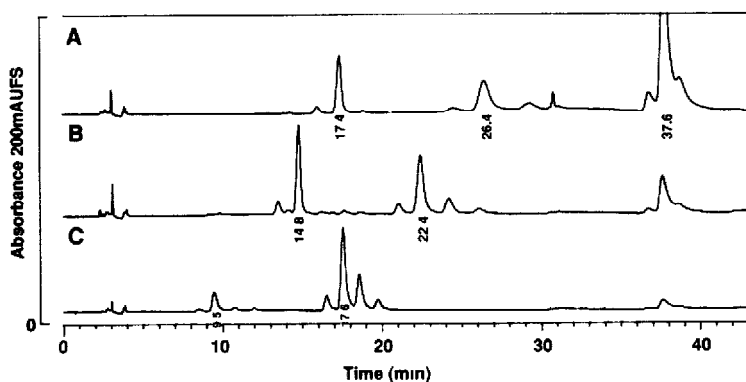

Fig. 4. Elution profiles of the bilirubin conjugates from the supernatants of rat liver suspensions of microsomal UDP-glucuronosyl-transferase incubated with bilirubin and UDP-xylose (A), UDP-glucose (B) and UDP-glucuronic acid (C). The bilirubin conjugates were chromatographed as their tetrapyrroles and monitored at 450 nm. The elution times of the IXα glycoside diesters and the C-8 IXα monoglycosides as well as the residual unconjugated bilirubin are indicated beneath their respective elution peaks.
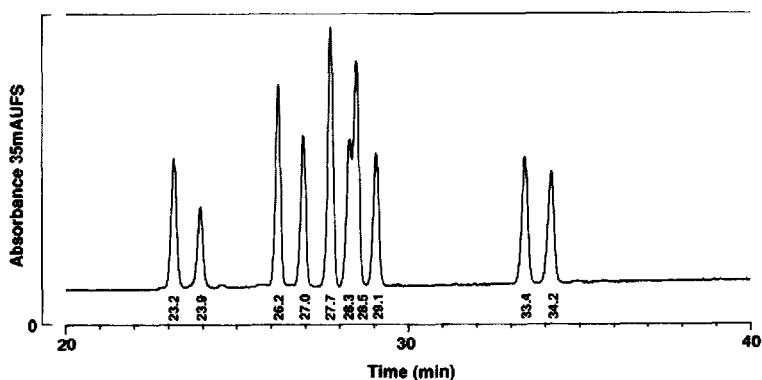
Fig. 5. Elution profiles monitored at 540 nm of the dipyrrolic azosulfanilate derivatives of the tetrapyrrole glycoside conjugates (glucuronide, glucoside and xyloside) of Fig. 4. The azodipyrroles elute as endovinyl and exovinyl pairs as illustrated. The endo- and exovinyl azodipyrrolic glucuronides elute at 23.2 and 23.9 min, respectively; the corresponding glucosides at 26.2 and 27.0 min and the xylosides at 27.7 and 28.5 min, respectively. The unconjugated endo- and exovinyl dipyrrolic azosulfanilates of bilirubin elute at 28.3 and 29.1 min, and the methylated endo- and exovinyl dipyrrolic azosulfanilates of dimethylbilirubin elute at 33.4 and 34.2 min, respectively.

exovinyl dipyrrolic azoglucuronides elute before those of the azoglucosides. The dipyrrolic endo- and exovinyl xylosides (27.7 and 28.5 min) overlap with the non-conjugated endo- and exovinyl azodipyrroles of bilirubin (28.3 and 29.1 min). The endo-and exovinyl azodipyrrolic methyl esters are last to elute (33.4 and 34.2 min, respectively) and are well separated. These latter methyl esters serve well as an internal standard. Except for the elution of the endovinyl azodipyrrolic sulfanilate of bilirubin and the exovinyl azodipyrrole of the xyloside conjugate of bilirubin, baseline separation of the conjugated glycosides is achieved. The separation of the endo- and exovinyl dipyrroles allows one to identify, in instances of monoconjugates of bilirubin, which dipyrrolic half of the molecule contains the conjugate. In the instance of rat bile where the C-8 IXα glycoside is the predominant monoconjugate, one can predict that the endovinyl azodipyrrole would be in greater concentration than the exovinyl dipyrrole containing the C-12 glycoside side-chain as seen in Fig. 5. In the instances of IIIα and XIIIα tetrapyrrolic isomers one expects only exo- and endovinyl dipyrrolic derivatives, respectively.

The quantitation of the chromatographic separation of bile pigments as their tetrapyrroles and dipyrrolic azosulfanilates is summarized in Fig. 6A and B, respectively. For these studies, 5–50 μl were injected using a 200-μl loop. The high correlations between the integrated area of the elution peaks and the n moles injected indicate the reliability of this method of analysis. However, the slopes varied on four consecutive days, from 5.77 to 3.99, indicating the need for daily calibration for accurate results. Although the precise extinction coefficients of the individual bilirubin conjugates as their tetrapyrroles is not known,
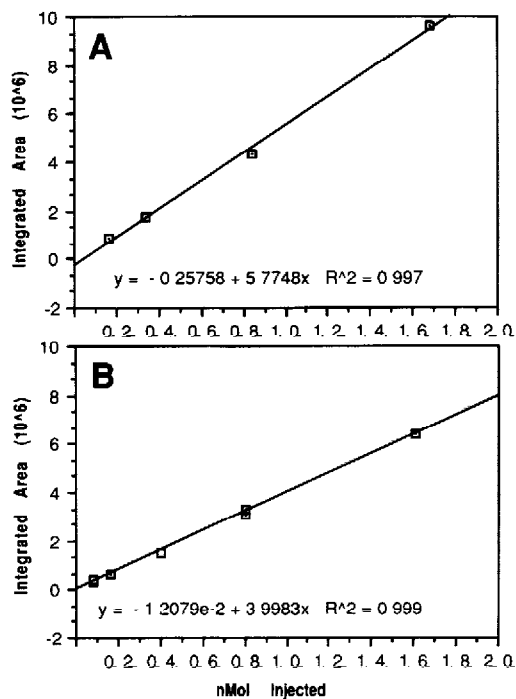
Fig. 6. Correlation between the integrated area of the elution peaks (y-axis) and the injected amount of bile pigment (x-axis). (A) Results with bilirubin injected as the tetrapyrrole, monitored at 450 nm; (B) derivatized dipyrrolic azosulfanilates, monitored at 540 nm.
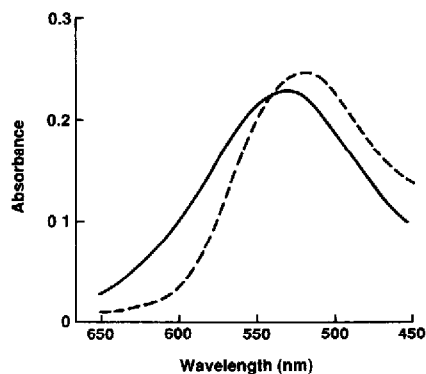

Fig. 7. Spectral absorbance of the dipyrrolic azosulfanilate derivatives of bilirubin in 50% methanol at pH 2.0 (solid line) and in a 60 40 mixture of 0.1 M ammonium acetate–acetonitrile at pH 4 85 (dashed line). There is an isosbestic point at 540 nm for the azodipyrroles in the two solvent systems at pH 2.0 and 4.85.

their conversion and measurement as their azodipyrroles as bilirubin equivalents at 540 nm permits a reasonable quantitation, because the chromophoric azo absorbance is little influenced by the presence of conjugates at the C-8 and C-12 propionic acid side-chains. We found (data not shown) that dissolution of 1.0 mg of recrystallized bilirubin and 1.05 mg of DMB after conversion to their dipyrrolic azosulfanilates had millimolar absorptivities of 60 in 50% methanol as bilirubin equivalent. Since the azochromophore is pH-sensitive, a comparison of the absorption spectra of the dipyrrolic azosulfanilates was made at pH 2.0 and 4.85 to reproduce the circumstances during chromatographic elution (Fig. 7). The extinction maximum of the azodipyrroles exhibits a shift from 533 at pH 2.0 to 520 nm at pH 4.85 with enhancement. As illustrated there is an isosbestic point at 540 nm which is the wavelength at which we monitored the chromatographic elutions of the azosulfanilates.

DISCUSSION

We previously reported when the polar elution solvent (0.1 M sodium acetate) was at pH 4.0 the C-12 IXα and IIIα monoglucuronides coeluted with the XIIIα and C-8 IXα monomethyl glucuronides [12]. By adjustment of the pH to 4.85 complete separation of the monoglucuronides from the monomethyl glucuronides was achieved (Fig. 2). Such adjustment reduced the ionization charge of the single carboxyl groups of the monomethyl glucuronides relative to the two carboxyl groups of the monoglucuronides such that the isomeric monomethyl glucuronides eluted as more apolar compounds. The substitution of ammonium acetate for sodium acetate permitted direct lyophilization of the chromatographically separated glucuronides, and thereby enabled greater purity for subsequent mass spectroscopy [16]. The advantage of the present methodology is that no prior solvent extraction of the bile is required and baseline separation of the tetrapyrroles can be achieved. Previous workers [8–11] pre-mixed the bile samples and baseline separation of the C-8 and C-12 IXα monoglucuronides was not demonstrable. The use of an isocratic elution rather than a linear gradient from 15 to 25 min (Table I), during the elution of the mono- and monomethyl glucuronides, provided the best separation of the conjugates.

Blanckaert and co-workers [17,18] have adapted alkaline methanolysis to the study of bile pigments as tetrapyrroles from biological fluids, but the conversion of the mono- and diglycosidic esters to bilirubin methyl esters does not allow identification of the original glycoside ester (e.g. glucuronic acid, glucose or xylose).

The current observations on the elution profiles of the glucuronide, glucoside and xyloside conjugate (Fig. 4) are comparable to those of Onishi et al. [9], with the overlap of the dixyloside IXα with that of the C-8 IXα monoglucuronide. However, by conversion of these mixed conjugates to their dipyr-

rolic azosulfanilates, separation for identification was easily obtained (Fig. 5). The dipyrrolic glucuronides and glycosides are readily separated from the xylosides.

Previous studies of bile pigments as their dipyrrolic derivatives has been reported after derivatization with diazotized ethyl anthranilate or p-iodoaniline [8,19]. We could not find comparable studies using diazotized sulfanilic acid. Since the azoanthranilates and azosulfanilates have similar absorptivities [20], we anticipated equal sensitivities for their quantitation by HPLC. The results indicate that the azosulfanilate derivatives separate the endo- and exovinyl conjugates as cleanly as the azo derivatives of ethylanthranilate and p-iodoaniline [19].

The linear correlations of the integrated areas of the elution peaks with the amount of pigment were excellent for both the tetrapyrrole and dipyrroles (Fig. 6A and B, respectively). Such results attest to the reliability of the assay technique. However, the slope of the tetrapyrrole correlation was steeper than that of the azodipyrroles when using common millimolar absorptivities of 60 for both the bilirubin at 450 nm and its azodipyrroles at 540 nm. The fact that the slopes are not identical suggests that the application of the common extinction coefficient is not completely justified. As demonstrated in Fig. 7, the measurement of the azosulfanilates at 540 nm is below the extinction maximum of the azochromophore at pH 2.0 and particularly at pH 4.85. This discrepancy can readily account for the lower slope value of the azodipyrroles relative to the tetrapyrrole.

When using methanolic diazotized sulfanilic acid to quantitate conjugated tetrapyrroles as their dipyrrolic azoderivatives, we have found it is important that they be analyzed immediately. Transesterification (methanolysis) of the acylglycosides occurs on standing, and sequential chromatograms reveal losses of the glycoside and appearance of the corresponding endo- or exovinyl methyl dipyrrolic azosulfanilates. The methanolysis obviously can also be used to advantage as evidence for acylglycosidic linkage.

The present HPLC method are suitable for the quantitative analysis of bile pigments in solutions that do not contain significant amounts of protein which bind and rapidly transacylate with bilirubin conjugates [4,5]. The application to bile samples has proven particularly useful in the analysis of duodenal bile of individuals with Gilbert's syndrome, where precise quantitation of the relative percentage of bilirubin mono- and diglucuronides is required [21].

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