

Application of a rapid and efficient h.p.l.c. method to measure bilirubin and its conjugates from native bile and in model bile systems

Potential use as a tool for kinetic reactions and as an aid in diagnosis of hepatobiliary disease

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We have developed an extremely rapid and efficient reverse-phase h.p.l.c. method for the measurement of bilirubin and its conjugates in human bile and in model bile systems. Our method involves the use of a Perkin–Elmer 3 μ C18 column and a methanol/sodium acetate/aq. ammonium acetate buffer system. Three isomers of bilirubin diglucuronide (BDG), two isomers of bilirubin monoglucuronide (BMG), three isomers of unconjugated bilirubin (UCB) and minor conjugates containing glucose and xylose were separated in 12 min. Initial quantification of BDG and BMG was based on the use of the ethyl anthranilate azo derivative of bilirubin (AZO UCB); however, the standard curves for BDG, BMG and UCB were similar enough to permit quantification to be later based on the UCB standard curve only, thereby simplifying the quantification process. Routine direct injection of 6 or 10 μ l of crude undiluted or diluted (1:1) bile sample was sufficient for analysis. The method was helpful in diagnosing biliary-tract obstruction in a newborn and a partial deficiency state of bilirubin conjugation (Crigler–Najjar syndrome) in a 10-year-old male. When the method was applied to biles of patients both with and without gallstones, levels of UCB were less than 2% of total pigment, consistent with previous reports. Because of its speed and efficiency, this method has the potential for a broad range of applications including enzymic, kinetic and bile sample analyses.

INTRODUCTION

In adult humans and in Sprague–Dawley rats, the major bile pigments are bilirubin diglucuronide (BDG) and bilirubin monoglucuronide (BMG) (Fevry *et al.*, 1972). These compounds are formed by the hepatic conjugation of bilirubin, a potentially toxic non-polar compound that is formed from the breakdown of haem.

There are species-specific differences in bilirubin conjugation (Cornelius *et al.*, 1975), and alterations can occur in the normal pattern of bilirubin conjugation within a given species. Unlike the adult human, who secretes 65–85% of his bile pigment as bilirubin diconjugates and the rest as monoconjugates, the deer mouse, guinea pig, hamster and prairie dog, all animal models of gallstone formation (Anderson *et al.*, 1966; Van der Linden & Bergman, 1979; Pitt *et al.*, 1984), excrete virtually all their bile pigment in the form of BMG (Spivak & Carey, 1985). The neonate (Onishi *et al.*, 1980) and patients with Gilbert's syndrome (Fevry *et al.*, 1977) secrete a greater proportion of their bile pigment in the form of BMG than the normal adult. Patients with Crigler–Najjar syndrome (a genetic form of hyperbilirubinaemia associated with a deficiency of glucuronyl-transferase) have striking alterations in the secretory

pattern of bilirubin conjugates (Gordon *et al.*, 1976; Fevry *et al.*, 1977).

Although UCB is present in only trace amounts in bile, several studies have suggested that alterations in the biliary secretion of bilirubin and its conjugates or post-secretory hydrolysis of bilirubin conjugates may be linked to the formation of gallstones (Soloway *et al.*, 1977; Masuda & Nakayama, 1979; Duvaldestin *et al.*, 1980; Spivak *et al.*, 1984).

As a result, accurate measurement of bilirubin and its conjugates has become extremely important in the understanding of the hepatic metabolism of bilirubin and the physical-chemical events that occur in the gall-bladder and biliary tree after bilirubin secretion. H.p.l.c. methods for bilirubin analysis (Onishi *et al.*, 1980; Chowdhury *et al.*, 1982; Gordon & Goresky *et al.*, 1982) have the distinct advantage over t.l.c. methods in that exposure to light, oxygen and an active surface, and cumbersome scraping of t.l.c. plates are eliminated. Spivak & Carey (1985) recently described a comprehensive and simple technique for the analytical separation and preparation of pure bilirubin conjugates using a 22 min reverse-phase h.p.l.c. technique. Although that method is considerably faster than previously described methods, further reductions in analysis time in analytical h.p.l.c. are

Abbreviations used: UCB, unconjugated bilirubin; BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; IPA, integrated peak area; AZO UCB, azo derivative of UCB; BMGGl, bilirubin monoglucuronide monoglucoside; BMGl, bilirubin monoglucoside; BMX, bilirubin monoxyloside; BDGl, bilirubin diglucoside.

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especially important because of the sequential processing inherent in h.p.l.c. analysis (i.e., only one sample can be run at a time, as opposed to t.l.c., where multiple samples can be processed in parallel). Rapid h.p.l.c. is particularly advantageous when multiple samples are to be processed or for monitoring kinetic reactions that require frequent sample injection.

Recent advances in h.p.l.c. column technology have resulted in the development of very short columns with small-diameter packing material. These columns permit more efficient separations in less time using less solvent. Using these new, more efficient columns, we report here an extremely rapid and efficient separation of bilirubin and its conjugates from bile and demonstrate the utility of the method for analysing human and animal bile samples and for kinetic studies.

EXPERIMENTAL

Chemicals

All chemicals were of the same grade and obtained from the same distributors as previously described (Spivak & Carey, 1985). Sodium acetate, which was not used in the previous h.p.l.c. solvent system, was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Equipment: h.p.l.c. apparatus

All h.p.l.c. apparatus was purchased from Perkin-Elmer (Elmwood Park, NJ, U.S.A.) (with exceptions as noted) and included a Series 4 LC with gradient control terminal, an LC85B detector with autocontroller and a 3600 data station with graphics printer. For analytical separations, a 6 or 10 μ l injector loop (Rheodyne, Berkeley, CA, U.S.A.) was completely filled with sample before injection, and a 3 μ C18 column with a length of 8.3 cm and an internal diameter of 4.6 mm was used. For preparative separations, a 500 μ l loop was filled with 300–500 μ l of sample, and a 5 μ l Altex Ultrasphere ODS column (Beckman Instruments, Fullerton, CA, U.S.A.) with an internal diameter of 10 mm and a length of 250 mm was used. Guard columns were fitted to each column to protect the columns from bile proteins that might degrade their separative capacity. The preparative column was fitted with a 50 mm Whatman Co. Pell ODS precolumn (Whatman, Clifton, NJ, U.S.A.) packed with 25–45 μ m C18 packing material; the analytical column was fitted with a 3 cm 3 μ C18 mini-column used as a precolumn.

Chemicals, buffers and h.p.l.c. solvents

Buffer solutions, diazo reagents and the crystalline ethyl anthranilate diazo derivative of UCB were prepared as previously described (Spivak & Carey, 1985). H.p.l.c. solvent A was prepared by dissolving 0.04 M (3.282 g) sodium acetate in 1000 ml of methanol. Solvent B was a 1% solution of ammonium acetate prepared by adding 10 ml of acetic acid to 1000 ml of distilled water and titrating to pH 4.5 with concentrated NH_3 (28–30%, w/v). Solvent C was 100% h.p.l.c.-grade methanol. Solvent D was prepared by adding 10 ml of concentrated (88% w/w) formic acid to 1000 ml of distilled water and titrating to pH 4.5 with concentrated NH_3 .

H.p.l.c. elution and quantification

Before each separation, the h.p.l.c. column was equilibrated for 5 min for the analytical separation and for 10 min for the preparative separation with the initial gradient mixture. Using solvents A and B for the analytical bile-pigment separations, a linear gradient of 65% A: 35% B to 95% A in 7 min, 95% A to 75% A in 5 min and then maintaining the gradient at 75% A for an additional 3 min was performed. BMG and BDG were quantified by using AZO UCB as a standard (Spivak & Carey, 1985). Azo pigments were eluted from the analytical column by using a gradient of 80% C: 20% B to 100% C in 5 min. Once we established that the standard curves for determining the concentration of BMG and BDG from their respective integrated-peak-area (IPA) values were virtually identical with the standard curve of UCB against UCB IPA (see the Results section), we used the UCB standard curve for determining BMG and BDG concentration. UCB was quantified by using recrystallized UCB (McDonagh & Assisi, 1972), which was dissolved in chloroform before h.p.l.c. injection. The preparative separation was performed in a manner identical with that previously described (Spivak & Carey, 1985) with a linear gradient of 60% C: 40% D to 100% C in 20 min. For analytical separations the flow rate was 1 ml/min, for preparative separations the flow rate was 2 ml/min.

Pigment identification

Identifications of BDG, BMG and UCB were based on the retention time of pure standards. Identification of other less common conjugates (such as bilirubin monoglucuronide monoglucoside, BMGGI) was based on their previously known h.p.l.c. elution pattern (Spivak & Carey, 1985). XIII α and III α isomers of UCB were identified using samples of these compounds kindly donated by Dr. Anthony McDonagh (Department of Medicine, University of California, San Francisco, CA, U.S.A.). Identification of BDG XIII α and III α isomers was based on their known formation from the IX α BMG isomer in the absence of ascorbic acid (Seig *et al.*, 1982), and on the assumption that the h.p.l.c. elution pattern of these isomers is similar to the h.p.l.c. elution pattern of UCB (XIII α is eluted before III α).

Preparation of pure BDG and pure BMG

BMG and BDG fractions eluted from the preparative column were evaporated to dryness under reduced pressure [26.6–33.3 Pa (200–250 mTorr)] with a Savant Speedvac evaporator (Savant Instruments, Hicksville, NY, U.S.A.) jacketing the specimens in a test-tube ice bath during solvent evaporation to prevent sample heating. Once visibly dry, if samples were subjected to prolonged exposure to reduced pressure (~ 6 h), sample degradation occurred. BMG and BDG prepared in this fashion were used for both quantification and for experiments dealing with 'molecular scrambling' of BMG.

Bile samples

Human bile samples were obtained with informed consent using a protocol approved by the New York Hospital Committee on Human Rights in Research. Gall-bladder bile samples were obtained after an overnight fast, either from the gall-bladder at the time of cholecystectomy or from the duodenum after the

Table 1. Bile pigment concentrations and identification of samples in the Figures

Values are concentrations expressed in μM ; percentage values are expressed as a percentage of the total bilirubin concentration (last column). Further abbreviations used: GBD, gall-bladder bile obtained from duodenum; GB, gall-bladder bile obtained at the time of cholecystectomy; STOMA, bile from hepatic portoenterostomy.

Scan (Fig. no.)	Source	Concn. (μM)							Total bilirubin
		Peak... 1 Pigment... BDG	2 BMGGI	3 BDGI	4 BMG	5 BMGI	6 BMX	7 UCB	
2(a)	Rat	104.6 44.6%	5.5 2.3%	0.0 0.0%	102.7 43.8%	4.9 2.1%	7.4 3.2%	9.2 3.9%	234.3
2(b)	GBD	53.5 77.6%	6.2 9.0%	1.0 1.5%	6.9 10.0%	Trace 0.0%	1.3 1.9%	Trace 0.0%	68.9
2(c)	GBD	0.0 0.0%	0.0 0.0%	0.0 0.0%	94.1 68.2%	13.0 9.4%	7.4 5.4%	23.4 17.0%	137.9
5(a)	GBD	399.0 63.5%	84.0 13.4%	36.6 5.8%	64.0 10.2%	22.0 3.5%	16.2 2.6%	6.6 1.1%	628.4
5(b)	GB	612.8 65.5%	87.2 9.3%	34.8 3.7%	97.6 10.4%	59.4 6.3%	29.4 3.1%	14.6 1.6%	935.8
5(c)	GB	319.8 64.4%	86.2 17.4%	11.8 2.4%	24.8 5.0%	33.2 6.7%	13.6 2.7%	6.8 1.4%	496.2
5(d)	GB	6280.0 95.1%	201.0 3.0%	0.0 0.0%	92.8 1.4%	0.0 0.0%	0.0 0.0%	31.3 0.5%	6605.1
6(a)	STOMA	18.2 47.5%	0.0 0.0%	0.0 0.0%	18.2 47.5%	0.0 0.0%	1.9 5.0%	0.0 0.0%	38.3
6(b)	STOMA	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	1.3 100%	1.3
6(c)	STOMA	38.6 78.6%	5.4 11.0%	Trace 0.0%	5.1 10.4%	0.0 0.0%	Trace 0.0%	0.0 0.0%	49.1
6(d)	GBD	17.3 100%	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	0.0 0.0%	17.3

intravenous administration of Sincalide (C-terminal cholecystokinin octapeptide obtained from E. R. Squibb and Son, Princeton, NJ, U.S.A.). Rat bile was enriched with bile pigments by intravenous infusion of UCB (Spivak & Carey, 1985). Once obtained, all bile samples were immediately placed on ice and protected from light. Samples were usually injected for h.p.l.c. analysis within 1 h of collection; if this was not possible, then the sample was diluted 1:1 (v/v) with aq. 10 mM-ascorbic acid and frozen overnight. The next day the sample was then thawed at room temperature and injected.

RESULTS

Table 1 lists the identity and peak elution of a total of seven pigments that could be separated by this method, not counting C-8 and C-12 isomers and III α and XIII α isomers, and gives the concentration in μM and the percentage concentration of each pigment shown in the Figures below.

The calibration curves for UCB, BMG, BDG are extremely similar, as can be seen in Fig. 1. Because of the extreme similarity between the three curves, we used only the UCB curve for routine standardization. This eliminated the need to prepare three standard curves on a daily basis.

Fig. 2(a) represents an analytical separation that demonstrates excellent separation of BDG, BMG and UCB from rat bile. Note that there is a clear separation of the BMG C-8 and C-12 isomers and baseline separation

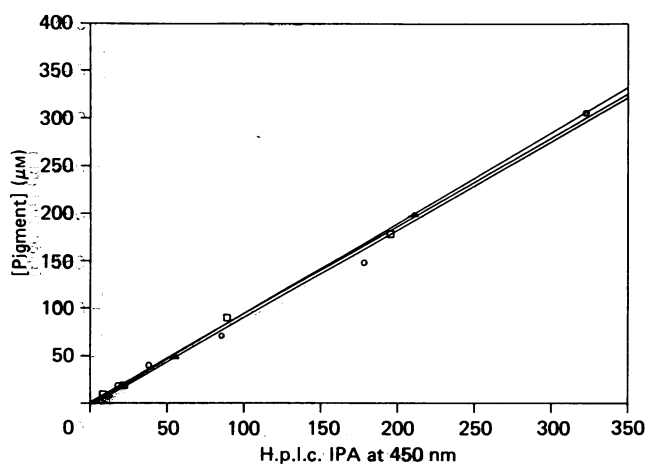


Fig. 1. Bile pigment calibration curve for determining BDG, BMG and UCB concentrations from their respective h.p.l.c. IPA ($10^{-3} \times$ arbitrary units) values measured at $\lambda = 450 \text{ nm}$

Concentrations of BMG and BDG were determined from their azo derivative concentration. The concentration of UCB was determined by using crystalline UCB (see the text). Δ , UCB; \circ , BMG; \square , BDG.

of BDG, BMG and UCB was achieved in only 12 min. As Fig. 2(b) demonstrates, normal human gall-bladder bile contains the additional pigments BMGGI, BDGI, BMGI

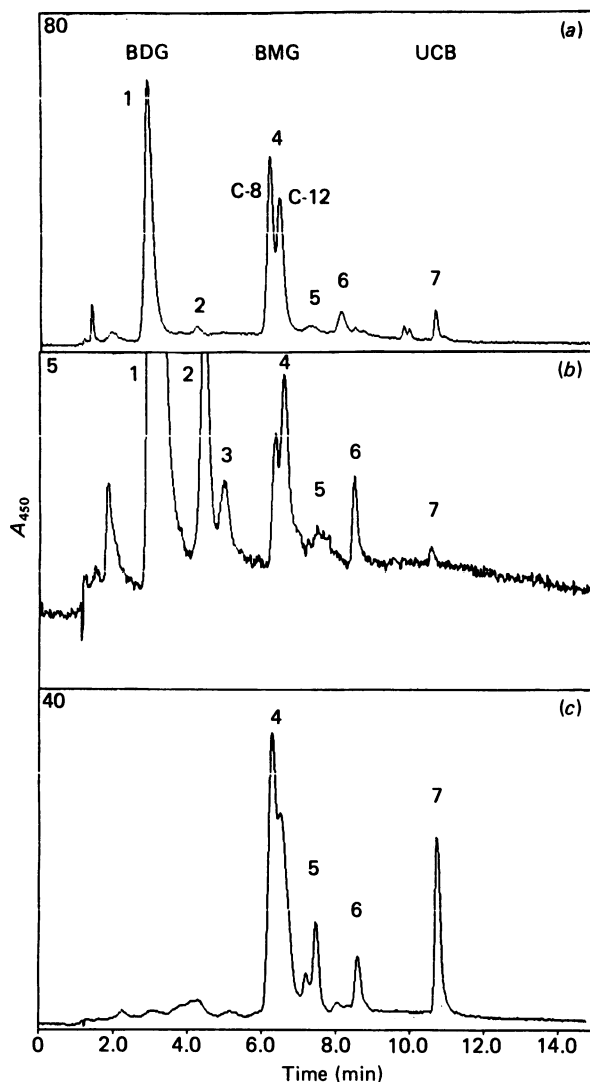


Fig. 2. Typical h.p.l.c. elution profiles demonstrating efficiency and rapidity of method

(a) Rat bile demonstrating BDG, BMG C-8 and C-12 isomers and UCB; (b) normal human bile with additional diconjugates consisting of BMGGI and BDGI, and BMX and BMX; (c) bile from a child with Crigler-Najjar syndrome. Note a large UCB peak and the absence of all diconjugates in (c). A 10 μ l portion of each sample was injected undiluted. The recorder output in mV full scale is in the upper left-hand corner of each scan.

and BMX. Fig. 2(c) is an h.p.l.c. scan of human gall-bladder bile from a 10-year-old boy with hyperbilirubinaemia from birth due to Crigler-Najjar syndrome. Unlike rat and normal human bile (Figs. 2a and 2b), it is clear that this child's bile has no bilirubin diconjugates, a characteristic finding in this disorder (Fevry *et al.*, 1977). This sample contains a mixture of monoconjugates, and more than 10 times the percentage concentration of UCB found in any other human bile sample (see Table 1).

Fig. 3 demonstrates an efficient and rapid baseline preparative separation of BMG and BDG from processed rat bile (see the Experimental section). Also note that the BMG peak in this preparative separation is split into its

C-8 and C-12 isomers. Although further purification of the C-8 and C-12 isomers was not attempted, some C-8 enrichment occurred fortuitously during normal sample collection, as demonstrated in Fig. 3. This degree of C-8-C-12 split could allow a second or third re-injection on the preparative column for further purification of these isomers. The upper inset of Fig. 3 demonstrates pure BDG isolated by this method and re-injected on the analytical column, whereas the lower inset represents pure BMG isolated from the preparative column and re-injected on the analytical column. Note the clear absence of pigment impurities in both insets.

Of particular importance in the measurement of bile pigments is the need to distinguish the IX α isomer that occurs naturally from the XIII α and III α isomers that occur by molecular scrambling; this is especially true for BMG that undergoes molecular scrambling quite readily in the absence of antioxidants. In the process of BMG scrambling, BDG and UCB can be formed. The overall reaction for this process is:

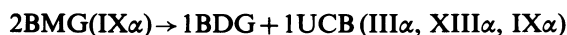


Fig. 4 depicts the capability of this h.p.l.c. system to monitor kinetically the non-enzymic rearrangement of 550 μ M-BMG to BDG and UCB at 37 $^{\circ}$ C at an average pH of 7.4 (the pH varied during the precipitation) in 0.05 M-Tris in the absence of ascorbic acid. Note that co-precipitation of BDG, BMG and UCB occurs after 4 h and that the supernatant and precipitate have similar proportions of pigments. Three isomers each of BDG and UCB are detected after 4 h of incubation. These isomers represent XIII α , IX α and III α isomers of BDG and UCB that form during free-radical isomerization of BMG (Jansen, 1973). At the end of 18 h, virtually all the pigment is in the precipitate. Although BDG is present in the initial precipitate, its notable absence in the precipitate at 18 h is presumably a result of non-enzymic hydrolysis of its glucuronic acid moiety at the solid/liquid interface. As a result of continued BMG rearrangement, and perhaps as a result of BMG hydrolysis to UCB, the amount of UCB in the precipitate at 18 h far exceeds that of BMG.

Figs. 5(b)–5(d) depict a series of human gall-bladder bile (obtained at the time of cholecystectomy) h.p.l.c. scans in patients with gall-bladder disease. For comparison purposes, the sample in Fig. 5(a) was obtained from the duodenum of a patient without gall-bladder disease after the administration of Sincalide to contract the gall-bladder. First, note the marked similarities in the appearance of bilirubin conjugates in the normal, Fig. 5(a) bile, and in the gallstone biles depicted in Figs. 5(b) and 5(c). All three have identical types of bilirubin conjugates and all three have similar, although not identical, proportions of bilirubin conjugates and unconjugated bilirubin. Note that the gallstone bile scan in Fig. 5(c) contains almost the same concentration of UCB as in that in Fig. 5(a), the normal, and although the gallstone bile sample in Fig. 5(b) has a higher concentration of UCB, none of the samples contains more than 2% UCB (see Table 1). Besides BDG, BMG and UCB, in Figs 5(a)–5(c), note that there are appreciable quantities of BMGGI and BDGI and trace amounts of BMGI and BMX. Presumably the split peaks for both the monoglucoside and the monooxyloside represent C-8-C-12 isomers for both of these conjugates. The scan in

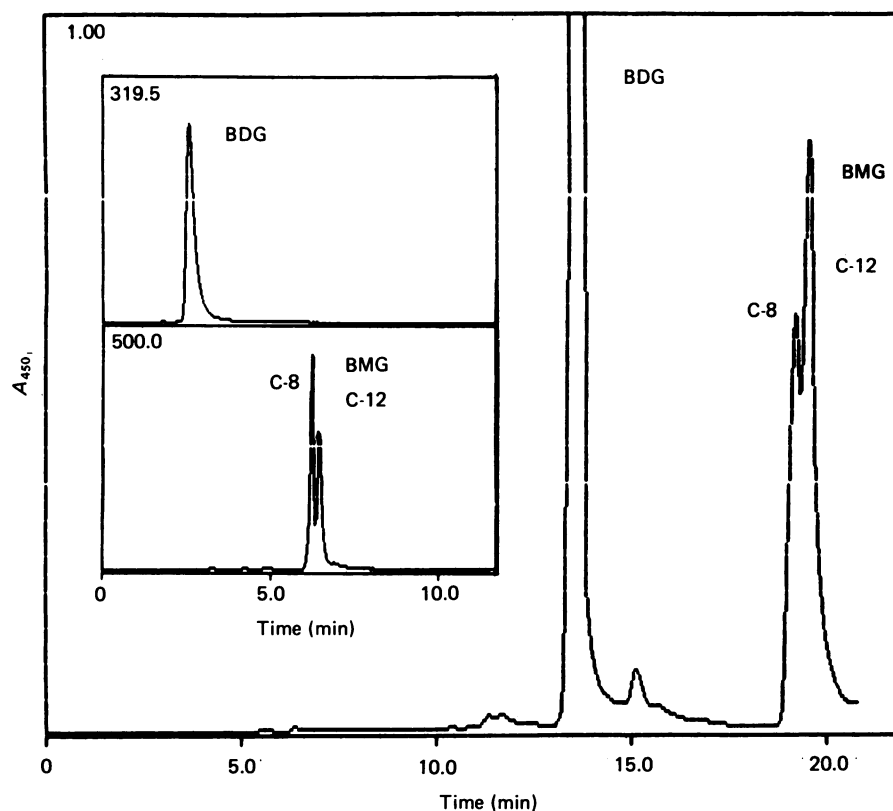


Fig. 3. Preparative h.p.l.c. separation of BDG from BMG

The sample was derived from pigment-enriched rat bile. Note the sharp separation of BDG and BMG. BMG C-8-C-12 isomers are separated, although not to baseline. Upper inset shows BDG isolated from preparative column and re-injected into the analytical column. Similarly, the lower inset shows BMG re-injected into the analytical column. Note absence of impurities in these re-injected samples. Although the C-12 peak is larger in the preparative separation, the C-8 peak is larger in the analytical separation. C-8 enrichment occurred as a result of terminating BMG sample collection before the C-12 peak reached baseline. Again the recorder outputs in V (main scan) or mV (insets) full scale are shown in the upper left-hand corners of the scans.

Fig. 5(d), obtained from the bile of a patient with acalculous cholecystitis, is clearly different from the previous scans in that extremely high concentrations of total pigment are present and that more than 98% of all pigment is in the form of diconjugates, with the rest in the form of small, but easily measured, levels of BMG and UCB. The inset of Fig. 5(d) ($\frac{1}{16}$ the attenuation of Fig. 5(d)) demonstrates that even with 6.28 mM-BDG and only 31.3 μ M-UCB (a 200:1 BDG/UCB ratio), UCB is separated to the baseline and that BMG is separated nearly to the baseline.

Applications to infant biles

Fig. 6 demonstrates the utility of this method in the analysis of bile samples from infants with a variety of hepatobiliary diseases. In Fig. 6(a) the presence of BDG, BMG and UCB can be detected in the initial biliary enterostomy output of a 6-week-old female who had undergone a successful hepatic portoenterostomy (surgical anastomosis between the remnant of the extra-hepatic biliary tree and the jejunum) for biliary atresia. After 6 weeks, as this infant's liver disease progressed and the anastomosis was no longer functioning, only unconjugated bilirubin could be detected in her bile (Fig. 6b). Fig. 6(c) is an enterostomy bile h.p.l.c. scan of a 18-month-old infant with biliary atresia who had undergone a successful hepatic portoenterostomy 16

months previously. Note a pattern of biliary bile pigments that is similar to that of adult bile, but with no detectable levels of UCB. Fig. 6(d) is a scan of bile taken from the duodenum of an infant with a gallstone after the administration of Sincalide to contract the gall-bladder. Note the predominance of BDG, with undetectable amounts of BMG and UCB.

DISCUSSION

We have described an extremely efficient 12-min h.p.l.c. method for the separation of native bile pigments without sample preparation. During the molecular-scrambling reaction of pure BMG, this method permitted the separation of BDG (three isomers), BMG (two isomers) and unconjugated bilirubin (three isomers) on only 6 or 10 μ l of sample (Fig. 4). This method is equally effective in detecting the formation of non-IX α isomers of UCB in native bile that is not properly preserved (W. Spivak & W. Yuey, unpublished data). Since there is considerable variation in the reported measured levels of UCB in bile (Soloway *et al.*, 1977), documentation that III α and XIII α isomers of UCB are not present would be important proof that oxidative formation of UCB has not occurred after sample collection, thereby falsely elevating UCB concentration. Of course, unconjugated bilirubin levels could also be falsely elevated after sample

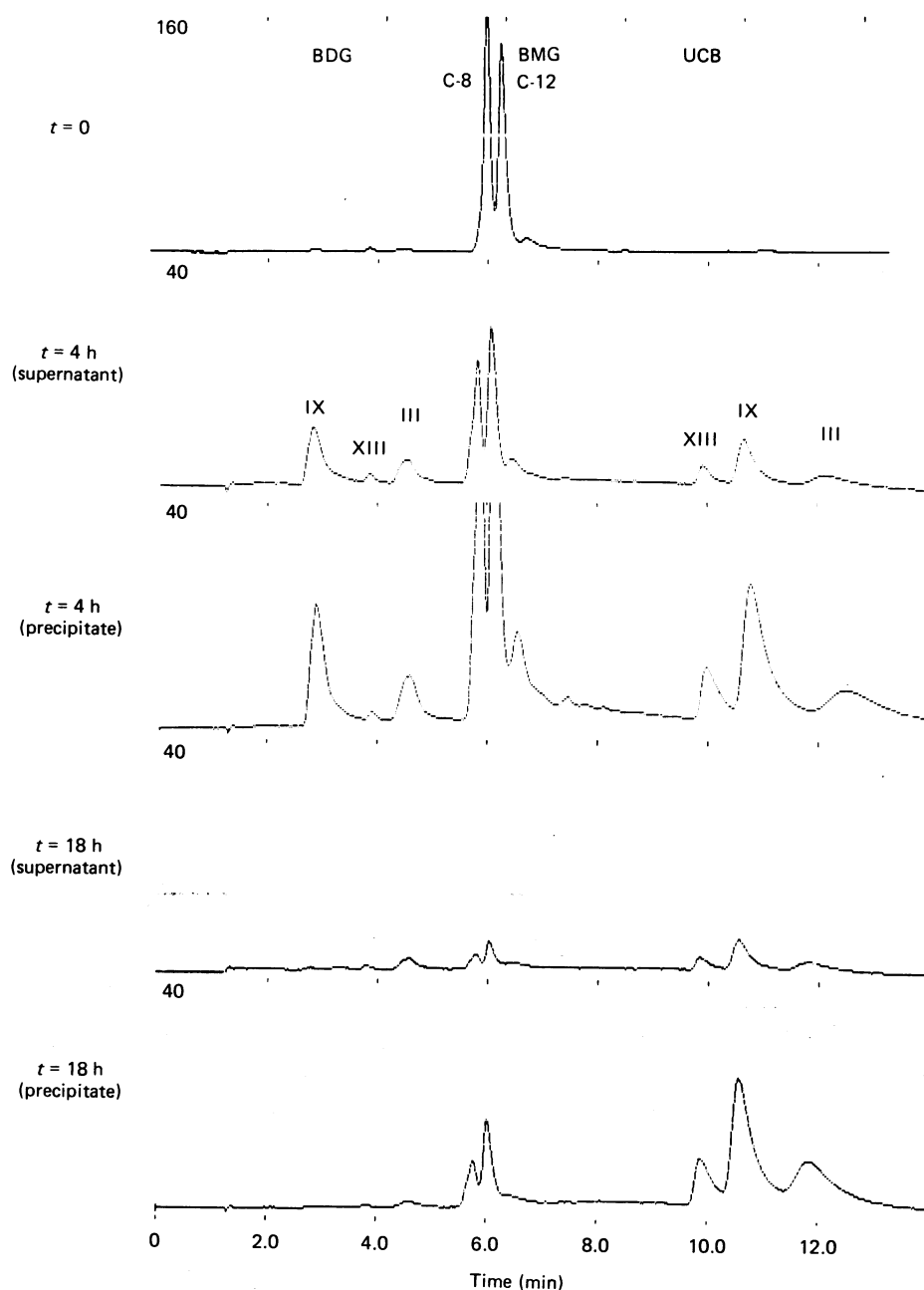


Fig. 4. Non-enzymic molecular scrambling of IX α BMG to BDG and UCB (XIII α , IX α , III α) at 37 °C in the absence of ascorbic acid

Co-precipitation of BDG, BMG and UCB occur at 4 h. At the end of 18 h, BDG is no longer present, and virtually all the pigment is in the precipitate. The precipitate was dissolved in methanol before injection. Absorbance was measured at 450 nm. The recorder output in mV full scale is given in the upper left-hand corner of each scan.

collection by non-enzymic hydrolysis of bilirubin conjugates, producing IX α UCB. By using sampling intervals as frequent as every 20 min, we have used this h.p.l.c. method to monitor the kinetics of such non-enzymic hydrolysis of BMG in model bile systems (Spivak *et al.*, 1984).

We have also shown here how this method can be easily applied to human bile samples. From these studies, it appears that two major patterns of bilirubin conjugation can exist in the normal human. One pattern shows a mixture of diconjugates of glucuronyl and glucosyl origin, with the monoglucuronide and traces of monoglucoside

and monoxyloside present (Figs. 5a–5c, and Fig. 6c). This pattern has been reported by others (Feverly *et al.*, 1972; Spivak & Carey, 1985). Another pattern seen here and not reported elsewhere is the pattern seen in Figs. 5(d) and 6(d) with diconjugates either the only form of bilirubin or > 97% of the total bilirubin. The significance of this is not clear. Perhaps there is a genetic polymorphism in the ability to diconjugate and a subset of the population are 'super diconjugators'. On the other side of the spectrum of bilirubin conjugation such genetic heterogeneity is seen in the common disorder of bilirubin conjugation, Gilbert's syndrome, where a higher per-

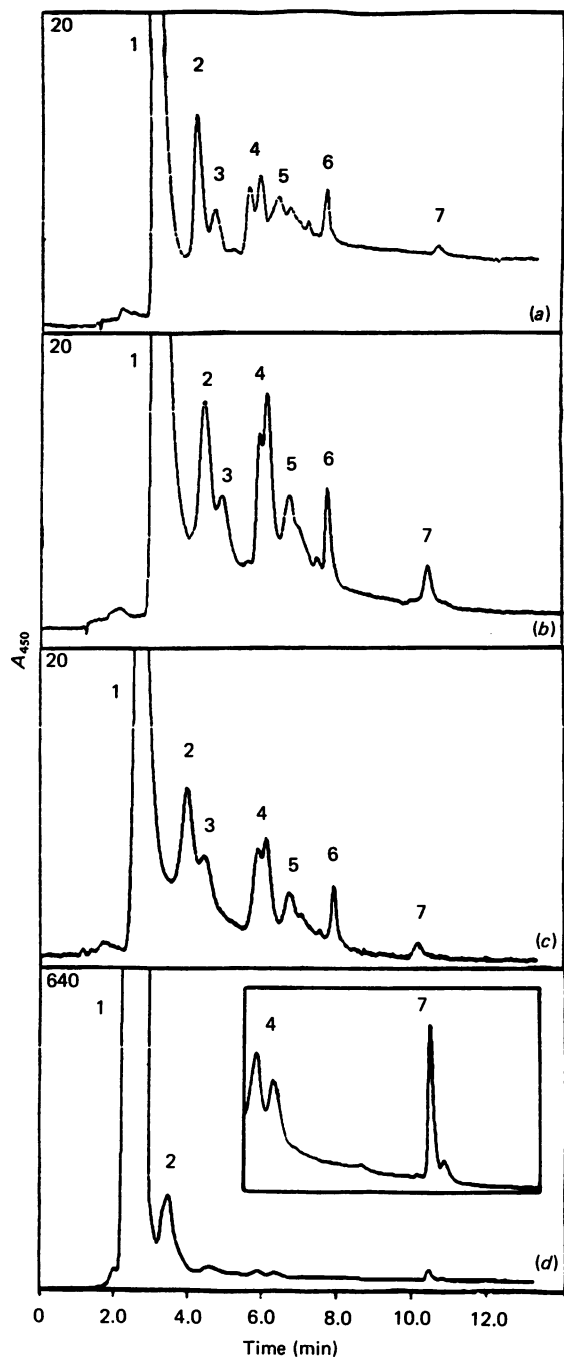


Fig. 5. Typical h.p.l.c. elution profiles of human bile

(a)–(c) are plotted at 20 mV output; (d) is plotted at 640 mV. (a) is a sample of normal human gall-bladder bile obtained from the duodenum after gall-bladder contraction. (b) and (c) were obtained directly from a gall-bladder with gallstones. All three have similar, although not identical, patterns of bilirubin conjugates. (d) is a sample from a patient with acalculous cholecystitis. Note the relative absence of monoconjugates. The inset to (d) is an electronic amplification ($\times 16$) of peaks from 5.5 to 12 min. (a)–(c) were diluted 1:1 with 10 mM-ascorbic acid before injection of $10 \mu\text{l}$. In (d), $10 \mu\text{l}$ of bile was injected undiluted. The recorder output in mV full scale is given in the upper left-hand corner of each scan.

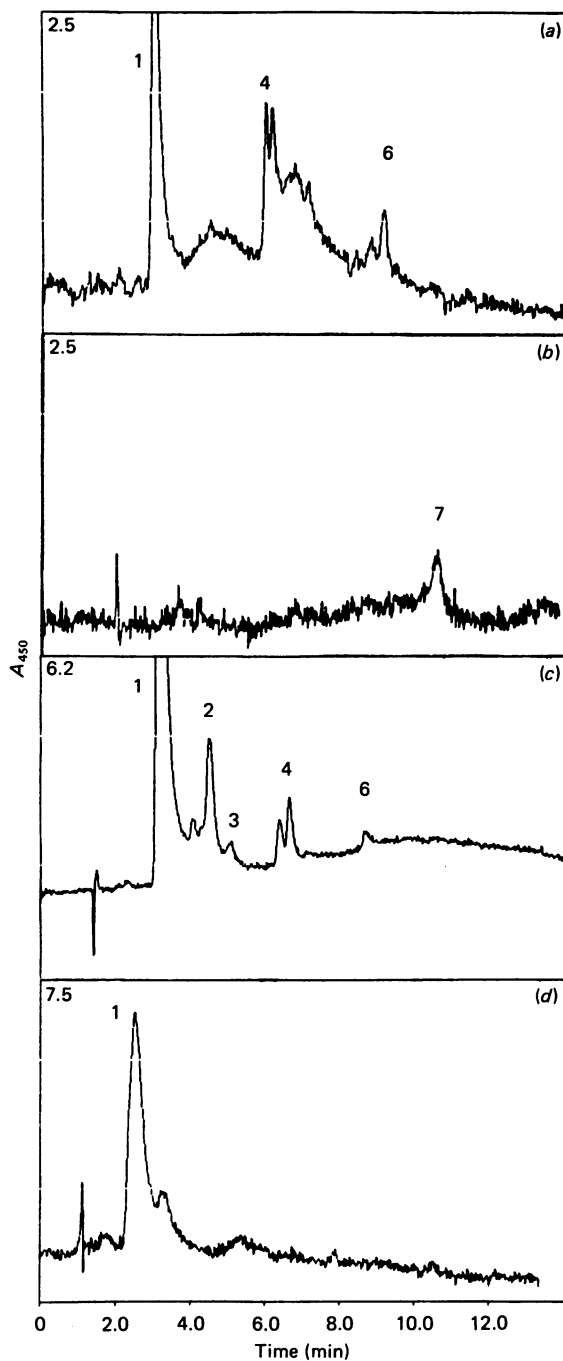


Fig. 6. H.p.l.c. elution profiles of biles from infants

Scan (a) is from the enterostomy drainage of an infant with biliary atresia with a functioning hepatic portoenterostomy, demonstrating bilirubin conjugates. (b) 6 weeks later, after obstruction of the surgical anastomosis, only UCB is detected in the enterostomy fluid. (c) Enterostomy drainage from an 18-month-old child with a functioning hepatic portoenterostomy for more than 16 months. (d) Bile from an infant with a gallstone. All samples were injected undiluted; for (a)–(c), $6 \mu\text{l}$ was injected; for (d), $10 \mu\text{l}$ was injected.

centage of bilirubin is excreted in bile in the form of BMG than in the normal population (Fevry *et al.*, 1977). The h.p.l.c. bile analysis depicted in Fig. 2(c) was obtained

from a child with an extreme form of conjugation deficiency. Since this boy had serum unconjugated bilirubin levels that were extremely high (37 mg%), the diagnosis of Crigler-Najjar syndrome was strongly considered before obtaining this bile sample. The presence of monoconjugated bilirubin, the absence of BDG and the increased percentage of UCB excreted in this child's bile confirmed this diagnosis (Gordon *et al.*, 1976; Fevery *et al.*, 1977). Later the diagnosis of Crigler-Najjar syndrome type II was diagnosed on the basis of a decrease in serum UCB levels after treatment with phenobarbital (Gollan *et al.*, 1975).

Accurate measurement of UCB in bile samples is extremely important, since precipitation of UCB may be the first step in the formation of gallstones (Masuda & Nakayama, 1979). UCB concentrations from the two normal samples here (Figs. 2*b* and 5*a*) are trace (< 0.5%) and 1.1% of total bile pigments respectively. These values are consistent with previous reports for normal gall-bladder bile (Boonyapisit *et al.*, 1978). Note that in gall-bladder bile from patients with gall-bladder disease (Figs. 5*b*–5*d*), less than 2% of bile pigment is in the form of UCB. Although the percentage concentrations are low (except for the anomalous secretion of 17% UCB in the patient with Crigler-Najjar syndrome), actual UCB concentrations range from < 0.5 μM (trace) up to 31.3 μM . From a physical-chemical standpoint, the absolute UCB concentration and the pH of bile may be more important in the formation of bile pigment precipitates than the percentage concentration of UCB (Ostrow *et al.*, 1977).

We have demonstrated the utility of this method for determining biliary obstruction. An infant with a previously successful anastomosis between the biliary outflow tract and the gastrointestinal tract was shown to have a distinct change in bile-pigment output once obstruction occurred. Fig. 6(*b*) demonstrates that bilirubin conjugates were no longer present in the ostomy output of this patient once obstruction occurred. The presence of yellow pigment by itself in the ostomy fluid would not be sufficient proof of continuity of the biliary anastomosis, since most body fluids are normally stained yellow in patients with jaundice. Although serum in patients with obstructive jaundice contains bilirubin conjugates, pigments secreted through the gastrointestinal tract mucosae in this jaundiced infant with biliary obstruction did not contain bilirubin conjugates. Presumably this is a result of hydrolysis of bilirubin conjugates by mucosal β -glucuronidase before secretion. Although further studies using this method for determining biliary obstruction are necessary, we have had additional success on other infants with this method.

Bilirubin and its conjugates are very labile: they readily undergo oxidation, dipyrrolic scrambling and photochemical breakdown (Lightner, 1982). In addition, the conjugates of bilirubin can undergo non-enzymic hydrolysis (Spivak *et al.*, 1984), and acyl shifts of the sugar moiety from the 1-*O*- to the 2-*O*- or 3-*O*-position (Blanckaert *et al.*, 1978). Any decrease in the time between analyses would therefore not only be beneficial in terms of reducing total h.p.l.c. time for sequential analysis of bile samples, but also decrease the possibility of bile-pigment degradation between samples. Decreased analysis time also makes h.p.l.c. more economically feasible, since less solvent is used for each analysis.

Since the analysis time for this method is half that of

any other published h.p.l.c. method (see the references cited in the Introduction), it is particularly suitable for sequential enzymic methods. This method may be particularly useful for kinetic studies that measure formation of BMG and BDG from UCB by the action of glucuronyltransferase in liver microsomes. Rapid detection of III α and XIII α isomers of BDG from BMG would indicate that the source of the BDG was from non-enzymic dipyrrole exchange of BMG and not from an enzymic reaction of native BDG. Enzymic studies involving preferential monoconjugation of the C-8 as against the C-12 propionic acid carbon atom of bilirubin could potentially use this method, since these isomers can be efficiently separated on the analytical column. Since the preparative column permits partial separation of the C-8 and C-12 BMG isomers with one injection, multiple re-injections of the partially purified C-8 or C-12 isomers may permit full separation of these isomers on a preparative scale. The use of pure C-8 or C-12 BMG as a substrate would then permit investigation of whether glucuronyltransferase has a higher affinity for diconjugating C-8 BMG than for C-12 BMG.

Note added in proof (received 9 December 1985)

Because of differences in solvents, gradient elution, h.p.l.c. equipment and columns, the calibration curves in Fig. 1 differ somewhat from those of Spivak & Carey (1985). In addition, in the present study, pure BMG was diluted in 100 mM-sodium taurocholate/1 mM-ascorbic acid in 0.05 M-Tris, pH 7.8, and kept at 4 °C before diazotization and h.p.l.c. calibration. We feel that this solution limits BMG aggregation and improves calibration, especially at higher BMG concentrations.

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