An Accurate and Sensitive Analysis by High-Pressure Liquid Chromatography of Conjugated and Unconjugated Bilirubin IX- α in Various Biological Fluids

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An accurate and sensitive method was developed for the complete separation of the native tetrapyrroles, such as bilirubin and its mono- and di-conjugates of glucuronic acid, glucose and xylose, by ion-pair reversed-phase high-pressure liquid chromatography. The application of this method was demonstrated by the analysis of bile pigments in human bile and urine, and the method also makes it possible to estimate very low UDP-glucuronyltransferase activity, such as is found in the human foetal and neonatal liver.

Van Roy & Heirwegh (1968) developed a t.l.c. method by which it was elucidated that the conjugates of bilirubin are composed of glucoside and xyloside in addition to glucuronide (Heirwegh *et al.*, 1973, 1974). This finding reflects the considerable progress being made in research techniques in the field of conjugated bilirubin structure. An accurate and sensitive analysis of bilirubin and its mono- and di-conjugates without the need to form derivatives is clinically very important to elucidate the pathogenesis of various diseases accompanying jaundice. But hitherto there has been no adequate analytical method for this purpose because these compounds are both photosensitive and easily oxidized, making their separation and analysis difficult. To overcome these difficulties, we introduced a new h.p.l.c. method in 1973 and reported our results at the Second International Symposium held in Jerusalem in 1974 (Onishi *et al.*, 1976). More recently, Billing (1978) has pointed out that the most promising approach would appear to be that of h.p.l.c. We concluded that h.p.l.c. is a useful method for separation of azo-pigments (Onishi *et al.*, 1979*b*), conjugated bilirubin or geometric isomers (Onishi *et al.*, 1979*c*) of bilirubin in biological fluids and have published a preliminary report of these results (Onishi *et al.*, 1979*a*). The present paper describes a complete method for the separation of bilirubin and its conjugates in biological fluids by ion-pair reversed-phase h.p.l.c. without azopigment formation.

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

Materials and Methods

H.p.l.c.

A Shimadzu LC-2FGU liquid chromatograph with an SPD-1 detector and Chromatopac C-R1A was used for all h.p.l.c. procedures. The relative proportions of the peaks were calculated electronically by the Chromatopac C-R1A, and related to the amounts of bilirubin chromatographed. Separation was achieved at room temperature, using two Shimadzu PCH-05/S2504 columns (25 cm $\times$ 4.6 mm) in series. The column used was packed with a reversed-phase packing material (5 μ m particles). The variable-wavelength detector was set at 455 nm. The flow rate was 1.0 ml/min. Separation was best achieved by using a linear gradient of acetonitrile in 0.1 M-acetate buffer, pH 4.0, containing 5 mM-sodium pentanesulphonate as counter-ion [20–48% (v/v) in 70 min, and then 48–60% (v/v) in 3 min]. To avoid loss by oxidation the h.p.l.c. solvent systems were degassed before use. All manipulations were carried out under subdued light.

Peak assignments were made by two methods: one was the t.l.c. method of ethyl anthranilate azo-pigments as described by Heirwegh *et al.* (1974) or the h.p.l.c. method for azopigments into which each peak was converted by diazotized ethyl anthranilate as described previously (Onishi *et al.*, 1979*a,b*); the other involved comparison with the retention time on h.p.l.c. of reference samples obtained from an incubation of bilirubin with UDP-glucuronic acid (or either UDP-glucose or UDP-xylose) and adult

human liver homogenate, yielding bilirubin glucuronides (or either glucoside or xyloside) respectively (Onishi *et al.*, 1979a).

Procedure

Bile samples obtained by duodenal aspiration were analysed immediately. Acetonitrile (300 μ l) was added to 500 μ l of the sample, which was vortex-mixed for 30s and then centrifuged for 5min at 1000 rev./min. The chromatograph was injected with 10–100 μ l of the supernatant.

Pigments and reagents

Bilirubin (E. Merck, Darmstadt, Germany), pentanesulphonate, which is offered for sale as PIC reagent B-7 (Waters Associates), and ethyl anthranilate (Kodak) were used without further purification. Pentan-2-one (Tokyo Kasei, Tokyo, Japan) was distilled before use. UDP-glucuronic acid, UDP-glucose and UDP-xylose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile was of analytical grade.

Results and Discussion

Fig. 1 shows the h.p.l.c. scan of bilirubin IX- α and its conjugates, which were enzymically prepared. Figs. 2 and 3 show the separation of bilirubin and its conjugates in the human adult and neonatal biles respectively. This h.p.l.c. method clearly demonstrated that the major conjugates of bilirubin in normal human bile are the mono- and di-glucur-

onides, with small amounts of other conjugates. The results show that human adult bile mainly contains diglucuronide and small amounts of monoglucuronide, whereas bilirubin monoglucuronide was the predominant bilirubin in bile of the newborn infant. This finding agrees well with the results of the t.l.c. method (Heirwegh *et al.*, 1973; Gordon *et al.*, 1976, 1977; Blumenthal *et al.*, 1979). Scramble and geometric isomerization, auto-oxidation and photo-oxidation are unlikely to occur under the deoxygenated closed condition used. No reconstitution of the parent tetrapyrrolic structures is required, even if complex mixtures are analysed. We have thus developed an innovative method to measure amounts of bilirubin conjugate rapidly and specifically by using h.p.l.c. This method separates bilirubin and its mono- and di-conjugates simultaneously on the h.p.l.c. chart, and it was also possible to assign the particular carboxy group of the B or C pyrrole ring with which the glucuronic acid moiety of monoglucuronide has an ester bond. As we used an acidic solvent system, we have demonstrated that the acid-catalysed rearrangement (Bonnett & McDonagh, 1970) does not occur during the h.p.l.c. analysis by

Fig. 1. H.p.l.c. scan of bilirubin IX- α and its mono- and di-conjugates of glucuronic acid, glucose and xylose obtained from enzymic synthesis

Peaks were numbered in order of retention time: 1, diglucuronide; 2, diglucoside; 3, dixyloside; 4, *endo*-vinyl monoglucuronide isomer (ring B); 5, *exo*-vinyl monoglucuronide isomer (ring C); 6, *endo*-vinyl monoglucoside isomer (ring B); 7, *exo*-vinyl monoglucoside isomer (ring C); 8, *endo*-vinyl monoxxyloside isomer (ring B); 9, *exo*-vinyl monoxxyloside isomer (ring C); 10, unconjugated bilirubin IX- α .

Fig. 2. H.p.l.c. separation of bilirubin and its conjugates in human adult bile

Fig. 3. H.p.l.c. separation of bilirubin and its conjugates in human neonatal bile

Fig. 4. H.p.l.c. scan of bilirubin IX- α with negligible accompanying III- α and XIII- α isomers

Fig. 5. H.p.l.c. scan of a separated mixture of bilirubin IX- α and bilirubin IX- α diglucuronide with negligible accompanying monoglucuronide

Fig. 6. H.p.l.c. separation of bilirubin IX- α and its di- and mono-glucuronide (rings B and C) in reaction medium for estimating UDP-glucuronyltransferase activity as described by Black *et al.* (1970)

showing chromatograms of bilirubin IX- α with negligible accompanying III- α and XIII- α isomers (Fig. 4) and a separated mixture of bilirubin and bilirubin

Fig. 7. Correlation between the electronically integrated peak area by Chromatopac RIA and the amounts of bilirubin loaded

diglucuronide with negligible accompanying monoglucuronide (Fig. 5).

We applied the h.p.l.c. method to the estimation of very low UDP-glucuronyltransferase activity. Fig. 6 shows the separation of bilirubin IX- α and its mono- and di-glucuronide in the reaction medium for estimating UDP-glucuronyltransferase activity as described by Black *et al.* (1970). In Fig. 7 the electronically integrated peak area is compared with the amounts of bilirubin chromatographed. This method makes it possible to measure with greater sensitivity systems with very low UDP-glucuronyltransferase activity and very low concentrations of reaction products (e.g. liver enzyme during perinatal development, Crigler-Najjar syndrome, Gilbert syndrome). The sensitivity of the h.p.l.c. method was increased over 100 times compared with the t.l.c. densitometric or spectrophotometric method (Black *et al.*, 1970; Strebel & Odell, 1971; Heirwegh *et al.*, 1974).

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