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Note

Analysis of bilirubin conjugates in human bile by column liquid chromatography

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Bilirubin is excreted into bile after conjugation by the liver The conjugated bilirubin species in human bile have long been analysed using thin-layer chromatography (TLC) [1], column liquid chromatography (LC) [2–4] and other methods, though many of the results reported do not seem to agree with each other. This report describes a simple and novel LC method for the separation, identification and quantitation of at least seven bilirubin species in human bile

EXPERIMENTAL

Bilirubin IX_{α}, UDP-glucuronic acid, UDP-glucose and UDP-xylose were obtained from Sigma (St Louis, MO, U S A) and 1-pentanesulphonic acid from Aldrich (Milwaukee, WI, U S A) All other reagent-grade chemicals were obtained from Wako (Osaka, Japan) Solvents, including acetonitrile, for LC were of HPLC grade

Apparatus

A C_{18} μBondapak column (30 cm × 3 9 mm I D., particle size 8–10 μm, Waters Assoc , Milford, MA, U S A) was used for the separation A Waters Model 510 liquid chromatography pump was used together with a Waters Lambda-Max Model 481 LC spectrophotometer–detector and a Data Module 730 recorder The eluent was monitored for absorbance at 450 nm. The flow-rate was 1 ml/min The mobile phase was acetonitrile–0 1 *M* sodium acetate (pH 4 0) containing 5 m*M* pentanesulphonate Gradient elution was done from

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20 to 48% acetonitrile in 35 min, from 48 to 90% in 10 min, and after that 90% acetonitrile was maintained for another 15 min

Procedure

Bile samples were obtained from seven healthy volunteers by duodenal aspiration Bile was collected on ice in the dark. Ascorbic acid was added to a final concentration of 4% Bile samples were analysed immediately or stored in liquid nitrogen until use Bile (1 vol) and acetone (3 vols) were mixed and centrifuged at 180 *g* for 5 min The supernatant was added with 4 vols. of 0.1 *M* sodium acetate and filtered through a Millex SR filter unit (0.5 μm, Millipore, Bedford, MA, U S A.) The filtrated mixture was injected into the column in aliquots of 10–100 μl.

The peaks obtained by LC were collected and re-injected into the LC column to see if there were any transformations of the peaks affecting other moieties of bilirubin Intra-day and inter-day variations were determined from ten measurements each

Microsomal suspension was obtained from a male Sprague–Dawley rat liver as described [5] The digitonin-activated microsomal suspension was used for the biosynthesis of bilirubin di- (BDG) and monoglucuronide (BMG), bilirubin di- and monoglucoside and bilirubin di- and monoxyloside [6] The enzyme reaction was stopped by adding 1 ml of 0 4 *M* glycine hydrochloride buffer (pH 1 8) saturated with sodium chloride, containing 4% ascorbic acid Each enzyme–substrate mixture was added with 1 ml of chloroform–ethanol (1:1) and shaken for 5 min at room temperature

The mixture was centrifuged at 1900 *g* for 5 min at 4°C The supernatant fraction was evaporated to reduce the volume, freeze-dried and stored in liquid nitrogen The dried samples were dissolved in the initial eluting buffer and injected into the LC column

Peak assignment

For peak collections, ammonium formate was used instead of sodium acetate. A 1-ml aliquot of the eluent of each peak and 0 5 ml of ethyl anthranilate diazo reagent [1] were kept at 25°C for 30 min in the dark. 4% Ascorbic acid solution (1 vol) was added and dried at room temperature using a rotary evaporator Then, the diazo derivatives of bile pigments formed were extracted into methylpropylketone–butyl acetate (17 3) and chromatographed on TLC plates (Kieselgel 60 F-254, Merck, Darmstadt, F.R.G.) or on LC columns (C_{18} μBondapak, Waters Assoc) Thin-layer chromatograms were developed by chloroform–methanol–water (65 25 3) over the first 6 5 cm, by chloroform–methanol (17.3) over the next 5 cm and by chloroform–methanol (19·1) over another 5 cm as reported [1]. After drying the methylpropylketone–butyl acetate extract, the diazo derivatives were dissolved in 0 1 *M* ammonium formate–acetonitrile (60 40). Reversed-phase LC of the diazo derivatives was performed from 0.1 *M* ammonium formate–acetonitrile (60 40) to 90% acetonitrile in 60 min [7] The quantitation of each peak was performed by measuring the absorbance at 450 nm with the ditaurine conjugate of bilirubin (Porphyrin Products, Logan, UT, U.S A) as the standard.

RESULTS

Fig. 1 shows the LC separation of bilirubin and its conjugates in normal human bile. At least seven peaks were found The diazotized LC peaks were developed on a TLC plate (Fig 2, Table I) Peak A mainly consisted of the δ

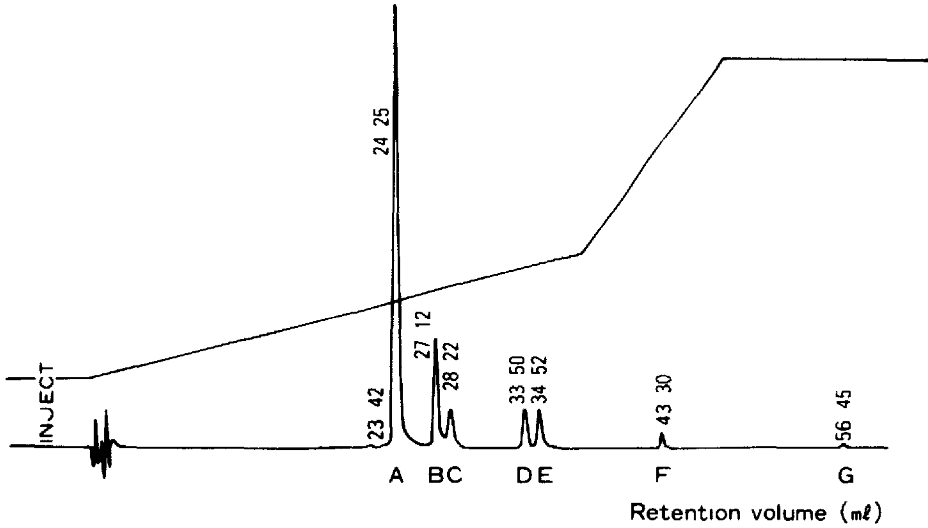

Fig 1 LC separation of bilirubin fractions from adult duodenal aspirate A 20-μl sample was injected Absorbance at 450 nm, 0 2 a u f s Numerals written on the peaks denote the retention volumes For peak identification, see Table I

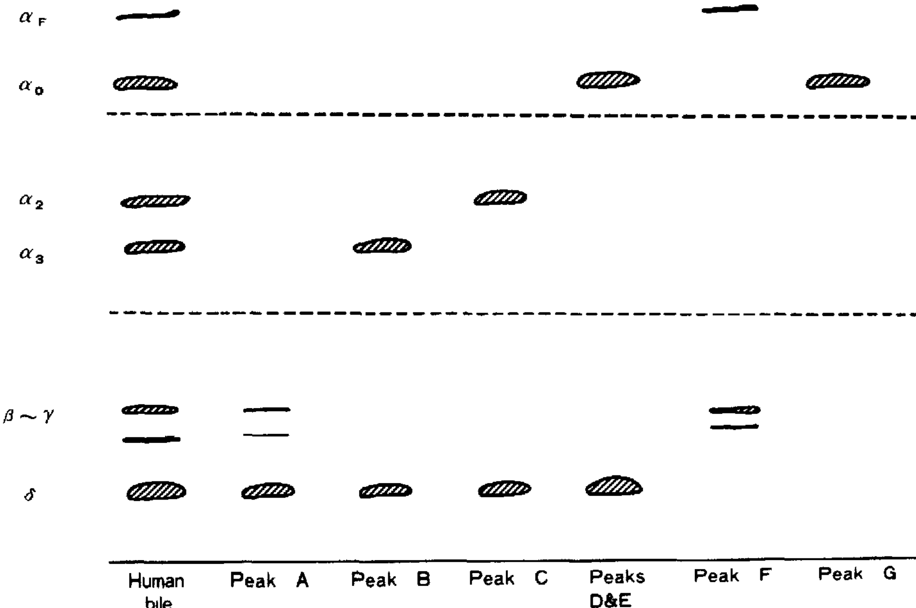

Fig 2 TLC separation of the azo derivatives of the LC peaks Each eluted peak obtained by LC was diazotized and analysed by TLC Peak A consisted of the δ band, B the δ and α_3 bands, C the δ and α_2 bands, D and E the δ and α_0 bands, F the β and α_F bands, G the α_0 band From these results, peak A was identified as BDG, B as GG, C as GX, D as endovinyl BMG, E as exovinyl BMG, F as bilirubin IX_β and/or IX_δ and G as bilirubin IX_α

TABLE I

STRUCTURAL ELUCIDATION OF THE SEVEN PEAKS SEPARATED BY LC

Peak	Retention volume (ml)	Azopigments	Bilirubin conjugate
A	24.3	δ, δ	Bilirubin diglucuronide
B	27.1	δ, α_3	Bilirubin monoglucuronide monoglucoside diester
C	28.2	δ, α_2	Bilirubin monoglucuronide monoxyloside diester
D	33.5	δ, α_0	Bilirubin monoglucuronide (endovinyl)
E	34.5	δ, α_0	Bilirubin monoglucuronide (exovinyl)
F	43.3	β, α_F	Bilirubin IX_β and/or IX_δ
G	56.5	α_0, α_0	Bilirubin IX_α

band on TLC and was identified as BDG. Peak B consisted of the α_3 and δ bands and was identified as bilirubin monoglucuronide monoglucoside diester (GG) Peak C consisted of the α_2 and δ bands and was identified as bilirubin monoglucuronide monoxyloside diester (GX). Peaks D and E consisted of the α_0 and δ bands and were identified as two BMG endovinyl and exovinyl isomers Peak F consisted of the α_F and β bands and was considered as bilirubin IX_β and/or IX_δ. Peak G consisted of the α_0 band and was identified as bilirubin IX_α BDG, BMG, GG, GX, bilirubin IX_β and/or IX_δ and bilirubin IX_α were recovered unchanged after the re-injection of each peak. The retention volumes of BDG and two BMG isomers, which were obtained by enzymatic reaction using rat microsomal preparation, were in good agreement with those of LC peaks A, D and E (Fig 1), respectively. The retention volumes of enzymatically obtained bilirubin diglucoside was 29.5 ml, two bilirubin monoglucoside isomers 38.8 and 40.2 ml, bilirubin dixyloside 31.9 ml and two bilirubin monoxyloside isomers 41.6 and 43.1 ml, and any LC peaks with these retention volumes were not found from normal adult human bile. LC of the diazotized derivatives of the bilirubin peaks obtained revealed that BDG (peak A) consisted of the two δ peaks with retention volumes of 17.6 and 19.3 ml, BMG (peak G) the δ (retention volume 17.6 ml) and α_0 (42.6 ml) peaks, BMG (peak F) the δ (19.3 ml) and α_0 (40.7 ml) peaks, GG the two δ and two α_3 (24.4 and 25.9 ml) peaks and GX the two δ and two α_2 (29.7 and 31.6 ml) peaks (Fig 3) Bile bilirubin fractions in seven normal adults were as follows (mean ± S D) BDG 76.5 ± 4.8%, GG 8.7 ± 2.0%, GX 3.7 ± 1.1%, BMG 9.8 ± 2.5%, bilirubin IX_β and/or IX_δ 1.1 ± 0.7% and bilirubin IX_α 0.2 ± 0.2%.

The intra-day and inter-day variations both for BDG and BMG determinations were 1.5 and < 3.7%, respectively.

DISCUSSION

LC analyses of bile bilirubin fractions were developed recently, because, compared with former methods [1], the intact tetrapyrroles can be analysed without any structural modification. Jansen and Tangerman [2], Onishi et al [3] and Gordon and Goresky [4] also reported the LC separations of bile pigments. However, the peaks of GG and GX were not identified by Onishi et al.

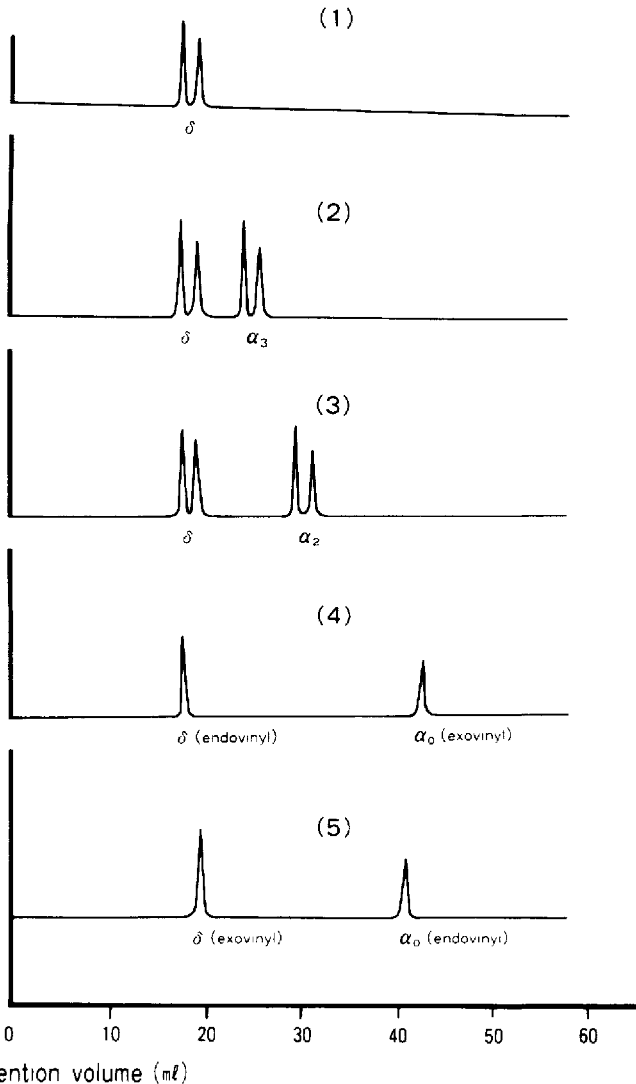

Fig 3 Further separation of the diazo derivatives of each LC peak Absorbance at 530 nm LC of the diazotized peak A (BDG) showed the two δ peaks (1), peak B (GG) the two δ and two α_3 peaks (2), C (GX) the two δ and two α_2 peaks (3), and D (BMG) the fast-eluted δ and slow-eluted α_0 peaks (4), E (BMG) the slow-eluted δ and fast-eluted α_0 peaks (5) and G (bilirubin IX_α) the two α_0 peaks The fast-eluted α_0, α_2, α_3 and δ peaks were confirmed to be the endovinyl azo derivatives of bilirubin, whereas the slow-eluted peaks were confirmed to be the exovinyl azo derivatives by nuclear magnetic resonance

[3] and the peak of GX was also not identified by Jansen and Tangerman [2] and Gordon and Goresky [4] The method described here can separate bile bilirubin conjugates into seven species, including GG and GX, and is simple and accurate. No transformation of any of the peaks was found after re-injection of the peaks obtained, which resulted in no significant changes for the other bilirubin moieties In normal adults, glucuronic acid is the major sugar donor of bilirubin conjugates GG and GX also exist, but their contribution to the total bile pigment is small From the results of LC of the diazotized derivatives, both endovinyl and exovinyl isomers of GG and GX exist Gordon and Goresky [4] reported that bilirubin diglucoside was found in dog bile, but we did not find

any bilirubin di- and monoconjugates with glucose and xylose in human bile Bilirubin UDP-glucuronyltransferase, bilirubin UDP-glucosyltransferase and bilirubin UDP-xylosyltransferase activities can be measured in the human liver and the proportion of these three enzymes has been reported as 1·0.07·0.6 by Fevery et al. [8] and 1 0 50·0 98 by the authors [9] The proportion of these three enzymes does not coincide with the results of biliary bilirubin fractionation reported here The predominance of BDG in bile is in favour of the predominance of bilirubin glucuronylation over glucosylation or xylosylation Trace amounts of bilirubin IX_{β} and/or IX_{δ} and bilirubin IX_{α} can be found in human bile In preliminary observations, the BDG fraction in bile decreased to ca 10% in Crigler-Najjar syndrome type II, and to ca 55% in Gilbert's syndrome and in haemolytic anaemia, the BMG fraction increased with a decrease in the BDG fraction (unpublished observation) This LC method seems to be useful for the observation of changes in the proportions of the bilirubin conjugates in liver diseases, especially in various hyperbilirubinaemias.

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