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Note

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

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Before excretion into bile, bilirubin is conjugated in the liver. Under physiological conditions, little unconjugated bilirubin appears in bile [1, 2]. Azopigment analysis has revealed that in man and rat, bilirubin mono- and diglucuronide are the main conjugates [3, 4]. Dog bile contains a mixture of glucuronide, glucoside and xyloside, mono-, di- and mixed conjugates [5]. These bilirubin conjugates react with diazo reagents, e.g. diazonium salts of ethyl anthranilate and *p*-iodoaniline, to form azodipyrrole pigments [6]. Azopigment analysis has led to the recognition that, besides bilirubin diglucuronide, other bilirubin conjugates exist [3—5]. A disadvantage of azopigment analysis is that through cleavage in dipyrryl derivatives, structural information about the composition of tetrapyrrole pigments is lost. Therefore methods are needed to separate the tetrapyrroles. Tetrapyrrole bile pigments can be separated directly by thin-layer chromatography (TLC) [3, 5, 7].

However, bile pigments are oxygen and light sensitive, and during work-up by TLC, considerable losses occur [4]. This paper describes a new method for the direct separation of bile pigments by means of high-performance liquid chromatography (HPLC). HPLC is particularly suited for bile pigment analysis because it is a rapid and simple technique and can be carried out under exclusion of light and oxygen.

MATERIALS AND METHODS

Chemicals

Tetrabutylammonium hydrogen sulphate was obtained from Fluka (Buchs, Switzerland). Acetonitrile came from Merck (Darmstadt, G.F.R.).

HPLC

A high-performance liquid chromatograph, Spectra Physics (Santa Clara, Calif., U.S.A.) Model 3500B, with a 20-μl injection loop was used, connected to a Spectra Physics variable-wavelength monitor (Model 770), set at 450 nm. A LiChrosorb 5 RP-18 column (150 mm × 4.6 mm I.D., particle size 5 μm; Chrompack, Middelburg, The Netherlands) was used.

Acetonitrile—0.01 *M* Tris—HCl buffer (40:60, v/v), containing 9.0 m*M* tetrabutylammonium hydrogen sulphate, was adjusted to pH 8.0 with 4 *M* sodium hydroxide and was used as the mobile phase. The flow-rate was 0.7 ml/min.

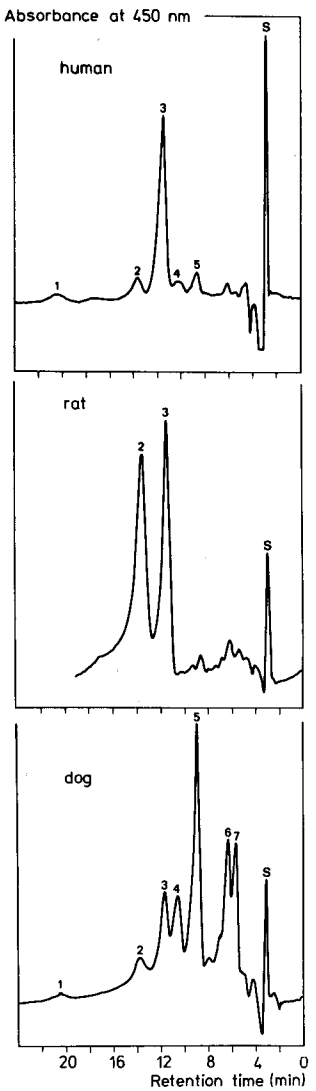

Fig. 1. HPLC analysis of human, rat and dog bile. Bile was diluted with citrate—phosphate buffer and injected directly into the HPLC column. S = solvent front, for peaks 1—7 see Table I.

Sample preparation

Rat bile was collected from fluothane anesthetized Wistar rats provided with bile duct cannulas. Dog bile was aspirated from the gall bladder of Labradors and Beagles. Human bile was obtained from a cholecystectomized patient with a bile duct drain. To prevent isomerisation [8], human bile and dog bile were diluted with 0.1 *M* citrate—0.2 *M* disodium hydrogen phosphate buffer (pH 4.0). Rat bile was collected in the dark, in ice, in tubes containing citrate—phosphate buffer. Diluted bile samples were injected directly into the HPLC column.

The preparation of reference material was carried out in the following way. After extraction from bile with chloroform, the bilirubin conjugates were separated using TLC [5]. The yellow pigments were eluted in methanol and injected into the HPLC column. Samples of the methanol eluates were diazotized with ethyl anthranilate as diazo reagent [6]. The azopigments were analysed by means of TLC [6].

RESULTS

Fig. 1 shows the HPLC analysis of human, rat and dog bile. Dog bile shows seven main peaks, enumerated 1—7. Peak 3 is the main pigment in human bile, peaks 2 and 3 in rat bile and peak 5 in dog bile. For identification of the

TABLE I

PEAK IDENTIFICATION

The tetrapyrrole pigments of human, rat and dog bile extracts were separated using TLC [5]. The R_F values of the pigments are shown in the table. The pigments were analyzed as follows. Samples of the separated pigments were submitted to azopigment analysis. According to the nomenclature proposed by Heirweigh et al. [5], α_0 is unconjugated azopigment; δ, azodipyrryl glucuronide; α_2, azodipyrryl xyloside; and α_3, azodipyrryl glucoside. In case two different azopigments were found, their ratio is shown within brackets. In addition, samples of the separated pigments were injected directly into the HPLC column. The retention times are shown. The pigment numbers correspond to the peak numbers in Fig. 1 and follow from comparison of the retention times.

Pigment	HPLC retention time (min)	TLC R_F value	Azopigments	Bilirubin conjugate
1	21	0.95	α_0	α_0—α_0, unconjugated bilirubin
2	13.5	0.58	α_0, δ (1:1)	α_0—δ, bilirubin monoglucuronide
3	11.5	0.38	δ	δ—δ, bilirubin diglucuronide
4	10.5	—	—	unknown
5	9.0	0.45	α_3, δ (1:1)	α_3—δ, bilirubin monoglucoside monoglucuronide
6	6.5	0.68	α_2, α_3 (1:1)	α_2—α_3 bilirubin monoxyloside monoglucoside
7	5.5	0.63	α_3	α_3—α_3, bilirubin diglucoside

peaks, bile was extracted in chloroform and the bile pigments were separated by means of TLC [5]. The separated pigments were analyzed by HPLC and diazotized as to study their azo-pigment composition.

Table I summarizes the results. Peak 2 is bilirubin monoglucuronide; peak 3, diglucuronide; peak 5, bilirubin monoglucoside monoglucuronide; peak 6, bilirubin monoxyloside monoglucoside; and peak 7, bilirubin diglucoside. Peak 4 could not be identified. This pigment is lost during extraction of bile with chloroform or during TLC.

DISCUSSION

HPLC is an excellent technique for the separation of tetrapyrrole bile pigments. With acetonitrile—water (70:30, v/v) as eluent, Lim [9] separated conjugated from unconjugated bilirubin, using a reversed-phase column. For the separation of mono- and diglucuronides Lim [9] used a μBondapak carbohydrate column. In the present paper a method is described for the separation of unconjugated bilirubin and several conjugates, in a single run on a reversed-phase column. Tetrabutylammonium hydrogen sulphate acts as a counter-ion. Without this compound all peaks run with the solvent front. At concentrations lower than 9.0 m*M*, the method can be used to separate the bilirubin IIIα, IXα, and XIIIα isomers (unpublished observations).

Gordon et al. [3] using a TLC technique, found that human bile contains 86% bilirubin diglucuronide, 7% bilirubin monoglucoside monoglucuronide diester, 4% bilirubin monoglucuronide and 3% bilirubin. According to the same authors, the main pigments in dog bile are bilirubin diglucuronide and bilirubin monoglucoside monoglucuronide diester [3]. Dog bile also contains bilirubin monoglucuronide, diglucoside, bilirubin and traces of xylose-containing conjugates [3]. Noir [4], also using a TLC method, found that rat bile contains mainly bilirubin mono- and diglucuronides. Our results are in agreement with these findings.

There is a current interest in the importance of unconjugated bilirubin in human bile for the formation of gall stones [10]. The HPLC method described in this paper seems to be well suited to study this problem.

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