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Note

Separation of bilirubin isomers and their conjugates by high-performance reversed-phase liquid chromatography

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Recently, several methods have been reported for the direct separation of bilirubin (BR) species by means of high-performance liquid chromatography (HPLC) [1—4]. At present, we need a more sensitive and precise assay method for direct determination of each BR isomer and its conjugates to obtain accurate information on the conjugation mechanisms.

In the present report, we describe a sensitive method for the specific determination of three isomers of unconjugated BR (IIIα, IXα, and XIIIα BR), four isomers of BR mono-glucuronide (IIIα, endovinyl IXα, exovinyl IXα, and XIIIα BMG), three isomers of BR di-glucuronide (IIIα, IXα, and XIIIα BDG), IXα BR mono- and di-glucosides, and IXα BR mono- and di-xylosides. This HPLC procedure is expected not only to serve as a valuable tool in the study of BR conjugation mechanisms but also to be clinically useful.

MATERIALS AND METHODS

Chemicals

Bilirubin (BR), UDP-glucuronic acid (UDPGa), UDP-glucose, UDP-xylose and glucaro-1,4-lactone were obtained from Sigma Chemical Company. IIIα, IXα and XIIIα isomers of BR were isolated from commercial BR and purified

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by the method of McDonagh and Assisi [5]. All other reagents were of analytical reagent grade. Glass plates precoated with silica gel (Merck, Kieselgel 60) were used for thin-layer chromatography.

High-performance liquid chromatography

The HPLC system was used was a Nippon Bunko (Tokyo, Japan) Tri-Rotar, equipped with a variable-wavelength spectrometric detector, Uvidex 100 III, and an electronic integrator, DP-L 200 data processor. The detector was set at 450 nm for analysis. Columns were of 4-mm bore polished stainless steel and 300 mm long. ODS gel, TSK-410 (particle size 5 μm, Toyo Soda, Japan) was used as packing material. Roughly 2 g of the packing material were suspended in 10 ml of chloroform, sonicated for 3 min and poured into the column. Then the packing material was packed with a constant pressure of 400 kg/cm^2 using methanol—water (1:1, v/v) as the pressuring liquid.

Two different solvent systems, A and B, were used. Solvent A, consisting of 0.05 *M* citrate buffer (pH 5)—methanol—acetonitrile (8:9:3, v/v), was used for the separation of BR conjugates. Solvent B, consisting of 0.05 *M* citrate buffer (pH 6.5)—methanol—acetonitrile (8:5:7, v/v), was used for the rapid analysis of unconjugated BR isomers. In most cases, after the elution of the conjugates with solvent A, the mobile phase was changed to solvent B for the separation of unconjugated BR isomers. The flow-rate was 0.6 ml/min and the paper speed 2 mm/min. All separations were carried out at room temperature (22—23°C).

Preparation of samples for HPLC

Amounts of 400 mg of crystalline ammonium sulfate, 20 mg of ascorbic acid and 0.2 ml of an extraction solvent consisting of isopropanol—methanol—dimethylsulfoxide (6:3:1, v/v) were added to 0.5 ml of each sample of bile and enzyme reaction mixture. The mixture was agitated vigorously on a Vortex mixer for 1 min, and centrifuged. An aliquot of the upper organic layer (5–20 μl) was injected into the column.

Biosynthesis of BR conjugates

One milligram of each of the purified BR isomers was dissolved in 0.05 *M* NaOH and the solution was diluted to the concentration of 200 μg/ml with 5.5% bovine serum albumin solution in 1.15% KCl. One volume of rat liver microsomal suspension (20 mg/ml in 1.15% KCl) was treated with 1 volume of digitonin solution (10 mg/ml in water), and the mixture was kept at 0°C for 30 min before preparation of the incubation mixtures.

The incubation mixture consisted of 0.05 ml of albumin-solubilized BR solution (final concentration 17.1 μM), 0.1 ml of digitonin-activated microsomal preparation (final protein concentration 2.0 mg/ml), 0.1 ml of aqueous solution containing UDPGa, $MgCl_2$ and glucaro-1,4-lactone (all final concentrations 10 m*M*), and 0.25 ml of 0.2 *M* phosphate buffer (pH 6.5). The mixture was incubated for 10 min at 37°C in a dark place. The reaction was stopped by adding 400 mg of ammonium sulfate and 20 mg of ascorbic acid, and the reaction tube was transferred into an ice bath. BR glucuronides biosynthesized were extracted with 0.2 ml of the extraction solvent. Glucose and xylose

conjugates of IXα BR were also synthesized enzymatically by the same procedure using UDP-glucose and UDP-xylose as the cofactors instead of UDPGa.

RESULTS AND DISCUSSION

The solvent systems A and B used here were adopted after a series of preliminary experiments. The solvent ratio in system A was chosen to obtain mainly the best resolution of the conjugates of BR isomers, and the ratio in solvent B was adopted to obtain a fast and good separation of unconjugated BR isomers.

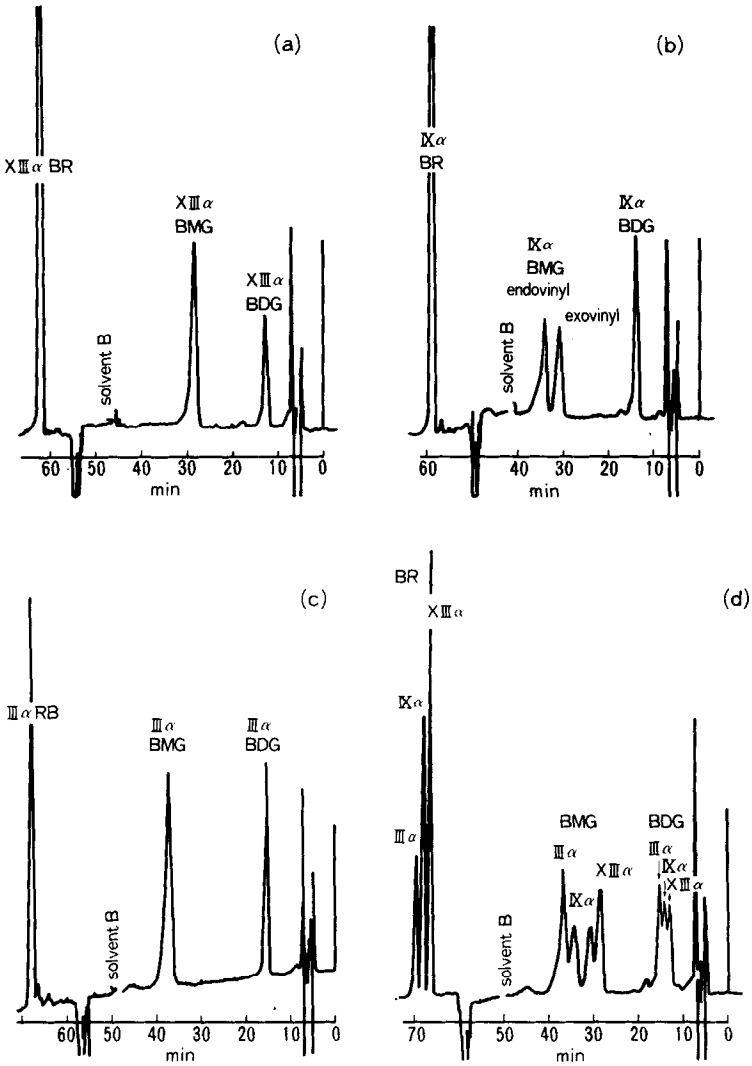

Fig. 1. HPLC separation of BR isomers and their glucuronides in enzymatic reaction mixture. (a) XIIIα species; (b) IXα species; (c) IIIα species; (d) mixture of XIIIα, IXα and IIIα species.

The elution order of BR isomers and their glucuronides was established by the standard samples synthesized enzymatically from specific substrates. Fig. 1 shows the HPLC analyses of three BR isomers and their mono- and di-glucuronides. It has been found that XIIIα isomers appeared first, followed by IXα and IIIα. IXα BMG was separated into two peaks corresponding to endo-vinyl and exovinyl isomers, respectively. The peak identification of the isomers was performed by subjecting the extract of BMG-enriched guinea-pig bile to HPLC analysis and azo pigment analysis [6]. As shown in Fig. 2, the guinea-pig bile extract mainly gave two peaks of IXα BMG isomers. The peak area of the first was about four times as large as that of the second. This was an identical value to the endovinyl/exovinyl isomer ratio obtained from the azo pigment analysis of the bile extract. From this, the former peak was identified as endo-vinyl isomer. Other investigators have also reported that the endovinyl isomer usually predominates in biological fluids [7].

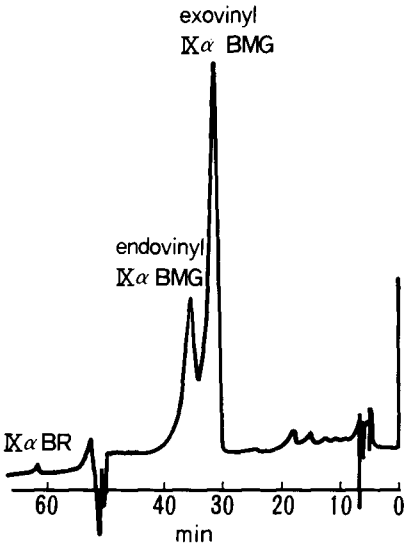

Fig. 2. Bilirubin metabolites in guinea-pig bile.

Fig. 3 shows HPLC analyses of IXα BR glucosides and xylosides. Mono-glucose and mono-xylose conjugates were clearly separated into two peaks corresponding to the endovinyl and exovinyl isomers.

The electronically integrated peak areas were compared with the amounts of each individual pigment. For each pigment, a linear relationship was observed between the amount of pigment injected (0—10 nmoles) and the corresponding peak area in the chromatogram. The repeatability of the peak areas for unconjugated BR isomers and their glucuronide species was also estimated. The relative standard deviation (S.D.) was less than 2% of each mean value (for five determinations) in all cases.

IXα BDG was the main component of human bile, and rat bile contained mainly IXα BMG and BDG (Fig. 4).

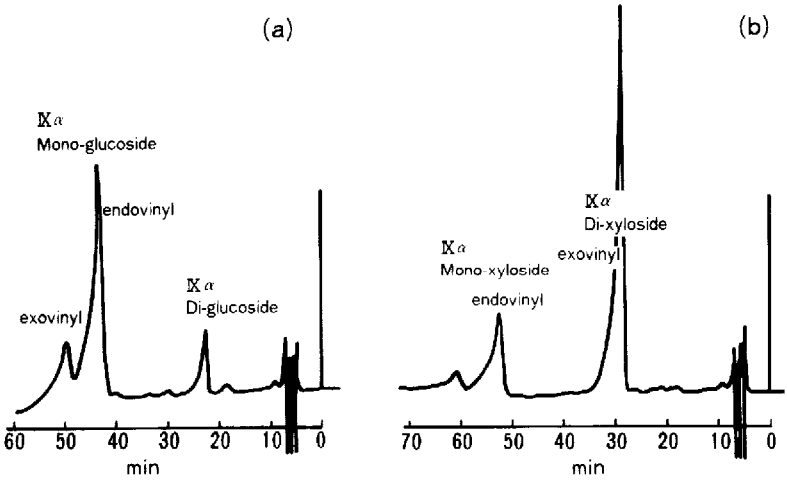

Fig. 3. HPLC separation of IXα BR glucosides and xylosides in enzymatic reaction mixture. (a) Glucosides; (b) xylosides.

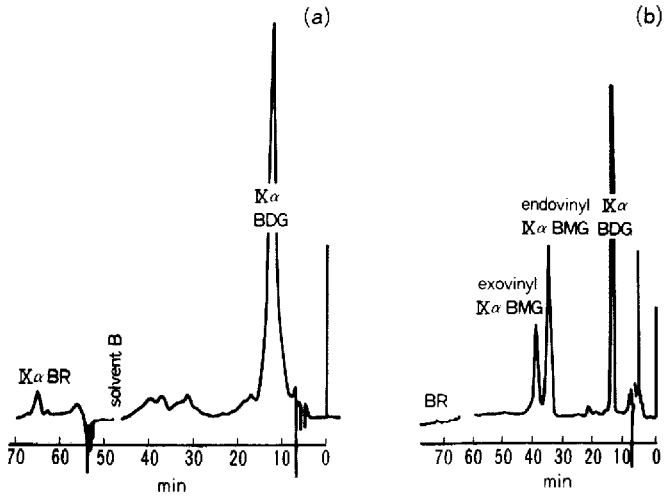

Fig. 4. Bilirubin metabolites in human and rat bile. (a) Human bile; (b) rat bile.

There is currently interest in the mechanisms of BR conjugation, especially BDG formation [8—13]. The HPLC method described here seems to be a useful tool to investigate these problems.

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