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An assay for separating and quantifying four bilirubin fractions in untreated human serum using isocratic high-performance liquid chromatography

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Abstract

Background: The quantification of serum bilirubin fractions has been widely performed with both the diazo-method and an enzymatic method; however, the accuracy of these methods has not been evaluated because quantitative fractional high-performance liquid chromatography (HPLC) reference methods have yet to be established.

Methods: Samples were analyzed using HPLC and Shodex® Asahipak GS-320HQ columns. Human serum was subjected to HPLC using direct injection, then eluted with acetonitrile: 0.3 mol/l phosphate buffer (pH 6.5) containing 1% Brij 35 and 0.08% sodium ascorbate (30 : 70, v/v).

Results: Serum bilirubin was separated into 4 fractions; retention times of 9.24, 19.92, 24.07, 35.75 min were identified as δ bilirubin, bilirubin diglucuronide, bilirubin monoglucuronide, and unconjugated bilirubin, respectively. Mean recovery was 93.0%–99.2%. Total precision of peak retention time, height and area exhibited <4.26% variation. Detection range was 3.1 to 348 mg/l. Hemoglobin (6 g/l) and immunoglobins produced a small positive interference. β-carotene (20 mg/l), vitamin-B_2 (370 μg/l) and B_{12} (9.5 μg/l) did not interfere with this analysis. Results ($n=30$) with this method were closely correlated to those by Adachi's HPLC method as $r=0.9941$ to 0.9960, slope = 0.88 to 1.27, intercept = −3.2 to +4.9, for each fraction.

Conclusions: Since this method was a precise quantitative HPLC method for serum bilirubin fractionation, it might be used to evaluate the accuracy and the characteristics of various routine methods for bilirubin measurement.

1. Introduction

Serum bilirubin is a good diagnostic marker for liver and bile duct diseases, congenital metabolic disorder diseases of bilirubin such as Gilbert's and hemolytic disease, and is used to monitor patient condition after liver transplantation. Bilirubin is also used to assess the severity of neonatal jaundice [1]. By various high-performance liquid chromatography (HPLC) analyses, it has been shown that bilirubin fractions in serum include a δ bilirubin (Bδ) form that is covalently bound to serum albumin, a bilirubin monoglucuronide (BMG) form, a bilirubin diglucuronide (BDG) form, and an unconjugated bilirubin (UCB) form [2–7]. Recently, it has been demonstrated that the ratio of conjugated bilirubin (CB)/Bδ and CB value are useful as a prognostic index after treatment such as percutaneous

Abbreviations: HPLC, high-performance liquid chromatography; Bδ, δ bilirubin; BDG, bilirubin diglucuronide; BMG, bilirubin monoglucuronide; TB, total bilirubin; CB, conjugated bilirubin; DCB, diconjugated bilirubin or di-taurobilirubin; DMSO, dimethylsulfoxide; HSA, human serum albumin; BSA, bovine serum albumin; Ig, immunoglobulin; PBS, phosphate-buffered saline.

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transhepatic cholangiodrainage and liver transplantation [8–10]. An accurate and precise method for the quantitation of bilirubin fractions would be of great benefit.

In 1981, Lauff et al. reported the accurate quantification of bilirubin species in serum using reverse-phase HPLC after the exclusive treatment of globulins from serum with sodium sulfate [2]. Then, Adachi et al. reported the facilitation of HPLC analysis by using a Micronex RP-30 column packed with particles made from polyacryl ester [3]. Many other workers have also reported on reverse-phase HPLC methods [5–7]. Since the reverse-phase HPLC methods described in these earlier papers are methods that use a linear gradient elution with an organic solvent, the eluent constitution tends to alter during analysis for bilirubin. Absorption spectrum and molar absorptivity of bilirubin species in solution tend to be easily modified by the ratio of organic solvent in the solution. Thus, it is difficult to accurately quantify serum bilirubin fractions. When analyzed by Lauff's HPLC method without exclusive treatment of globulins from serum with sodium sulfate, the separation efficiency of the column decreased as the number of analyses increased, because increasing amounts of serum protein were becoming adsorbed to the silica gel particles in the column. Meanwhile, Mizobe et al. have analyzed various sera using HPLC on an internal surface reversed-phase silica support column and an acetonitrile eluent: 0.5 mol/l Tris–HCl buffer (20:80, v/v, pH 7.2) [4]. Using Mizobe's analysis, bilirubins in human and domestic animal sera were separated into 4 major fractions, however the fractionation of human serum was not complete.

2. Materials and methods

2.1. Materials

NIST SRM916a (bilirubin standard) was from the NIST center (Gaithersdurg, MD). Pure UCB and diconjugated bilirubin (DCB: di-taurobilirubin) were from Merck (Darmstadt, Germany) and from Scripps Laboratories (San Diego, CA), respectively. Human serum albumin (HSA, essentially globulin free) and immunoglobulin G (IgG), IgA and IgM were from Sigma (St. Louis, MO). Bovine serum albumin (BSA, fraction V) and Woodward's reagent k were from Oriental Yeast Co., Ltd. (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan), respectively. Triethylamine, sodium carbonate, sodium dihydrogenphosphate, disodium hydrogen phosphate, acetonitrile (HPLC grade) and Brij 35 were from Kishida Reagent Chemicals (Tokyo, Japan). EDTA disodium salt, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and dimethylsulfoxide (DMSO) were from Dojindo Laboratories (Kumamoto, Japan). Sodium ascorbate, sodium acetate, sodium pentanesulfonate, caffeine, imidazole, β-carotene, vitamin B_2 and vitamin B_{12} were from Wako Pure Chemicals (Osaka, Japan). Conditioned hemoglobin was from Sysmex Corporation (Kobe, Japan). All chemicals were of reagent grade. NIST bilirubin, UCB and DCB were dissolved in DMSO (at a final concentration of 1%) and 0.1 mol/l sodium carbonate (final concentration, 2 mmol/l), and diluted with 4% BSA solution (previously adjusted to pH 7.4). These bilirubin solutions were stored at −80 °C in the dark until using. The NIST bilirubin solution was used as a bilirubin standard throughout this study and also used for quantifying all the bilirubin fractions. Bδ was synthesized using the method reported previously [11]. In brief, UCB and Woodward's reagent k were mixed and incubated in acetonitrile containing triethylamine. After removing the acetonitrile and triethylamine, phosphate-buffered saline (PBS) and HSA were mixed and incubated to covalently bind the UCB and HSA. The reaction mixture was washed with a solution containing caffeine and sodium benzoate (3.75% caffeine, 5.6% sodium benzoate, 5.6% sodium acetate, 0.1% EDTA disodium salt) using a centriprep® YM-30 centrifugal filter device (Millipore Corporation, Bedford, MA) and subsequently exchanged with PBS. The synthesized Bδ solution contained 220 mg/l of bilirubin and 33 g/l of albumin. Bile was obtained from humans.

2.2. Chromatography

We used Shodex® Asahipak GS-320HQ column (7.6 × 300 mm, Showadenko, Tokyo, Japan) joined in tandem with a GS-2G 7B grad column (7.6 × 50 mm, Showadenko, Tokyo, Japan), packed with 6 μm diameter particles of polyvinylalcohol gel. This column is routinely used to analyze drugs in serum by virtue of 3 functional modes: exclusion (gel filtration), absorption (reverse phase), and ion-exchange. We used a Tosoh 8020 automatic analyzer consisting a PX-8020 pump, SD-8020 solvent de-gasser, AS-8020 auto-sampler, CO-8020 column oven, and a PD-8020 [detector cell exchanged with semi-micro cell (SUS, 4 mm), Tosoh, Tokyo, Japan]. Other analysis conditions were as follows: flow rate, 0.7 ml/min; column temperature, 30 °C; injection volume, 10 μl (direct injection); light source, deuterium lamp. The chromatogram data (from 0 to 47 min at intervals of 400 ms) and wavelength data (from 300 to 500 nm with 2 nm bandwidth) were collected using the PD-8020 program.

2.3. Eluent

Eluent contained 30% acetonitrile and 70% 0.3 mol/l phosphate buffer (pH 6.5) containing 1% Brij 35 and 0.08% sodium ascorbate (v/v). The eluent was prepared as follows. Brij 35 was dissolved in 0.3 mol/l phosphate buffer (adjusted to pH 6.5) and sodium ascorbate was added to the buffer when used in the examination, since sodium ascorbate is unstable in the buffer at pH 6.5. Finally, the conditioned buffer was filtered with a membrane filter (pore size: 0.22 μm) and the resulting buffer mixed with acetonitrile.