Available online at www.sciencedirect.com

SCIENCE DIRECT®

Clinica Chimica Acta 366 (2006) 146 – 155

www.elsevier.com/locate/clinchim

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

Susumu Osawa [a,*], Shin Sugo [c], Toshihiko Yoshida [b], Toshihiko Yamaoka [c], Fumio Nomura [d]

[a] *Division of Biological Sciences and Technology, Department of Health Sciences, School of Medicine, Kyushu University, 3-1-1, Maidashi, Higasshi-Ku, Fukuoka City, 812-8582, Japan*

[b] *Clinical Laboratory of Medicine, University Hospital, Chiba University, Japan*

[c] *Biochemical Research Laboratory, Research and Development Headquarters, Eiken Chemical Co., Ltd., Japan*

[d] *Department of Molecular Diagnosis Graduate School of Medicine, Chiba University, Japan*

Received 4 June 2004; received in revised form 19 September 2005; accepted 20 September 2005
Available online 19 January 2006

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.

* Corresponding author. Tel.: +81 92 642 6712; fax: +81 92 642 6712.
E-mail address: osawas@shs.kyushu-u.ac.jp (S. Osawa).

0009-8981/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2005.09.031

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.

2.4. Comparison of HPLC methodology

We used Adachi's HPLC method as a direct comparison to our own method; analysis was performed under the same conditions and procedures reported by Adachi [3]. The column, the eluent, and the associated analytical conditions were as follows: column, Micronex RP-30 (6 × 150 mm; Sekisui Chemical Co., Osaka, Japan); eluent A, 5 mmol/l pentanesulfonic acid solution containing 0.1 mol/l of acetic acid; eluent B, acetonitrile; column temperature, 37 °C; flow rate, 1 ml/min; injection volume, 20 μl (direct injection); detection wavelength, 450 nm. We used a linear gradient of eluent: from 100% eluent A to 50% eluent A: 50% eluent B over 20 min, and then finally to 100% acetonitrile in 7 min (the latter being maintained for a total of 5 min).

2.5. Identification of each fraction

Human serum (140 mg/l of total bilirubin (TB) value) was separated into separate fractions using the following methodology. Fractions were identified by retention time and the absorption spectrum; the retention times of each fraction were compared with the retention times of HSA, IgG, IgA, IgM, synthesized Bδ, bile (mainly containing BMG, BDG) and UCB. Sodium ascorbate was added to the eluent to avoid the oxidative degradation of bilirubin during the HPLC analysis (data not shown). Since sodium ascorbate in the eluent had high absorption at 280 nm, it was not possible to detect protein absorption. Even though buffer was prepared without sodium ascorbate and used as eluent, the retention time, and the shape of the absorption spectrum (from 320 to 500 nm) in each fraction peak were the same as that if sodium ascorbate was added to the buffer. We therefore used the absorption spectrum (from 250 to 500 nm) whose fraction was separated with eluent without sodium ascorbate, for identification purposes.

2.6. Molar absorptivity of Bδ, BDG, BMG, DCB and UCB in the eluent

NIST (3.318 μmol/l) was dissolved in the eluent from the present method, and absorptivity at 450 nm was measured. After weighing and correcting for purity, the molar absorptivity was calculated from the molecular weight. Using the same method, molar absorptivity was calculated from 4.616 μmol/l DCB. To obtain the molar concentration of synthesized Bδ, synthesized Bδ was added to phosphate buffer (0.134 mol/l, pH 7.4) as reported by Doumas et al. [12], and an absorbance spectrum was prepared. The molar concentration of this Bδ was then calculated from the previously reported molar absorptivity (ε = 72,400) of Bδ at 440 nm. Synthesized Bδ was mixed with the eluent from the present method, an absorbance spectrum was prepared, and the molar absorptivity was calculated from the previously obtained molar concentration and absorptivity at 450 nm. Bile was hydrolyzed by adding 0.02 ml bile to 0.8 ml of 0.1 mol/l KOH solution and heating at 37 °C for 5 min. Then 0.2 ml of 1 mol/l BES (pH 6.0) was added, after which the solution was heated at 37 °C for 5 min and the fraction was measured using HPLC.

The molar absorptivity of BDG and BMG was obtained after 2 types of bile with different compositions were converted to UCB by alkaline hydrolysis described above, and chromatograms of each of the 3 components were measured at 450 nm. The areas of the chromatograms of BDG, BMG, and UCB for the 2 types of bile were obtained, and the molar absorptivity of BDG and BMG was calculated using simultaneous linear equations.

2.7. Recovery rate of various bilirubin species

UCB, DCB, bile and synthesized Bδ were added to human serum at the appropriate concentration, and recovery rate was calculated by using the following formula:

$$\text{Recovery rate } (\%) = (B - C)/A \times 100$$

Here, A is the solvent used for dissolution mixed with each type of undiluted bilirubin solution at a ratio of 9 : 1 in the added bilirubin solutions. B is the respective added bilirubin solutions mixed and prepared with human serum at a ratio of 9 : 1 in bilirubin-added serum. C is the solvent of each additive-use bilirubin solution mixed and prepared at a ratio of 9 : 1 in 9 dissolutions of human serum (140 mg/l with total bilirubin value).

The solvent described above was 4% BSA solution containing 2 mmol/l sodium carbonate and 1% DMSO. Bilirubin solution for addition was prepared by dissolving UCB, DCB or synthesized Bδ in BSA solution. The recovery rate of UCB, bile and synthesized Bδ was calculated from the area exchange of fractions corresponding to each bilirubin, and that of DCB was from area exchange of CB (the sum of BDG and BMG) fraction. Each sample was analyzed twice.

2.8. Precision of each peak

Within-run and day-to-day precision was obtained in conformance with NCCLS protocol EP5. For within-run precision and day-to-day precision, human serum was analyzed ten times with continual measurement and over 24 days with the detection wavelength at 450 nm. Precision evaluated with the mean, SD, and CV of the retention time, the peak height and the area in each fraction.

2.9. Interferences of hemoglobin, β-carotene, vitamin B_2, vitamin B_{12}, IgG, IgA and IgM

Hemoglobin, β-carotene, vitamin B_2 and vitamin B_{12}, which are biological pigment species, IgG, IgA and IgM were added and mixed into human serum at concentrations of 0–10 g/l, 0–20 mg/l, 0–370 μg/l and 0–9.5 μg/l, 0–85.0 g/l, 0–20.5 g/l, 0–13 g/l, respectively, and these mixtures

were subsequently analyzed. The effects of the pigments described above were evaluated by the concentration of bilirubin, which was calculated by using the following formula. The equivalent concentration of bilirubin (mg/l)=$D/E \times 203.3$: D, area of the fraction peak (by addition of pigments and Igs) which appeared near the retention time of each serum bilirubin fraction; E, fraction area of NIST bilirubin standard; 203.3, bilirubin concentration (mg/l) in NIST bilirubin standard.

2.10. Minimum detection limit and linearity

To evaluate the minimum detection limit, samples were prepared by the dilution of 4.07 mg/l NIST bilirubin solution (1/8–8/8 dilution). Samples were analyzed 3 times with continual measurement, and the mean and SD of the peak areas detected were calculated. The minimum detection limit was defined as the concentration where the value of the mean −3.0 SD calculated from separated NIST bilirubin peak areas was larger than that of the mean+3.0 SD obtained from the dilution solution for preparation of NIST bilirubin (bilirubin concentration: 0 mg/l); this is because 3 SD statistically accounts for 99% of all data. We examined the linearity of the samples prepared by dilution of 300 mg/l bile and 348 mg/l UCB solution (1/5–5/5 dilution) and its ten fold diluted solution. Samples were analyzed in duplicate.

2.11. Correlation

Statistical analysis for correlations with Bδ, BDG, and BMG values with the present method was done using calculated values as NIST bilirubin concentrations based on the ratio of molar absorptivity obtained in the eluent used. Sera was collected from a total of thirty patients. Using these sera, which contained various concentrations of bilirubin, we estimated the correlation between the Asahipak GS-320HQ method and Adachi's method, for each fraction and TB value. The bilirubin value of each fraction separated from these sera was calculated from the following formula: each fraction area/NIST bilirubin standard fraction area × 203.3 (bilirubin concentration in NIST bilirubin standard), in the present method moreover multiplied by respective conversion factor from Bδ, BDG and BMG to UCB. CB values in HPLC methods were calculated from the sum of BDG and BMG values, and TB values were obtained from the sum of Bδ, CB and UCB values.

Correlation was assessed by simultaneous measurement of TB and DB samples with the present method and the diazo-method (Bilirubin HR II-test Wako, Osaka, Japan; modification of Doumas's method) using serum from 73 patients (including 15 neonates). Moreover, sera was collected from a further twenty patients and used to evaluate the association between the Asahipak GS-320HQ method and a method utilizing a commercially available reagent for CB determination (Ekdia L 'Eiken' D-BIL, enzymatic method using bilirubin oxidation). Bilirubin measurements were obtained using Ekdia L 'Eiken' D-BIL, Bilirubin HR II-test Wako and a Roche Hitachi®717 analyzer using the manufacturer's instructions. Diazo-methods (Bilirubin HR II-test Wako) were calibrated with the same NIST bilirubin standard as that used in HPLC methods, and Ekdia L 'Eiken' D-BIL was calibrated with the DCB traced by the NIST bilirubin standard.

2.12. Statistical analysis

The correlation coefficient (r) and its linear regression formula between bilirubin values obtained by our own method, those by Adachi's method, diazo-method (Bilirubin HR II-test Wako) and by the Ekdia L 'Eiken' D-BIL method, were calculated using Microsoft Excel software (Microsoft Corporation, Redmond, WA), and the significant differences between 2 methods analyzed using the t-test ($p<0.05$). Differences between methods were evaluated further by a Bland–Altman plot analysis [13].

3. Results

3.1. Human serum bilirubin analysis

Using the Asahipak GS-320HQ method, patient serum was separated into 5 major fractions with retention times of 7.77 min (1; first peak), 9.24 min (2; second peak), 19.92 min (3; third peak), 24.07 min (4; 4th peak) and 35.75 min (5; fifth peak) at the detection wavelength of 450 nm, as shown in Fig. 1. The peak 1 (7.77 min), and the peak 2 (9.24 min), were closely eluted and slightly overlapped with each other (the peak 1 being 5.8% of the height of the peak 2), and the retention time corresponded to that of IgG, IgA, IgM (7.76 min) and HSA (9.22 min), respectively. The peak 3 (19.92 min) was broad and slightly overlapped with the peak 4 (6.8% of the height of the peak 4), and retention times of peak 3 and 4 were nearly in accord with those of the bile. The retention time of the peak 5 (35.75 min) corresponded to that of the UCB. The spectrum of the peak 1 (7.77 min) was in the shape of the peak having λ_{max} at 280 nm, however absorption was not observed near 450 nm to appear yellow, which is the distinctive color associated with bilirubin, and the absorption spectra of IgG, IgA, and IgM had the same shape as the first peak. The spectrum of the peak 2 (9.24 min) was in the shape of the 2 peaks possessing λ_{max} at 280 and 425 nm, and its absorption at 280 nm was much higher than 425 nm. The absorption at 425–450 nm of Bδ in serum samples was similar to that of synthesized Bδ, however, since the samples included free albumin, the absorption at 280 nm was high. The spectrum of the peak 3 was biphasic in form and exhibited λ_{max} at 415, 445 nm with slight absorption at 280 nm. The wavelength of λ_{max} for the peak 4 was at 420 and 450 nm. These absorption curves were the same as the absorption of bile. The

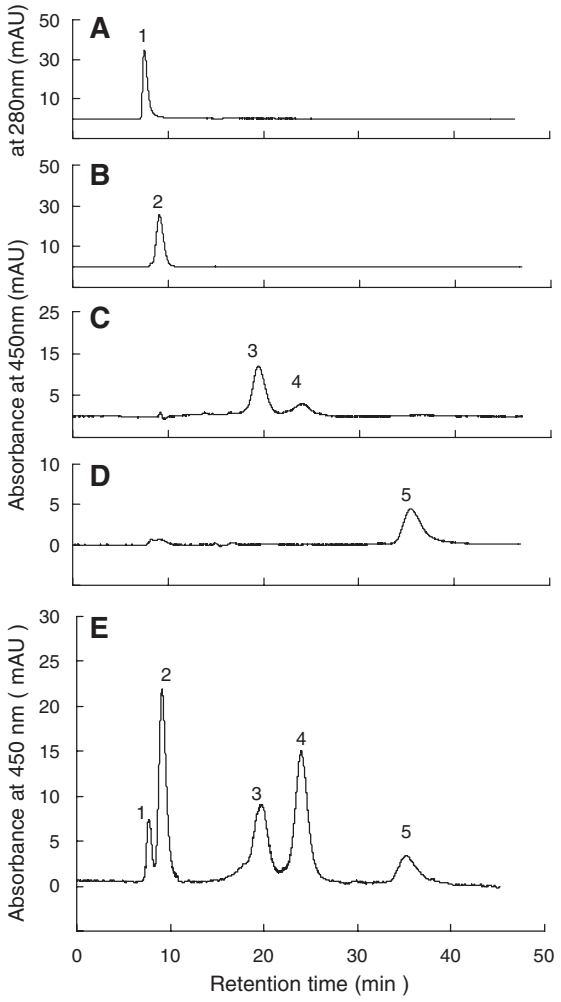

Fig. 1. A typical chromatogram of high-performance liquid chromatography (HPLC) analysis for human serum. Human serum (10 μL) was subjected to HPLC by a direct injection method. Column: Shodex® Asahipak GS-320HQ column (7.6 × 300 mm) joined with a GS-2G 7B grad column (7.6 × 50 mm). Solvent: acetonitrile/0.3 mol/l phosphate buffer (pH 6.5) containing 1% Brij 35 and 0.08% sodium ascorbate (30/70, v/v). Flow rate: 0.7 ml/min. Column temperature: 30 °C. Figures indicate the chromatograms of A) immunoglobulin G, B) synthesized δ bilirubin (Bδ), C) human bile; peak 3 and peak 4 corresponding to bilirubin diglucuronide and bilirubin monoglucuronide, D) unconjugated bilirubin (UCB) and E) human serum. The numbers in figure E indicate peak numbers at retention times of 7.77, 9.24, 19.92, 24.07 and 35.75 min in turn. AU indicates absorbance unit defined in the PD-8020 detector.

wavelength of λ_{max} for the peak 5 (35.75 min) was at 445 nm, and the absorption approximated that of UCB. These results indicate that the peak 1 is not associated with any bilirubin species.

3.2. Molar absorptivity of Bδ, BDG, BMG, DCB and UCB in the eluent

The absorptivity of NIST SRM916a (UCB) at 450 nm was 0.2069 and the molar absorptivity was induced as 62,340 l mol^{-1} cm^{-1}. The absorptivity of DCB at 450 nm was 0.2241, and the molar absorptivity was 48,550 l mol^{-1} cm^{-1}. The 440 nm absorptivity of synthesized Bδ in pH 7.4, 0.134 mol/l phosphate buffer was 0.2525, so the concentration was calculated to be 3.488 μmol/l. This calculation is based on the 440 nm Bδ molar absorptivity of 72,400 reported by Doumas et al. [12]. Therefore, since the absorptivity of synthesized Bδ at 450 nm in eluent was 0.2700, the molar absorptivity was calculated to be 77,390 l mol^{-1} cm^{-1}. A sample of BDG, BMG, and UCB fractions in bile had areas of 1275, 440, and 105 mAU × s, respectively. When the sample underwent alkaline hydrolysis, the fraction areas changed to 65, 59, and 2264 mAU × s, respectively. An other sample of BDG, BMG, and UCB fractions had areas of 317, 269, and 84 mAU × s, and following the same treatment the areas became 33, 31, and 767 mAU × s. Using simultaneous equations of the changes in area for each peak after alkaline hydrolysis of 2 types of bile, the conversion factor from BDG to UCB was calculated to be 1.411, and that from BMG to UCB to be 1.186. The 450 nm molar absorptivity UCB in eluent with the present method was 62,340, so the respective molar absorptivities of BDG and BMG were 44,170 and 52,520 l mol^{-1} cm^{-1} (Table 1).

3.3. Accuracy and precision

The recovery rate (mean ± SD) of UCB, DCB, Bδ and bile was 93.0 ± 4.7%, 95.0 ± 1.0%, 97.2% ± 3.5% and 99.2 ± 1.5%, respectively. In the analysis taken 10 times with continual measurement using pooled serum (peak 2 Bδ, 36.5 mg/l; peak 3 BDG, 37.3 mg/l; peak 4 BMG, 49.0 mg/l; peak 5 UCB, 14.6 mg/l; TB, 137.4 mg/l), the CVs of the retention time, height and area for five major peaks were evaluated to be 0.12%–0. 37%, 1.28%–2.63%, 1.31%–2.76%, and the CV of total area was 1.35%. The day-to-day precision over 24 days with pooled serum (peak 2 Bδ, 24.2 mg/l; peak 3 BDG, 11.6 mg/l; peak 4 BMG, 8.2 mg/l; peak 5 UCB, 8.7 mg/l; TB, 52.7 mg/l) is indicated by the CV of the retention time, the height and the area of each peak on the chromatogram of 0.36–1.02%, 2.50–4.26%, and 1.44–3.27%, respectively. The CV of the TB area was 1.32%.

Hemoglobin added into a serum(TB; 6.8 mg/l, Bδ; 2.9 mg/l, BDG; 0 mg/l, BMG; 1.7 mg/l, UCB; 2.2 mg/l) eluted with a retention time near to that of Bδ, and the concentration of 2, 4, 6, 8 and 10 g/l was evaluated to be in equivalent bilirubin concentrations of 0.4, 0.3, 1.5, 4.7

Table 1
Molar absorptivities (ε) of unconjugated bilirubin, δ bilirubin, bilirubin diglucuronide and bilirubin monoglucuronide in eluent

Bilirubin	ε (l mol^{-1} cm^{-1})
Unconjugated bilirubin	62,340
δ bilirubin	77,390
Bilirubin diglucuronide	44,170
Bilirubin monoglucuronide	52,520
Di-taurobilirubin	48,550

Each value represents ε of wavelength at 450 nm.

and 5.9 mg/l, respectively, while β-carotene, vitamin B_2 and vitamin B_{12} added into serum were not detected as fraction peaks. IgG, IgA, and IgM were added to pooled serum (TB; 4.94 mg/l, Bδ; 2.25 mg/l, BDG; 0 mg/l, BMG; 1.19 mg/l, UCB; 1.5 mg/l, IgG; 14.0 g/l, IgA; 2.9 g/l, IgM; 0.9 g/l) at 49.6, 20.5, 13.0 g/l, respectively. The resulting rise in addition concentration led to an increase in the area of the first fraction peak, and the bilirubin conversion concentration of the second fraction also increased to 3.3, 0.3, and 1.3 mg/l, respectively. However, this effect is slight enough to be ignored in ordinary patient serum.

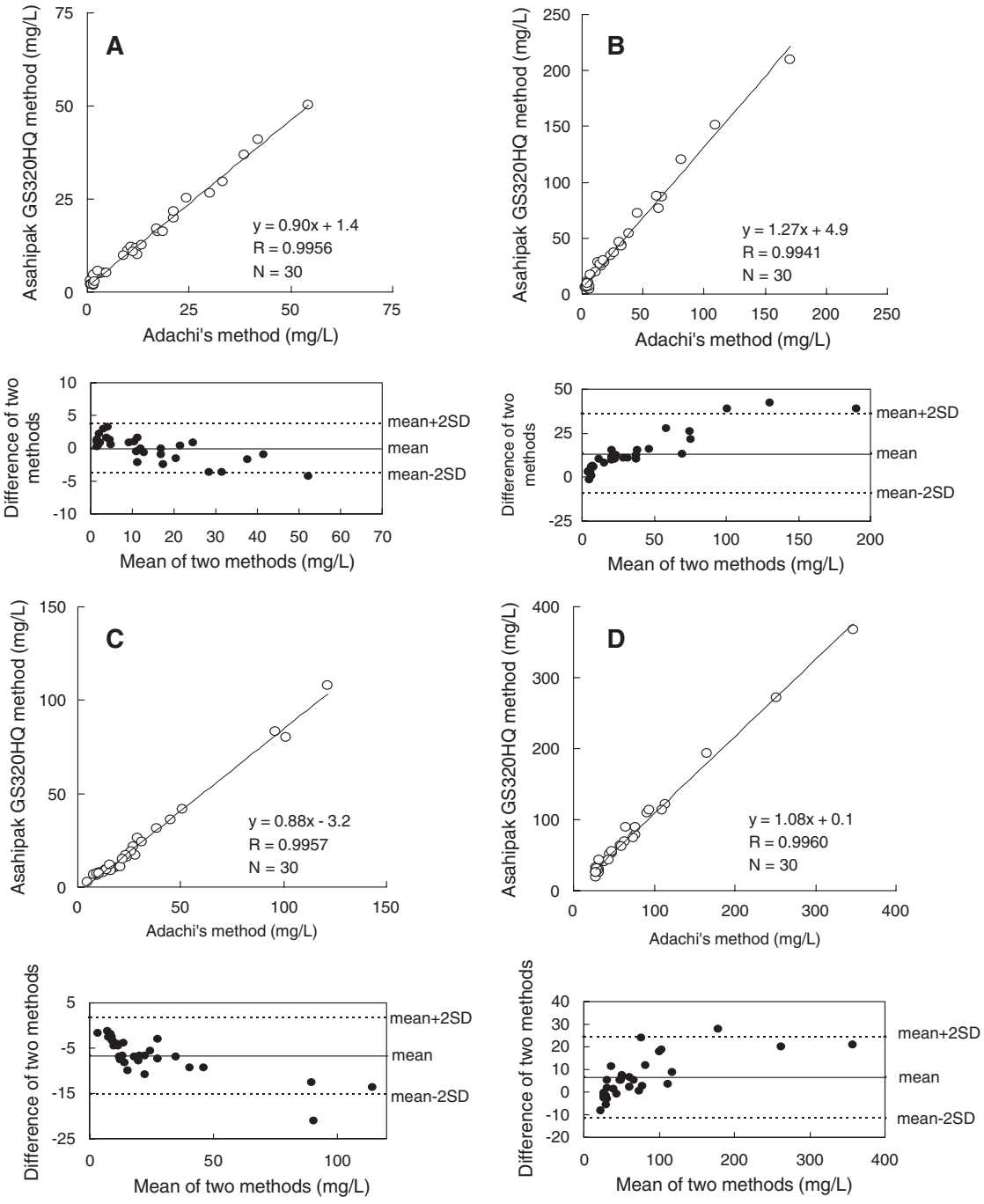

Fig. 2. Correlation and Bland–Altman analysis between this HPLC method and the conventional HPLC method for the determination of various bilirubin fractions. Adachi's HPLC method was used as the conventional HPLC method. Thirty individual human serum samples were analyzed by direct injection. Concentration of fraction was calculated from each fraction peak area using NIST SRM916a bilirubin as a standard. The TB values of this HPLC method are the sums of concentrations of each fraction calculated by multiplying the value from (area of each fraction/area of NIST SRM916a) × (concentration of NIST 916a) times the conversion coefficient for UCB. The CB (the sum of BDG and BMG) and Bδ value are the values corrected with the same UCB conversion factor. A, B, C and D show the correlation between 2 HPLC methods for the determination of Bδ, conjugated bilirubin (CB), UCB and TB, respectively. The unit of difference of 2 methods in Bland–Altman plot Fig. (bottom): mg/l.

3.4. Range of detection

The area value (mean±3SD) of 0, 1.0, 1.6, 2.1, 2.6, 3.1, 3.7 and 4.1 mg/l UCB solution was 30.73±20.71, 33.97±16.38, 52.54±16.50, 66.20±15.16, 79.39±29.72, 91.84±24.98, 111.09±27.77 and 134.95±31.61. The minimum detection limit, by utilizing NIST bilirubin solution, was estimated to be at the concentration of 3.1 mg/l. Above the detection limit, the peak area of NIST bilirubin fraction increased with bilirubin concentration in a linear fashion. The CB (bile) and UCB concentration was closely associated with the fraction area and linearity was confirmed up to 300 and 348 mg/l passing through a zero point. Based on these data, measurement by this method was evaluated to range from 3.1 to 348 mg/l.

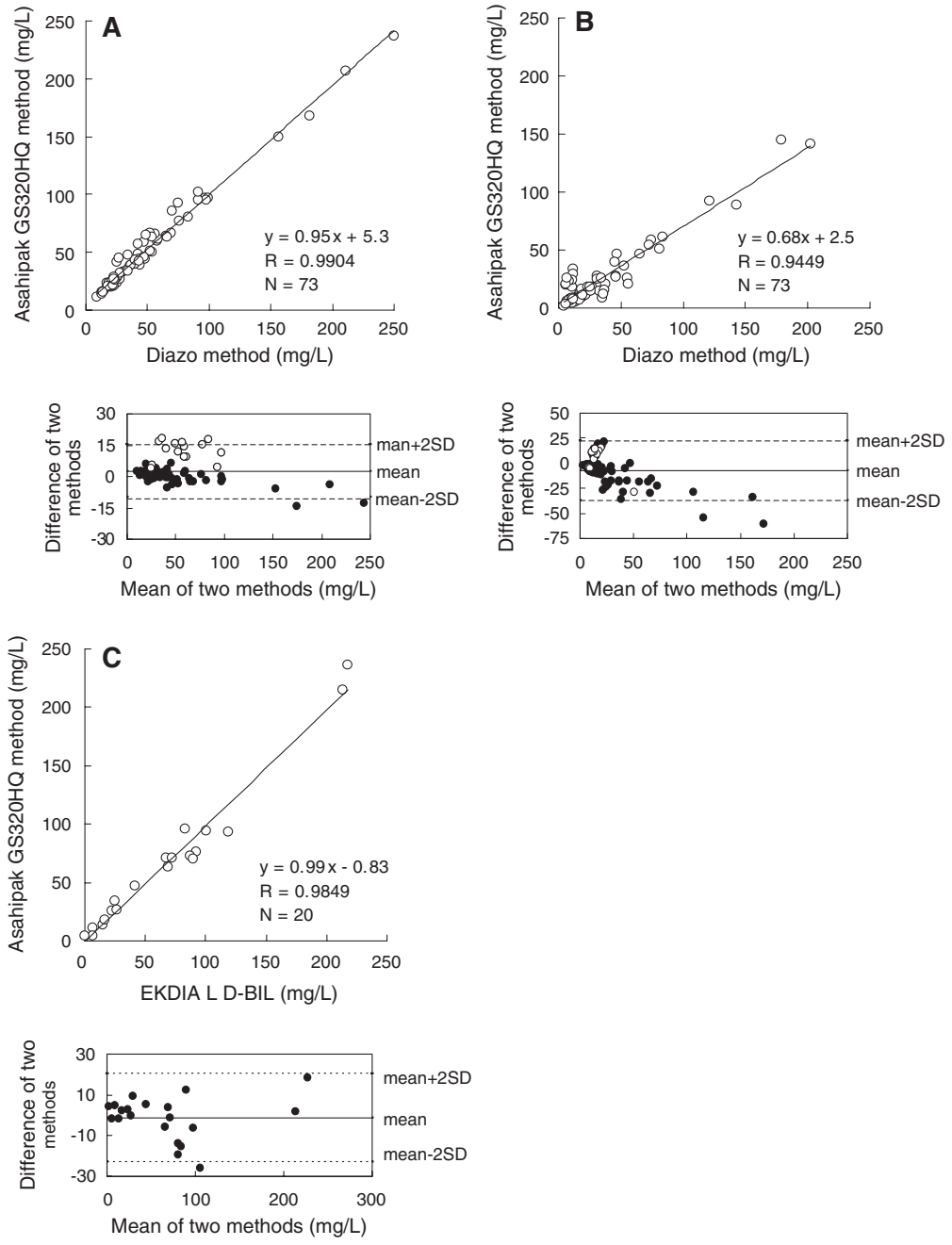

Fig. 3. Correlation and Bland–Altman analysis between this HPLC method, diazo-method and an enzymatic method for the determination of various bilirubin fractions. A and B represent the correlation between this HPLC method and diazo-method of total bilirubin (TB), and direct bilirubin (DB) for measurement using 73 human serum samples, respectively. Open circles in Bland–Altman plot Figs. (bottom of A and B) show neonatal samples. Fig. 3C represents the correlation between HPLC methodology and a commercially available reagent method for CB measurement using 20 human serum samples. The unit of difference of 2 methods: mg/l.

3.5. Correlation between the Asahipak GS-320HQ method and conventional HPLC methodology, diazo-method and an enzymatic method

Comparisons between measurements taken using the Asahipak GS-320HQ method and those by Adach's method gave the following linear regression formula for Bδ, CB, UCB and TB (intercepts in mg/l): for Bδ, $r=0.9956$, $y=0.90x+1.4$; for CB, $r=0.9941$, $y=1.27x+4.9$; for UCB, $r=0.9957$, $y=0.88x-3.2$; for TB, $r=0.9960$, $y=1.08x+0.1$ (Fig. 2A–D). There were no significant differences between each fraction because the calculated t-values were from −1.180 to 0.969 and these values were smaller than the t-boundary-value of 2.00. The mean and 2SD value of difference between the two methods by Bland–Altman plot analysis were as follows: for Bδ, −0.05, 3.7 mg/l; for CB, 13.11, 22.6 mg/l; for UCB, −6.74, 8.4 mg/l; for TB, 6.32, 18.1 mg/l. The correlation between the diazo-method TB and the present method was $r=0.9904$, $y=0.95x+5.3$ (Fig. 3A), and the t-value of −0.237 was smaller than the t-boundary-value of 1.976, although the difference was not significant. The mean difference between the two methods by Bland–Altman plot analysis was 2.68 mg/l and the 2SD value was 12.5 mg/l. 12 of 15 neonatal samples were able to leave plot in the high level side of Bland–Altman plot. Conversely, the correlation of the diazo-method DB and the present method CB (BDG+BMG) was $r=0.9449$, $y=0.68x+2.5$ (Fig. 3B), and the t-value of 1.610 was smaller than the t-boundary-value of 1.978, however the difference was not statistically significant. The mean difference between the 2 methods by Bland–Altman plot analysis was −7.50 mg/l and the 2SD value was 30.1 mg/l. However, many inconsistent results were seen. Values obtained using this method were correlated with those obtained by Ekdia L 'Eiken' D-BIL ($r=0.9849$ and $y=0.99x-0.8$, Fig. 3C); there were no significant differences between the methods as the calculated t-value was 0.068 and this value was smaller than the t-boundary-value of 2.02. The mean difference between the 2 methods by Bland–Altman plot analysis was −1.35 mg/l and the 2SD value was 21.6 mg/l.

4. Discussion

The clinical value of serum bilirubin fraction quantification has been confirmed by HPLC. In the routine methods for direct bilirubin measurement, such as the diazo-method and enzyme method, UCB or Bδ may be measured [14]. In the newly reported enzyme method conjugated bilirubin is thought to be specifically measured [15]. However, there is no way to test the accuracy of these common test methods. HPLC is considered to be the most likely method for comparisons. However, serum bilirubin fractions cannot be accurately quantified because the respective molecular weights and optical characteristics differ. Moreover, methods reported to date require elution of fraction components using a linear gradient, so the composition of the solvent is not homogeneous. We selected a multi-mode column that can isolate 4 fractions of serum bilirubin by isocratic elution, and established the conditions for this method. With the use of Shodex® Asahipak GS-320HQ columns and 0.3 mol/l phosphate buffer (pH 6.5) containing 1% Brij 35 and 0.08% sodium ascorbate as the eluent, it is possible to elute 4 serum bilirubin fractions with the addition of 30% acetonitrile. Moreover, bilirubin can be stabilized during the analysis by adding 0.08% sodium ascorbate to the eluent, and the stability of bilirubin can be assured during the measurement time.

As shown in Fig. 1E, human serum was separated into 5 major fractions by using the Asahipak GS-320HQ method. The retention time of the peak 2 (9.24 min), the peak 3, 4 (19.92, 24.07 min) and the peak 5 (35.75 min) fractions corresponded to that of synthesized Bδ, bile and UCB, and distinct absorption at 420–450 nm based on bilirubin species was observed in the absorption spectrum associated with each peak. Thus, we concluded that when separating with the Asahipak GS-320HQ method, fraction numbers 2–5 correspond to the Bδ (δ), BDG (γ), BMG (β) and UCB (α) fractions previously reported [2–7].

We used an HPLC column packed with particles possessing multi-modal characteristics (exclusive, adsorptive, and possessing ion-exchange properties). Since the molecular size exclusion of the column was approximately 40 kDa, it was deduced that globulins such as IgG (molecular weight: 150 kDa), IgA (150 kDa), IgM (600 kDa), and the turbidity associated with their proteins, eluted with the earliest retention time (peak 1 in Fig. 1E) by the exclusivity of the column, and that subsequent to this, the albumin (the major serum component) of 60 kDa was eluted. As Bδ is a covalently bound complex of bilirubin and albumin [7], it is suggested that Bδ also eluted with the same retention time as that of albumin. Since 30% acetonitrile is added to the eluent in this method, denaturing components of protein (globulin) appear in peak 1, which is thought to affect the Bδ fraction. From an experiment in which immunoglobulin was added to pooled serum, immunoglobulin from healthy individuals was added to 4 times the upper limit of the reference intervals to investigate interference by IgG, IgA and IgM of the Bδ fraction. However, the influence was a bilirubin equivalent concentration of 3.3 mg/l at maximum, which is a level of error that may be considered small in ordinary patient serum. The Asahipak GS-320HQ column has a molecular exclusion limit molecular weight of 40 kDa, so immunoglobulin denaturing substances from acetonitrile and Bδ isolation are insufficient. However, improvement of the isolation ability of peaks 1 and 2 of the serum chromatogram was achieved with a molar concentration of the eluent of 0.3 mol/l and the addition of 1% Brij 35. Then, BDG, BMG and UCB, whose molecular weights are <kDa, were eluted as BDG, BMG and UCB fractions in turn, and in proportion to the

hydrophilic and adsorptive nature of the column. These facts strongly suggest that 4 bilirubin species of major serum bilirubin were fractionated accurately by our method.

Accurate measurement of the 4 main fractions in serum requires a reference substance for each fraction component, but since BMG and BDG are unstable there are no products on the market for this. In accordance with the method of synthesizing Bδ [11], we obtained the coefficient in the present eluent from the previously reported molar absorptivity [12]. The molar absorptivity of BMG and BDG was obtained using a simultaneous equation from the respective chromatogram areas of BMG, BDG, and UCB before and after alkaline hydrolysis of 2 types of bile. As a result, the conversion factor for BDG to UCB was calculated to be 1.411, and that from BMG to UCB to be 1.186. Since the 450 nm molar absorptivity of UCB in eluent with this method was 62,340 l mol^{-1} cm^{-1}, the molar absorptivities of BDG and BMG were determined to be 44,170 and 52,520 l mol^{-1} cm^{-1}, respectively. The molar absorptivities of NIST SRM916a (UCB) and DCB at 450 nm were 62,340 and 48,550 l mol^{-1} cm^{-1}, respectively. The molar absorptivity at 450 nm of synthesized Bδ in eluent of this method was 77,390 l mol^{-1} cm^{-1}. Using the molar absorptivity of each fraction, it becomes possible to take the fraction concentration as the UCB concentration, and more accurately quantify these components. Moreover, the recovery rates of the various bilirubin species added into human serum were >93%, indicating a rate approaching 100% efficiency. Therefore, it is possible to quantify the 4 main fractions in serum with greater accuracy than with the previously reported HPLC.

In the present method, interference of the yellow components in serum for detection at 450 nm is a problem. Of the pigment constituents examined (contained in vivo), high concentrations of hemoglobin (8 g/l) appeared to be related to measurement errors for Bδ value, while β-carotene, vitamin B_2 and vitamin B_{12} did not appear to affect fraction measurement at all. Therefore, the detection method at 450 nm in the present method can specifically detect bilirubin when serum is the sample. The CVs of the retention time, height, and area of each fraction by continuous analysis were <3%, by day-to-day precision were <4.3% and scatter was small. Based on these data, we conclude that this method fractionates in a precise manner and accurately measures bilirubin species in human serum. The CV value for TB measurement was <50% of the physiological fluctuating range in a healthy individual [16], thereby providing further evidence of the precision obtained with this method.

The measurement range of this method was estimated to be from 3.1 to 348 mg/l by using NIST bilirubin solution and human bile. The minimum detection limit was higher than that (about 0.4 mg/l) of a diagnostic reagent used for routine bilirubin measurement: which suggests that the detection level of our system was inferior to that of the diagnostic reagent. However, we propose that this range is suitable enough for the diagnostic range for bilirubin measurement, since the standard value of direct bilirubin in human serum is reportedly from 0 to 3 mg/l, and that of TB from 2.0 to 10.0 mg/l [17]; thus bilirubin level in patient sera is rarely >300 mg/l.

Values of each bilirubin fraction and TB, determined by the Asahipak GS-320HQ method, were strongly correlated with those determined by Adachi's HPLC method. However, the slope of a linear regression formula was approximately 0.90 for Bδ fraction, 1.27 for CB fraction, and 0.88 for the UCB fraction and 1.08 for TB. The value for the each fractions differed by −10% and 27%. Two factors may be considered to account for these differences. One is that concentration of each bilirubin fraction in the present method was converted to a UCB equivalent from the molar absorptivity of the bilirubin fraction. Thus the Bδ fraction was 20% lower. In contrast, BDG and BMG were 40% and 18% higher, respectively, which is thought to have been because they were measured as UCB concentration. Another factor may have been that with Adachi's method, UCB absorptivity at 450 nm in 100% acetonitrile, which elutes UCB, was 10% higher than the eluent with the present method. Since every fraction separated by this method was detected under similar conditions during analysis, we conclude that the detection accuracy of the Asahipak GS-320HQ method is superior to that of Adachi's method.

The correlation between the diazo-method and the present method was $r=0.99$, $y=0.95x+5.3$ indicating good association. However, 12 samples were able to leave plot in the high level side of Bland–Altman plot in 15 neonatal samples (Fig. 3A). The neonatal sample was divided into UCB fraction mainly and photobilirubin fraction (closely BMG elution position) by the present method. It is speculated that, since the photobilirubin concentration was calculated with the molar absorption coefficient of BMG in the present method, the difference between two methods was induced. In addition, degradation product except photobilirubin is reported on a light bombardment to bilirubin [18]. It was thought that these portions did not cause the diazo-reaction from the chemical constitution, and it was speculated that the diazo-method showed a low value than the present method. Actually, TB value by this method became higher than that by the diazo-method in proportion to the ratio of the photo bilirubin content in the neonatal samples. Therefore, it is desirable that neonatal sample is excluded when the present method is used for evaluation of accuracy for other methods.

The CB values measured by the Asahipak GS-320HQ method (over twenty sera samples) were strongly associated with those of the Ekdia L 'Eiken' D-BIL enzyme method. The tendency of the regression equation for correlation was 0.99, indicating no comparative systematic error. Compared with conventional enzyme methods, this enzyme method has improved specificity for conjugated bilirubin. Moreover, the calibrator in this system uses DCB, but since calibration is determined by NIST SRM916a, it is thought to be possible to accurately measure CB.

Reverse-phase HPLC methods for serum bilirubin analysis with 2 eluents create the problem that they have poor spectroscopic quantification and that serum proteins are liable to adsorb to the particles in a column. Using the Asahipak GS-320HQ method, the major serum bilirubin fractions were separated and quantified almost completely. Human serum can be analyzed without a pre-treatment procedure; globulin species and the albumin components of serum can be excluded by molecular exclusion. The column used in this method displayed considerable endurance and measurements could be performed over one hundred times by direct injection of patient serum samples. Even if serum was measured with HPLC columns from different Lot (production) numbers, the detected peaks of serum bilirubin fractions eluted with the same retention times, and the spectrums of each fraction were also similar.

When serum was analyzed using HPLC on an OH pak SB-802.5HQ column (molecular exclusion size, 10 kDa; Showadenko, and very similar to the Asahipak GS-320HQ column), the first and second fraction peaks separated by Asahipak GS-320HQ method were detected as only one fraction peak (data not shown). Since the molecular exclusion size of the OH pak SB-802.5HQ column was approximately 10 kDa, the results described above may be due to the inability to separate the globulin species from albumin. These facts further suggest that the first fraction peak obtained by this method might be separated further from the second peak by using a column possessing a larger molecular exclusion size than that of the Asahipak GS-320HQ.

Our method can detect each fraction in isocratic eluent, and quantify the fractions as UCB concentration corrected by the UCB concentration conversion coefficient obtained from the molar absorptivity of each fraction. At present this method may be considered a comparative method or reference method for quantifying serum bilirubin fractions. Furthermore, our method may be used as a comparative method to evaluate fractional characteristics in a chemical or an enzymatic measurement used as part of routine diagnostic tests [19]. This is especially interesting in the case of the Bδ fraction in human serum, which was quantified with great precision; this might be considerably useful from the clinical point of view [20].

References

[1] American Academy of Pediatrics. Practice parameter: management of hyperbilirubinemia in the healthy term newborn. Pediatrics 1994; 94:558–65.

[2] Lauff JJ, Kasper ME, Ambrose RT. Separation of bilirubin species in serum and bile by high-performance reverse-phase liquid chromatography. J Chromatogr 1981;226:391–402.

[3] Adachi Y, Inufusa H, Yamashita M, et al. Clinical application of serum bilirubin fractionation by simplified liquid chromatography. Clin Chem 1988;34:385–8.

[4] Mizobe M, Kondo F, Toyoshima C, Kumamoto K, Terada T, Nasu H. Rapid analysis of 4 bilirubins in domestic animal sera using high-performance liquid chromatography. J Vet Med Sci 1996;58:495–9.

[5] Goresky CA, Gordon ER. High-performance liquid chromatographic separation of bilirubin conjugates. Anal Biochem 1989;183:269–74.

[6] Onishi S, Kawade N, Itoh S, Isobe K, Sugiyma S. High-pressure liquid chromatographic analysis of anaerobic photoproducts of bilirubin-IXα in vitro and its comparison with photoproducts in vivo. Biochem J 1980;190:527–32.

[7] Lauff JJ, Kasper ME, Wu TW, Ambrose RT. Isolation and preliminary characterization of a fraction of bilirubin in serum that is firmly bound to protein. Clin Chem 1982;28:629–37.

[8] Arvan D, Shirey TL. Conjugated bilirubin: a better indicator of impaired hepatobiliary excretion than direct bilirubin. Ann Clin Lab Sci 1985;15:252–9.

[9] Wu TW, Levy GA, Yiu S, et al. Delta and conjugated bilirubin as complementary markers of early rejection in liver-transplant recipients. Clin Chem 1990;36:9–14.

[10] Akamatsu Y, Ohkohchi N, Seya K, Satomi S. Analysis of bilirubin fraction in the bile for early diagnosis of acute rejection in living related liver transplantation. Tohoku J Exp Med 1997;181:145–54.

[11] Ihara H. Isolation of delta bilirubin and its chemical synthesis. Igakukensa 1994;43:1253–5 [in Japanese].

[12] Doumas BT, Wu TW, Jendrzejczak B. Delta bilirubin: absorption spectra, molar absorptivity, and reactivity in the diazo reaction. Clin Chem 1987;33:769–74.

[13] Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–10.

[14] Doumas BT, Wu TW. The measurement of bilirubin fractions in serum. CRC Crit Rev Clin Lab Sci 1991;28:415–45.

[15] Kurosaka K, Senba S, Tsubota H, Kondo H. A new enzymatic assay for selectively measuring conjugated bilirubin concentration in serum with use of bilirubin oxidase. Clin Chim Acta 1998;269:125–36.

[16] Ricos C, Alvarez V, Cava F, et al. Current databases on biologic variation: pros, cons and progress. Scand J Clin Lab Invest 1999;59: 491–500.

[17] Doumas BT, Kwok-Cheung PP, Perry BW, et al. Candidate reference method for determination of total bilirubin in serum: development and validation. Clin Chem 1985;31:1779–89.

[18] Bonnet R, Ioannou S. Phototherapy and the chemistry of bilirubin. Mol Aspects Med 1987;9:457–71.

[19] Osawa S, Yoshida T, Nomura F, Sugo S, Yamaoka T. A new high-performance liquid chromatography using isocratic condition eluent for determination of 4 bilirubins in human serum as comparative method. 2005 IFCC/AACC meeting in Orlando, Florida (presentation A-95). Clin Chem 2005;51:A90 suppl.

[20] Weiss JS, Gautam A, Lauff JJ, et al. The clinical importance of a protein-bound fraction of serum bilirubin in patients with hyperbilirubinemia. N Engl J Med 1983;309:147–50.