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Liquid-Chromatographic Assay and Identification of Mono- and Diester Conjugates of Bilirubin in Normal Serum

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This liquid-chromatographic procedure for determining bilirubin mono- and diester conjugates in normal serum is based on pre-analysis conversion of bilirubin monosugar and disugar conjugates to the corresponding methyl esters by alkaline methanolysis. Here, extracted unconjugated bilirubin, bilirubin monomethyl esters, bilirubin dimethyl ester, and internal standard are separated on a reversed-phase column within 15 min, detected in the effluent at 436 nm, and quantified from their peak areas. Carotenoids do not interfere. Within-day and day-to-day CVs range from 5 to 13%. The smallest concentrations of monoconjugated and diconjugated pigments that are detectable and measurable are about 10 and 20 nmol/L, respectively. Such data are given for sera from 43 healthy adults. Total bilirubin concentrations in serum tended to be lower in women than in men, but the relative amounts of the various bilirubin fractions in sera from men and women were comparable. Analysis of ethyl anthranilate azoderivatives from serum permitted identification of the bilirubin ester conjugates in normal serum as bilirubin 1-*O*-acyl glucuronides.

Additional Keyphrases: *normal values · distribution of bilirubins in serum*

The full potential value of serum bilirubin assay as an aid to diagnosis of liver disease cannot be realized because specific detection, let alone measurement of small concentrations of esterified bilirubins in the presence of unconjugated bilirubin, is impossible, at least with current nonisotopic methods (*1*). In fact, even the recently devised alkaline methanolysis procedure (*2*) combined with normal phase "high-pressure" liquid chromatography (HPLC), which permits specific measurement of individual bilirubin esters in serum, is too insensitive to detect conjugated bilirubins unambiguously in serum of healthy adults (*3*).

We describe here a liquid-chromatographic method for the specific detection and measurement of bilirubin esters in normal serum, and we have tested the method by assaying serum from healthy men and women. The bilirubin esters in normal sera were identified as bilirubin 1-*O*-acyl glucuronides.

Materials and Methods

Apparatus

We used a liquid chromatograph, consisting of the following components: a Model U6K injector, a Model 660 solvent programmer, two pumps (Models 6000A and 45), and a Model 440 absorbance detector set at 436 nm. The 300 × 3.9 mm column containing C_{18} μBondapak, 10-μm nominal particle size, was maintained at 40 °C with an aluminum column heating block connected to a Paratherm U2 water circulator (Juchheim Labortechnik KG, Seelbach, F.R.G.). A stainless-steel guard precolumn packed with approximately 500 mg of 35–70 μm Bondapak C_{18} bulk packing was mounted in line between the injector and the analytical column. Except for the water circulator, all the above materials were from Waters Associates, Milford, MA 01757. The precolumn contents were replaced every 30 runs. We used an electronic integrator (Model 3390A; Hewlett-Packard, Palo Alto, CA 94304) to record chromatograms and measure peak areas. Glass 10- or 45-mL centrifuge tubes with ground-glass stoppers and a standard laboratory centrifuge (1000 × g_{av}) were used in the alkaline methanolysis procedure. Glass plates, precoated with silica gel (60F254, 5761/0025 or 5763/0025; Merck AG, Darmstadt, F.R.G.) were used for thin-layer chromatography. Absorbances were measured in a Model SP8-250 spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.).

Chemicals, Standards, Preparation Procedures

All solvents and reagents were analytical grade. Chloroform (containing 5–10 mL of ethanol per liter as stabilizer), methanol, and ethanol were from Merck AG. Ethyl anthranilate was from Fluka AG, Buchs, Switzerland. Pentan-2-one, dried on $CaSO_4$ and redistilled, was from Union Chemique Belge, Drogenbos, Brussels, Belgium. Tetrabutylammonium phosphate solution (PIC ion-pair reagent A) was from Waters Associates.

Hemin was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Bilirubin ($\varepsilon_{452(\lambda_{max})}$ in chloroform: 61.0×10^3 $L \cdot mol^{-1} \cdot cm^{-1}$, containing the following proportions of isomers: 3% IIIα, 94% IXα, and 3% XIIIα), lycopene, and β-carotene (type IV) were from Sigma Chemical Co., St. Louis, MO 63178.

Crystalline preparations of bilirubin dimethyl ester and monomethyl esters were synthesized as previously reported (*2*). The crystalline xanthobilirubinic acid methyl ester ($\varepsilon_{412(\lambda_{max})}$ in methanol: 36.2×10^3 $L \cdot mol^{-1} \cdot cm^{-1}$) that we used as internal standard was prepared as described by Grünewald et al. (*4*).

Stock solutions of the internal standard contained about 100 μg of the pigment per liter of methanol. The pigment solution was stored in the dark at −15 °C and handled under dim light.

[^{14}C]Bilirubins were prepared biosynthetically from δ-amino[4-^{14}C]levulinic acid (58 Ci/mol; New England Nuclear, Boston, MA 02118). We used bile collected from rats infused with the labeled heme precursor to prepare [^{14}C]bilirubin as described by Ostrow et al. (*5*). For preparation of [^{14}C]bilirubin mono- and dimethyl esters, we extracted [^{14}C]bilirubin mono- and diglucuronides from bile and isolated them by thin-layer chromatography as described by Fevery et al. (*6*). Isolated pigments were individually converted to the corresponding methyl esters by alkaline methanolysis, and ^{14}C-labeled mono- and dimethyl esters were isolated by thin-layer chromatography on silica gel plates as described previously (*2*). Specific activities of the [^{14}C]biliru-

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bins were 13 000 dpm/nmol for bilirubin, 20 590 dpm/nmol for the bilirubin monomethyl ester preparation (consisting of 58% C-8 isomer and 42% C-12 isomer), and 22 630 dpm/nmol for bilirubin dimethyl ester.

Bilirubin 1-*O*-acyl β-D-mono- and diglucuronides were prepared from rat bile and isolated by thin-layer chromatography as described (*7*). Purity of these preparations was assessed by the alkaline methanolysis procedure followed by normal-phase HPLC (*3*). The monoglucuronide preparation contained 92% authentic monoglucuronide, 6% diglucuronide, and 2% unconjugated bilirubin. Neither unconjugated bilirubin nor monoglucuronides were detectable in the diglucuronide preparation. Isomeric composition (IIIα, IXα, XIIIα) of the aglycone as determined by alkaline hydrolysis (*3*) and isomeric analysis by thin-layer chromatography (*10*) was 3% IIIα, 89% IXα, and 8% XIIIα in the monoglucuronide preparation, and 2% IIIα, 91% IXα, and 7% XIIIα in the diglucuronide preparation.

Analytical Procedures

All work with bilirubins was done under dim light. Ethyl anthranilate azo derivatives were prepared, analyzed by thin-layer chromatography, and their structure verified according to published techniques (*1*).

Preparation of reference solutions. Commercial bilirubin, lycopene, β-carotene, and crystalline bilirubin mono- and dimethyl esters and xanthobilirubinic acid were dissolved in chloroform for use as chromatographic references.

Clear, non-hemolyzed specimens of sera from healthy individuals were pooled and used as diluents for calibration reference solutions as follows. Pooled serum was exposed to fluorescent light for 6 h, the photosensitive bilirubin pigments being degraded and no longer detectable by HPLC assay ("serum diluent").

Reference solutions of unconjugated bilirubin were prepared by dissolving 5 mg of the pigment in 1.0 mL of a 50 mmol/L NaOH solution containing 5 mmol of disodium EDTA per liter. The solution was then quantitatively transferred to a 20-mL volumetric flask and diluted to volume with serum diluent. This stock solution was then diluted with serum diluent to give standards with final concentrations ranging from 0.2 to 172 μmol/L.

Similarly, calibration reference solutions of bilirubin 1-*O*-acyl monoglucuronide and bilirubin 1-*O*-acyl diglucuronide were prepared by dissolving about 0.35 μmol [estimate based on spectrophotometry of a methanolic solution of the pigment, assuming a molar absorptivity (ε) at 450 nm of 60 × 10^3 $L \cdot mol^{-1} \cdot cm^{-1}$] of the corresponding dry pigment glucuronide in 20 mL of serum diluent. The concentrations of pigment in these stock solutions were determined with a diazo method (*vide infra*) and with alkaline methanolysis combined with normal-phase HPLC (*3*). Results by these two methods agreed within 3%. Calibration reference solutions with pigment concentrations ranging from 0.04 to 17 μmol/L were prepared by appropriate dilution of the stock solution with serum diluent.

Collection of serum samples. Blood samples from healthy blood donors and laboratory staff members were centrifuged within 3 h of collection and the separated sera were stored at −70 °C for a maximum of one month.

Alkaline methanolysis. About 60 mg of sodium ascorbate and 2 to 3 mg of disodium EDTA were mixed with 0.6 mL of serum; 5 mL of methanol and 1 mL of methanol containing the internal standard were added; and the mixture was treated with 6 mL of a 20 g/L solution of KOH in methanol and promptly vortex-mixed. After 60–90 s at 20–25 °C, 6 mL of chloroform and 12 mL of glycine/HCl buffer (0.4 mol/L HCl adjusted to pH 2.4 with solid glycine) were added sequentially, and the mixture was shaken and centrifuged. The organic phase was transferred to a dry 10-mL conical tube and evaporated under nitrogen at 30 °C. The residue was stored under argon at −20 °C and analyzed within two weeks.

In selected cases, the identity of the bilirubin ester peaks in the chromatogram was verified by taking a duplicate sample through the alkaline methanolysis procedure, except that methanol replaced the KOH/methanol reagent. In this "sample blank," transesterification does not occur and peaks corresponding to methyl esters derived from sugar conjugates do not appear in the chromatogram.

Reversed-phase chromatography. The pigment residue was dissolved in 80 μL of chloroform, the solution was kept at 20–25 °C for 10 min, the solution was concentrated by evaporation under nitrogen to about half the initial volume, and a 15–25 μL aliquot was injected into the liquid chromatograph. (Injection of a larger volume resulted in splitting of the unconjugated bilirubin peak.) The pigments were separated by eluting the column at the rate of 1.5 mL/min with a convex gradient (curvature 9 of Waters Solvent Programmer), starting with methanol/water/PIC reagent A (65/35/1.1 by vol)—"solvent A"—and ending after 9 min with methanol/ethanol/water/PIC reagent A (75/10/15/1.1 by vol)—"solvent B." Both solvents contained 2 mmol of sodium ascorbate per liter. Elution was continued for 6 min with solvent B, and then the column was re-equilibrated for 5 min with solvent A. Column temperature was kept at 40 °C. The absorbance of the eluted pigments was monitored at 436 nm, and the area under the peak was integrated electronically.

The concentration, in micromoles per liter, of each bilirubin fraction in the samples was calculated with the equation:

$$(area_{pigment}/area_{internal\ std.}) \times RF \times (IS/SV)$$

where IS is the micrograms of internal standard added to the sample, SV is the milliliters of sample, and RF is the response factor. RF values for each pigment were determined by performing the alkaline methanolysis and the chromatographic procedure on serial dilutions of serum samples containing known amounts of unconjugated bilirubin and of bilirubin mono- and diconjugates (*vide supra*). The values obtained were 1.102 for unconjugated bilirubin, 1.279 for bilirubin monoconjugate, and 1.329 for bilirubin diconjugate.

Structural identification of the bilirubin ester conjugates in serum. The *p*-iodoaniline (*9*) and ethyl anthranilate (*10*) procedures were used to convert the bilirubin ester conjugates in 2 mL of serum to azo derivatives, which were separated by thin-layer chromatography. Chromatographic fractions are denoted according to Blanckaert et al. (*11*); ethyl anthranilate azopigment reference materials are designated as proposed by Bergstrom and Blumenthal (*12*). Relative amounts of azoderivatives in chromatograms were determined by densitometry at 536 nm.

Auxilary methods. Total bilirubins were determined by the diazo method (*13*). Assay of bilirubin and its esters by alkaline methanolysis and normal-phase HPLC was done as described elsewhere (*3*), as was measurement of ^{14}C (*2*).

Results

Optimization of Reversed-Phase HPLC of Bilirubin and Its Methyl Esters

Attempting to improve the sensitivity of the existing alkaline methanolysis–HPLC method for detection of bilirubin esters in serum, we substituted reversed-phase for

normal-phase chromatography because this obviated separation of isomeric bilirubin methyl esters and interference by carotenes. The following procedural variables were tested and optimized during development of the present chromatographic method: the concentrations of tetrabutyl ammonium phosphate ion-pair reagent, methanol, ethanol, and ascorbate in the mobile phase, the eluting solvent gradient programming and the re-equilibration time, the flow rate of the mobile phase, and the temperature of the column.

Figure 1 shows a chromatogram of the reference pigments. With this system, interfering compounds from serum are eluted ahead of the unconjugated bilirubin peak. Figure 2A shows a chromatogram of the bilirubins in the serum of a healthy adult. Heating the column to 40 °C improved peak resolution, decreased column back-pressure, and shortened total chromatography time to 15 min. The added sodium ascorbate minimizes breakdown of the bilirubins in the mobile phase.

Evaluation of Analytical Variables

Precision. Reproducibility of the assay was assessed from 10 analyses of a single serum (Table 1). The within-day CV ranged from 5 to 8%, the day-to-day CV (assessed during two weeks) ranged from 6 to 13%.

Accuracy. Serial dilutions of serum containing known amounts of unconjugated bilirubin and bilirubin mono- and diglucuronides were analyzed, and the results were evaluated by linear regression analysis. Each individual pigment fraction showed a linear relation between peak area in the chromatogram and amount of pigment injected for the range of serum concentrations tested, which was 0.04–17 μmol/L for bilirubin mono- and diconjugate and 0.2–90 μmol/L for unconjugated bilirubin (Table 2).

Analytical recovery of injected pigment through the reversed-phase chromatographic procedure was estimated for each individual bilirubin fraction by injecting known amounts of ^{14}C-labeled reference pigment in the liquid chromatograph and determining the radioactivity accounted for in the effluent fraction corresponding to the appropriate peak in the chromatogram. Mean recoveries (and SD) were 94% (4%) (n = 6; amounts injected: 2–10 nmol) for unconjugated bilirubin, and 97% (3%) (n = 4; amounts injected: 0.2–0.8 nmol) for the mixture of C-8 and C-12 isomers of bilirubin monomethyl ester and also for the dimethyl ester.

Further to assess the accuracy of the new reversed-phase method, we compared our results (y) for unconjugated bilirubin in normal sera with the values (x) obtained with the normal-phase HPLC procedure (*3*). The linear regression analysis data comparing the two methods were: n = 19; r^2 = 0.916, y-intercept = 0.219 μmol/L, and slope = 0.954.

Interference. The carotenoids β-carotene and lycopene were not eluted. Samples of normal human serum containing hemoglobin from erythrocyte lysate in concentrations ranging from 3 to 12 mg/L gave a peak in the chromatogram corresponding with that produced by reference hematin, but it did not interfere with the assay of bilirubin pigments.

Sensitivity. With a 0.6-mL sample of serum, the signal-to-noise ratio was approximately 4:1 at 0.06 μmol of bilirubin methyl ester per liter. The detection limit was about 0.15 μmol/L for unconjugated bilirubin, 0.01 μmol/L for bilirubin monoester conjugate, and 0.02 μmol/L for the di-ester conjugate.

Detection and Identification of Bilirubin Glucuronides in Serum of Healthy Adults

Thin-layer chromatography of the *p*-iodoaniline azoderivatives prepared from serum of 25 healthy adults (14 women;

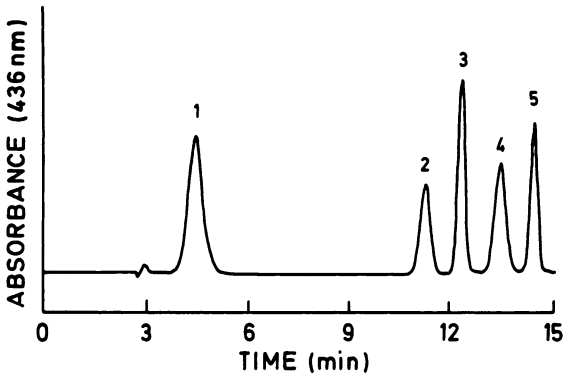

Fig. 1. Chromatogram of reference compounds

A mixture of the reference bilirubins, internal standard, and hemin, dissolved in chloroform, was injected at time zero. *1*, unconjugated bilirubin-IXα; *2*, internal standard; *3*, bilirubin monomethyl esters (C-8 and C-12 isomers); *4*, hemin; *5*, bilirubin dimethyl ester

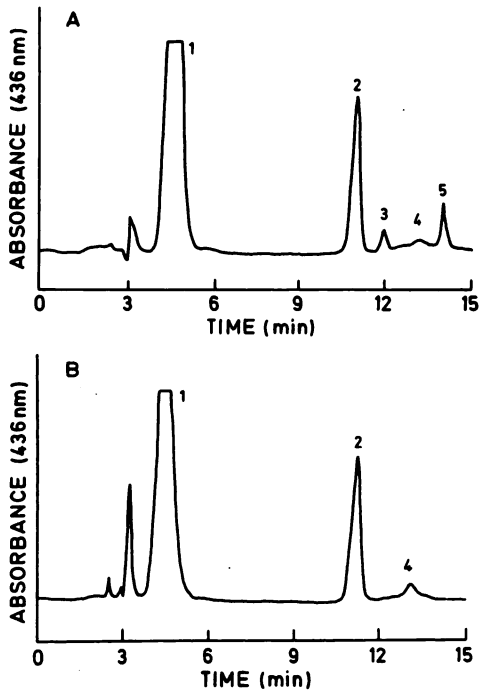

Fig. 2. Chromatograms of pigments from serum of a healthy adult male

A, composition of the pigments extracted from serum following alkaline methanolysis. *B*, same serum sample subjected to alkaline methanolysis procedure, except KOH/methanol reagent replaced by plain methanol. Pigment peaks denoted as in Fig. 1

Table 1. Precision of the Present Method

Pigment	Mean pigment concn, μmol/L (and CV, %)	
	Within-day	Day-to-day
Unconjugated bilirubin	13.20 (8)	12.40 (13)
Bilirubin monoconjugate	0.10 (5)	0.10 (6)
Bilirubin diconjugate	0.13 (5)	0.11 (11)

Table 2. Linearity of the Present Method[a]

Pigment	Intercept, μmol/L	Slope	r^2
Unconjugated bilirubin	0.05	0.97	0.99
Bilirubin monoconjugate	−0.09	0.98	1.00
Bilirubin diconjugate	−0.16	0.98	1.00

[a] Data obtained with calibration standards subjected to linear-regression analysis.

11 men) showed a predominant α_0 fraction (88–98% of total azopigment) and small amounts of the β_x (1–9%) and δ (0.2–2.3%) fractions, suggesting that bilirubin glucuronides were present in each of these serum specimens. The relative amounts of these azopigments on the thin-layer chromatography plates could not be determined accurately because of the considerable background of yellowish non-azopigment in the chromatogram.

To structurally identify the δ fraction, we prepared ethyl anthranilate azo derivatives from five specimens of serum—three from healthy men and two from healthy women—and analyzed them individually by thin-layer chromatography. Each chromatogram showed one major (α_0, 50 to 70% of total azo pigment) and two minor fractions (β_x and δ, 24 to 39% and 5 to 15%, respectively). Treatment of the α_0 fraction with diazomethane completely converted it to two reaction products, which by thin-layer chromatography along with appropriate reference compounds we identified as azo derivatives Azpm-8(Me) and Azpm-12(Me) (Figure 3). Therefore, the α_0 fraction corresponded to a mixture of Azpm-8 and Azpm-12, the two isomeric unconjugated azo derivatives obtained when unconjugated bilirubin reacts with diazotized ethyl anthranilate. In these serum samples, Azpm-8 and Azpm-12 probably were derived principally from unconjugated bilirubin, but partly from monoconjugated bilirubin.

Most importantly, the so-called δ fraction, moving chromatographically as authentic azopyrromethene glucuronide, was detected in all five chromatograms. The identity of this δ azopigment with a mixture of the endovinyl and exovinyl isomers of azopyrromethene 1-*O*-acyl glucuronide Azpm-8(GlcU) and Azpm-12(GlcU) was verified as follows. Methylation and subsequent acetylation of the δ pigment resulted at each step in the formation of derivatives corresponding to those obtained from the authentic reference azopyrromethene glucuronides after identical derivatization. Alkaline ethanolysis of each isomeric acetate derivative yielded the corresponding endovinyl and exovinyl ethyl ester isomers Azpm-8(Et) and Azpm-12(Et).

Methylation of the β_x fraction with diazomethane yielded a single derivative, identified by thin-layer chromatography as Azpm-3,7(Me;Me). Therefore, β_x-pigment was identified as Azpm-3,7 and probably was derived from bilirubin-IXβ, because we also detected trace amounts of 13,18-Vn_2-Azpm (α_F-fraction)—the other, unstable dipyrrolic half of bilirubin-IXβ—in the chromatograms.

Collectively, these results and the findings obtained with alkaline methanolysis and HPLC demonstrate that small amounts of bilirubin 1-*O*-acyl glucuronides are present in normal serum, and also are consistent with the presence in normal serum of trace amounts of unconjugated bilirubin-IXβ. However, unequivocal identification of the latter pigment would require isolation of the parent intact tetrapyrrole, which was impossible with available methodology.

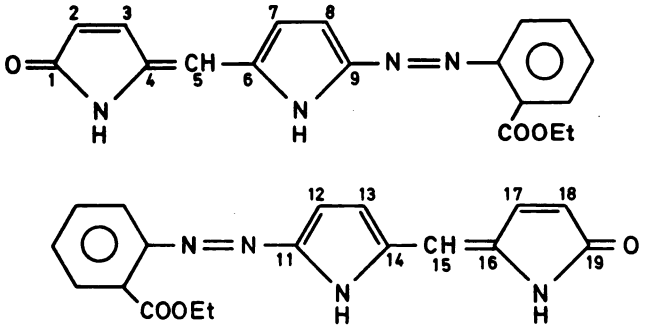

Fig. 3. Structures of ethyl anthranilate azopigments derived from normal serum

The common skeletal structures, their carbon atom numbering, and nature of the β-substituents are shown. Me = CH_3, Vn = $-CH=CH_2$; Et = CH_2–CH_3; CEt = $-CH_2-CH_2-COOH$; GlcU = glucuronosyl

Azopyrromethene abbreviation	Chromatographic fraction	Side chains
Azpm-8[a]	α_0	Me at 2 and 7; Vn at 3; CEt at 8
Azpm-12[b]	α_0	Vn at 18; Me at 13 and 17; CEt at 12
Azpm-8 (Me)	α_0 derivative	same as Azpm-8, but $-CH_2-CH_2-COOCH_3$ at 8
Azpm-12 (Me)	α_0 derivative	same as Azpm-12, but $-CH_2-CH_2-COOCH_3$ at 12
Azpm-8 (GlcU)	δ	same as Azpm-8, but $-CH_2-CH_2-COO$ GlcU at 8
Azpm-12 (GlcU)	δ	same as Azpm-12, but $-CH_2-CH_2-COO$ GlcU at 12
Azpm-3,7	β_x	Me at 2 and 8; CEt at 3 and 7
13,18-Vn_2-Azpm	α_F	Me at 12 and 17; Vn at 13 and 18

[a] Corresponds to so-called endovinyl isomer. [b] Corresponds to so-called exovinyl isomer.

Bilirubin in Serum of Healthy Men and Women

Peaks corresponding to bilirubin monomethyl and dimethyl esters were detected in chromatograms of all the 43 sera tested, and the identity of these peaks with methyl esters derived from corresponding bilirubin monosugar and disugar conjugates in serum was verified by demonstration of their absence from the chromatograms when the KOH/methanol reagent was replaced by plain methanol (Figure 2). Our results for 29 men and 14 women are shown in Table 3. Mean concentrations of all three serum bilirubin fractions were lower in women than in men, and in both sexes the total concentration of bilirubin esters averaged about 3.5% of the total bilirubin concentration. However, considerable interindividual variation in the proportions of conjugated bilirubins was observed for both sexes, the range being 1.2 to 9.9%.

Discussion

The alkaline methanolysis procedure combined with the present reversed-phase chromatography method provides a highly specific, relatively simple, and rapid assay of bilirubin and its ester conjugates in serum. The accuracy of the new method is well validated, and the imprecision (CV) of about 10% is acceptable. The new assay is sensitive enough to detect and measure the monoconjugated and diconjugated bilirubins in serum of healthy men and women, something not possible by non-isotopic methods hitherto. Indeed, even unambiguous detection of conjugated bilirubins in normal human serum is impossible with the previously reported normal-phase HPLC methods (*3, 14*), largely because carot-

Table 3. Bilirubins in Serum of Healthy Men and Women

Mean concn ± 1 SD, μmol/L (and range)			Conjugated bilirubins
UCB	BMC	BDC	(% of total bilirubins)
Men (n = 29)			
6.79 ± 2.62	0.10 ± 0.04	0.12 ± 0.06	3.5 ± 1.9
(1.76–12.26)	(0.03–0.22)	(0.04–0.26)	(1.2–9.3)
Women (n = 14)			
5.21 ± 2.95	0.09 ± 0.06	0.10 ± 0.07	3.7 ± 2.3
(2.42–12.27)	(0.03–0.25)	(0.03–0.25)	(1.7–9.9)

UCB, unconjugated bilirubin; BMC, bilirubin mono-ester complex; BDC, bilirubin di-ester complex.

enoids significantly interfere at these extremely low pigment concentrations and separation of C-8 and C-12 isomers of bilirubin monomethyl ester decreases the sensitivity for detection of monoconjugated bilirubins. An alternative but more elaborate and less specific method for estimation of conjugated bilirubins in normal serum is provided by the diazotized [^{35}S]sulfanilic acid radioisotope dilution method reported by Brodersen (*15*). This assay permits determination of the total amount of sulfanilic acid azopyrromethene ester conjugates derived from human serum. It therefore gives an estimate of the sum of bilirubin monoester and diester conjugates. However, actual concentrations of the parent bilirubin conjugates cannot be calculated with this method, because the relative amounts of mono- and diconjugates remain undetermined.

On analysis of 43 specimens of normal serum we found bilirubin ester conjugates in all, which agrees with Brodersen's findings (*15*). Bilirubin-IXβ previously has been identified in bile of mammals, including humans, and its presence probably reflects a minor pathway of heme degradation in which heme is cleaved at the β-meso bridge instead of at the α-bridge (*16, 17*).

In agreement with previous reports (*18, 19*), values for mean total serum bilirubin found with the new method were lower in healthy women than in men (Table 3), but the composition and proportions of the bilirubins were about the same for men and women. Total conjugated bilirubins (mean values: 0.22 μmol/L in males, 0.19 μmol/L in females) averaged about 3.5% of total serum bilirubins, with large interindividual variations (between 1.2% and 9.9%) observed in both sexes. Using the radioisotope dilution method, Brodersen (*15*) calculated lower total conjugated bilirubin concentrations (0.21 μmol/L for males and 0.15 μmol/L for females), but his values are too low because he assumed that all conjugated azopigment is derived from diconjugates. However, our results show that mono- and diconjugates were present in approximately equal amounts. Therefore, roughly a third of the conjugated azo derivatives have been derived from monoconjugates. This correction of Brodersen's calculated values for the presence of monoconjugates gives 0.28 μmol/L and 0.20 μmol/L of total conjugated pigment in serum of men and women, respectively, in good agreement with our results.

The new assay reported herein provides a powerful tool for examination of various aspects of bilirubin metabolism. However, the value of this type of sophisticated analysis of serum bilirubins for diagnostic purposes in patients with hepatobiliary or hematologic disease and (or) disordered bile pigment metabolism still remains to be established and currently is under investigation.

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