Clinical Application of Serum Bilirubin Fractionation by Simplified Liquid Chromatography

Yukihiko Adachi, Hiroko Inufusa, Masaki Yamashita, Akira Kambe, Kazutoshi Yamazaki,1 Yoshihide Sawada,1 and Toshio Yamamoto

Serum bilirubin was fractionated by a new reversed-phase "high-performance" liquid-chromatographic (HPLC) procedure, on Micronex RP-30, a polycryl ester. The five fractions were: \( \delta \) (\( \delta \)-bilirubin, B\( \delta \)), \( \gamma \) (bilirubin diglucuronide, BDG), \( \beta \) (bilirubin monoglucuronide, BMG), \( \beta' \) ([\( \Delta \),\( \Sigma \)]- and \( \alpha \) ([\( \Delta \),\( \Sigma \)]-bilirubin IX\( \alpha \]). We found close correlation with results of the modified HPLC fractionation of Lauff et al. (J Chromatogr 1981;226:391–402), except for the \( \beta' \) fraction, which was eluted after \( \beta \). The Micronex HPLC involves a simple pretreatment of serum samples, in contrast with the complex preparation described by Lauff et al., and is convenient for routine use in the clinical evaluation of hyperbilirubinemia. We could quantify B\( \delta \), BDG, BMG, and unconjugated bilirubin even in sera with normal values for total-bilirubin concentrations. Photodervatives of bilirubin such as the \( \beta' \) fraction could be separated and quantified by the same procedure, making the method feasible for pediatric research.

Additional Keyphrases: jaundice • hyperbilirubinemia • lumburin • delta bilirubin • pediatric chemistry • chromatography • reversed-phase • conjugated bilirubin

In 1966, Kuenzel et al. (1) first reported the column-chromatographic fractionation of serum bilirubin, without precipitation of serum proteins, into four fractions: \( \alpha \) (unconjugated bilirubin; Bu), \( \beta \) (bilirubin monoglucuronide; BMG), \( \gamma \) (bilirubin diglucuronide; BDG), and \( \delta \) (tightly protein-bound bilirubin or delta bilirubin, B\( \delta \)).1 In 1981, Lauff et al. (2) showed the presence of the above four fractions in icteric sera by HPLC after precipitating serum globulins, and Weis et al. (3) reported on the diagnostic significance of this fractionation. More recently, Singh and Bowers (4), with a less complicated treatment, also successfully obtained four fractions of serum bilirubin by HPLC with a wide-pore column. In 1984, Wu et al. (5) and Sundberg et al. (6) described the multilayer film process (the "Ektachem method"), with which serum bilirubin can be resolved into three fractions: B\( \delta \), conjugated bilirubin; Bc, which is BMG + BDG; and Bu. However, results so obtained do not totally agree with those obtained by HPLC in evaluating B\( \delta \) in sera, whether sera with low bilirubin concentration or with unconjugated hyperbilirubinemia (7).

Materials and Methods

Chemicals. Bilirubin and purified human serum albumin were from Sigma Chemical Co., St. Louis, MO; 1-pentane-2-sulfonic acid was from Aldrich Chemical Co., Milwaukee, WI. The bilirubin was >98% bilirubin IX\( \alpha \) as assessed by HPLC with a C\( \gamma \) \( \mu \)Bondapak column (8). "HPLC grade" solvents, including acetonitrile, were from Wako Junyaku, Osaka, Japan.

Column. We used Micronex RP-30 column (6 mm i.d. × 150 mm; Sekisui Chemical Co., Osaka, Japan), an M600 pump (Waters Associates, Milford, MA), and a Type 490 detector (Waters Associates). The column is packed with porous 8- to 10-\( \mu \)m-diameter particles of polymer gel, cross-linked synthetic organic polymers: 80% polyethylene-glycol dimethylacylate and 20% tetramethylmethane tetra-acrylate. This material has predominantly hydrophobic interactions for solute retention. At an acid pH, albumin is weakly retained by the column, and noncovalently bound bilirubin is dissociated from albumin and adsorbed to the column, whereas bilirubin is not dissociated from B\( \delta \).

Assay procedure. Mix serum with an equal amount of 0.1 mol/L acetic acid reagent and pass the mixture through a 0.45-\( \mu \)m (pore-size) filter to eliminate any fibrin precipitates. Inject 10- to 50-\( \mu \)L (usually 10 \( \mu \)L) of the filtrate onto the column. Pass 5 mmol/L pentanesulfonic acid solution containing 0.1 mol of acetic acid per liter (PAA) through the column for 3 min. Then, for reversed-phase HPLC, use a linear gradient of the eluent at room temperature: from 100% PAA to 50% PAA:50% acetonitrile in 20 min, and then to 100% acetonitrile in 7 min; maintain the latter for 5 min. The shorter run time results in poorer resolution of peaks, but the peaks can still be measured reasonably accurately. Keep flow rate at 1 mL/min throughout. Monitor bilirubin and protein in the eluate by their absorbances at 450 and 280 nm, respectively.

Comparison methods for fractionation of serum bilirubin. The diazo reaction, a modified Jendrassik–Gröf (J-G) method (9); the Ektachem method, with the Ektachem 400 multilayer film analyzer; and Lauff's HPLC after pretreatment of serum as reported by Nakamura et al. (modified Lauff's method) (10) were performed in parallel with the Micronex HPLC.

Identification and quantification of HPLC bilirubin fractions. To determine the composition of each peak eluted from the Micronex HPLC, we collected in liquid nitrogen in subdued light (red light) the fractions derived from 200 \( \mu \)L of icteric serum and lyophilized them. We added an equal-volume mixture of water and dimethyl sulfoxide (DMSO) to the lyophilized eluate, then added 0.5 mL of freshly prepared ethylenethanlylate diazo reagent to 0.1 mL of the mixture and allowed the diazo reaction to proceed at 25°C for 30 min. The azo derivatives from the first bilirubin fraction (B\( \delta \), located at the same elution position as albumin, were applied directly to silica gel (Kieselgel 60 F-254; E. Merck, Darmstadt, F.R.G.) for thin-layer chromatography. Azo derivatives from the other fractions were applied to silica gel after extraction with a mixture of methyl propyl ketone/butyl acetate, 85/15 by vol (8).

The serum bilirubin fractions eluted from the Micronex HPLC were also analyzed by Lauff's HPLC. A bilirubin–albumin solution was prepared by adding 0.5
mL of bilirubin solution in 0.05 mol/L NaOH to 9.5 mL of human serum albumin solution and dialyzing the mixture against 1 mmol/L sodium EDTA solution (pH 7.4) for 24 h (11) (final bilirubin and albumin concentrations: both 0.15 mmol/L). We exposed the bilirubin–albumin solution or a solution of bilirubin in DMSO (bilirubin concentration: 0.15 mmol/L) to light under an argon atmosphere for up to 18 h (12) and subsequently analyzed these by Micronex and Lauff’s HPLC. The bilirubin in DMSO was not pretreated for assay by Lauff’s HPLC.

The bilirubin–albumin solution was used as the reference solution for quantifying bilirubin fractions from HPLC.

Analytical conditions. All steps were performed in subdued light (red light). Collected sera were analyzed without delay or within a week, with storage in liquid nitrogen. We used two human serum specimens for determining the within-day and day-to-day CV for the Micronex HPLC. We also compared the results of bilirubin fractionation in 44 human serum samples by the Micronex HPLC with those by the modified Lauff’s HPLC and the Ektachem method.

Results

Figure 1 illustrates a chromatogram obtained with the Micronex HPLC method. Bilirubin in icteric sera was fractionated into five elution peaks: δ, γ, β, β′, and α. Minimal detection limits of the fractions were about 170 nmol/L when 5 μL of serum was injected into the column. Four fractions of δ, γ, β, and α from Micronex HPLC were eluted in the same order by Lauff’s HPLC. Azodipyroles formed from each of the five peaks by diazo HPLC were applied to thin-layer chromatography plates. The δ fraction consists of two fractions, one bound to albumin and the other being free unconjugated dipyrrole. The γ fraction is mainly gluconic acid-containing dipyrrole. The β fraction was split into unconjugated and gluconic-acid-containing dipyrroles. β′ and α are formed by a single unconjugated dipyrrole moiety. We identified the δ, γ, β, β′, and α fractions of human serum bilirubin separated by Micronex HPLC as Bδ, BDG, BMG, and two fractions of unconjugated bilirubin IXα, respectively. Micronex HPLC revealed the β’ fraction from bilirubin–albumin solution or bilirubin solution in DMSO exposed to light for just 1 min. The β′ fraction was eluted immediately after the β fraction also in the modified Lauff’s HPLC method. The peaks of new photoderivatives appeared between γ and β fractions and between δ and γ fractions in both bilirubin solutions, and were named γ’ and δ’ fractions, respectively. The addition of a trace of trifluoroacetic acid eliminated β’ and δ’ fractions in Micronex HPLC (Figure 2). The β′ fraction was identified as (E,E)-and (E,Z)-bilirubin IXα and the δ’ fraction as (E,E)-bilirubin IXα, according to the previously published reports of Onishi et al. (12, 13) and McDonagh and Palma (14). The γ’ fraction, which was not eliminated by added trifluoroacetic acid, showed negative diazo reactivity and was suggested to be cyclobilirubin (lumirubin) (13). Continued illumination of bilirubin–albumin solution yielded δ fraction (2.5% of initial bilirubin content after 6 h and 4.6% after 18 h; means of two separate experiments). In the present study, γ’ and δ’ fractions were not found in all the adults’ sera tested.

Micronex HPLC showed within-day CVs <7.2% and day-to-day CVs <6.5% for Bδ, BDG, BMG, and Bu (corresponding to β’ fraction + α fraction) fractions, respectively. Modified Lauff’s HPLC and the Ektachem method gave comparable data, as previously described (7). The correlation between Micronex and modified Lauff’s HPLC (Figure 3) was excellent. Modified Lauff’s HPLC tended to give slightly higher results for BMG and BDG, while Micronex HPLC gave slightly higher values for Bu. The correlation between Micronex HPLC and the Ektachem method was also studied. Because the fractionation of bilirubin in sera with normal bilirubin concentrations and with unconjugated hyperbilirubinemia by the Ektachem method was difficult (7), we used sera with conjugated hyperbilirubinemia (total bilirubin (TB) >22 μmol/L as measured by the J-G method) for this comparison. Correlations were excellent between the Micronex HPLC and the Ektachem method for TB (r = 0.98), Bu (r = 0.93), Bc (BMG + BDG in Micronex HPLC) (r = 0.84), and Bδ (r = 0.98) (24 determinations). Micronex HPLC gave a slightly higher value for Bc and a slightly lower Bδ than did the Ektachem method. We used the Micronex HPLC method to assay more than 300 serum samples with the same column, with marginal chromatographic changes.

Discussion

Micronex HPLC is a simple, fast, and reliable method that can directly quantify all the different bilirubin fractions, δ, γ, β, β′, and α, both in normobilirubinemic and in conjugated and unconjugated hyperbilirubinemic samples. We identified the β’ fraction in Micronex HPLC as the
modified Lauff's HPLC (only 10% to 20% of that of the Micronex HPLC) as well as the partial thermal conversion of (E, Z)- and (or) (Z, Z)-bilirubin (β’ fraction) to (Z, Z)-bilirubin (α fraction) (12). The undetectability of photoderivatives in HPLC originally reported by Leuff et al. (2) may also derive from the thermal conversion of photoderivatives. The Micronex HPLC showed lower concentrations of BMG and BDG and a higher concentration of Bu in comparison with modified Lauff's HPLC. This may be explained at least in part by the observation that in modified Lauff's HPLC the β’ fraction was undetectable and was partly included in BMG. With the HPLC technique of Singh and Bowers (4) the quantitative analysis of sera with low bilirubin concentration seems rather difficult because of the small sample size, as in the case of modified Lauff's HPLC. The alkaline methanalysis–HPLC method developed by Muraca and Blanckaert (15) also separates bilirubin fractions selectively, but it does not measure B5.

Development of δ’ and γ’ fractions by continuing light exposure suggests that Micronex HPLC can be used to monitor phototheraphy of neonatal jaundice. According to our experience, Micronex HPLC was convenient and practical for clinical use. The results of clinical observations will be reported elsewhere.

We are grateful to Dr. M. Muraca of Università di Padova and to Dr. C. Tiribelli of Università degli Studi di Trieste for critical reading of the manuscript.

References

CLINICAL CHEMISTRY, Vol. 34, No. 2, 1988 387
We examined a new colorimetric homogeneous immunoassay for carbamazepine based on the apo-enzyme reactivation immunoassay system (ARIS) principle. The test, in dry-reagent strip format, is to be used with the Ames Seralizer reflectance photometer. Within-run CVs (n = 20) were 3.0%, 2.7%, and 2.8% at 3.0, 6.1, and 12.1 mg/L; between-run CVs (n = 15, in 15 days) were 4.1%, 2.7%, and 1.9% at 6.0, 9.1, and 12.1 mg/L. Mean analytical recovery was 99.9 (SD 2.3%). Results by this test (y) for clinical plasma specimens (n = 96) compared very well with those obtained by fluorescence polarization immunoassay (y = 1.01x - 0.02, r = 0.995) and by liquid chromatography (y = 0.99x + 0.14, r = 0.990). Bilirubin (45 mg/L), uric acid (145 mg/L), and various anticoagulants at about fourfold the usual concentrations did not interfere. High concentrations of cholesterol (4.9 g/L), triglycerides (3.8 g/L), and hemoglobin (4 g/L) caused slight positive interference. Carbamazepine-10,11-epoxide cross reacted only at ≥5 mg/L. The two-point calibration line was validly stored for at least three weeks. Free carbamazepine also can be measured. The test is convenient and rapid (test time 80 s), and thus is particularly useful in all clinical settings where prompt testing is needed.

Additional Keyphrases: anticonvulsant drugs · apo-enzyme reactivation immunoassay system · reflectance photometry · monitoring therapy

A Dry-Reagent Strip for Quantifying Carbamazepine Evaluated

Danilo Croci,1 Angelo Nespolo,1 and Giordano Tarenghi2

Carbamazepine (CBZ) is one of the major drugs used in the treatment of epilepsy. Originally introduced for treating trigeminal neuralgia (1, 2), in the last 10 years it has become one of the anticonvulsants most commonly used for controlling secondarily generalized tonic–clonic seizures, as well as mixed and partial seizures with complex symptomatology (3, 4). In humans, CBZ is metabolized mainly to carbamazepine-10,11-epoxide (CBZ-E), which also has anticonvulsant properties (5–7). Because about 70–80% of the drug is bound to plasma proteins, interindividual variations of its free fraction can lead to marked differences in the drug's therapeutic effect (5, 8). The most significant adverse effect (pancytopenia) is not strictly related to drug concentrations in plasma (9); however, other toxic side effects (ataxia, lethargy, hirsutism) are dose dependent and account for the need to monitor the concentrations in plasma of total CBZ and, when there are concomitant physiopathological dysprotidemias (8, 10, 11), the free fraction also.

In recent years, many analytical methods have been described for monitoring total and free carbamazepine concentrations in plasma (12, 15). Most are fast, accurate, precise, and simple, but only a few are suitable for use in urgent analyses, assay of small-medium batches of samples, and direct use in outpatient neurology clinics.

A dry-reagent strip test satisfying such requirements has been described (16) and recently introduced for use with the...