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QUANTITATIVE FRACTIONATION OF SERUM BILIRUBIN SPECIES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid high-performance liquid chromatographic method is described for separating and quantifying four bilirubin species present in serum: bilirubin, bilirubin monoglucuronide, bilirubin diglucuronide, and bilialbumin. Sample preparation consists of dilution with ascorbic acid and dimethylsulfoxide and filtration to remove solid materials. The diluted serum was injected directly onto a wide-pore (300 Å) reversed-phase column and the bilirubin species eluted with a water—isopropanol gradient. Excellent agreement was found between the total bilirubin concentration measured by the high-performance liquid chromatographic method and a diazotized sulfanilic acid procedure.

INTRODUCTION

Bilirubin is a catabolic product of hemoglobin and heme proteins formed in the reticuloendothelial system. This species of bilirubin that leaves the reticuloendothelial system en route to the liver for extraction is referred to as unconjugated or α-bilirubin. It is a hydrophobic molecule and is, therefore, transported in blood complexed to albumin [1]. The enzymatic conjugation of bilirubin with glucuronic acid occurs in the hepatocytes prior to the excretion of conjugated bilirubin in bile. The predominant species of bilirubin in bile is the diconjugated or γ-bilirubin with small amounts of monoconjugated or β-bilirubin being present [2—4].

The current measurements of serum bilirubin to assess liver function are based on the quantitation of diazo derivatives formed in the reaction of serum bilirubin with diazotized sulfanilic acid [5]. In these measurements, it is

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assumed that the unconjugated bilirubin fraction is incapable of reacting with the diazo reagent unless small quantities of organic reagents, such as alcohol or caffeine (accelerators), are used to solubilize and/or displace it from albumin [6, 7]. The conjugated bilirubins being more hydrophilic in nature are capable of reacting with the diazo reagents directly without the need for the presence of an accelerator. These reactions with diazo reagents have generated the familiar concept of direct-reacting bilirubin and total bilirubin, the former being associated with conjugated bilirubin species and the latter being the sum of conjugated and unconjugated bilirubin.

The strict association of the direct-reacting fraction with mono- and diconjugated bilirubin has been challenged in view of the recent observation that a fourth species of bilirubin that is covalently bound to albumin commonly referred to as δ-bilirubin or biliprotein is capable of a direct reaction with diazo reagents [8]. Furthermore, it has also been documented that depending on the reaction conditions, approximately 10% of the unconjugated bilirubin is also capable of a direct reaction with diazo reagents [9, 10]. Such limited precision of the diazo reactions, especially in the 20—50 mg/l [11, 12] total bilirubin region would complicate the diagnosis of certain syndromes where the differentiation between conjugated and unconjugated bilirubin is necessary.

To overcome some of the limitations of the diazo techniques for quantifying serum bilirubin, a number of liquid chromatographic techniques have been developed over the years that are capable of resolving bilirubin into its different species [13—20]. However, to-date, only two such procedures [13, 17] have the capability of resolving bilirubin into the four fractions found in serum: namely, unconjugated bilirubin (α-fraction), monoconjugated bilirubin (β-fraction), diconjugated bilirubin (γ-fraction) and biliprotein (δ-fraction). The superiority of these two techniques over others has been attributed to the fact that serum used in these procedures was not totally deproteinated prior to chromatography. These techniques ensured that the biliprotein was not lost during precipitation of the serum proteins.

Using an open silica column, Kuenzle and co-workers [17, 21], were able to resolve and quantify the four fractions of serum bilirubin. Because of the rather involved nature of this procedure, this technique is now only of historical interest and is not applicable to the routine analysis and separation of bilirubin.

Accurate measurements of each of the four species of bilirubin have been achieved by reversed-phase high-performance liquid chromatography (HPLC) developed by Lauff et al. [13, 22]. Their procedure involves the pretreatment of serum with sodium sulfate to selectively precipitate high-molecular-weight proteins prior to chromatography. This pre-chromatographic procedure of selectively and carefully precipitating large proteins, makes this an elaborate procedure not suitable for use in analyzing large numbers of clinical samples.

In this paper, a simple reversed-phase HPLC procedure is described that is capable of resolving serum bilirubin into its four fractions. This procedure involves minimal handling and experimental manipulation of samples prior to chromatography.

EXPERIMENTAL

Materials

Standard unconjugated bilirubin was obtained from the National Bureau of Standards (Washington, DC, U.S.A.). The standard solution was prepared in serum from healthy donors using the procedure described by Routh [23]. Human serum albumin was obtained as a 30% solution (Pentex) from Miles Labs. (Kankakee, IL, U.S.A.). Cellulose acetate filters (0.45 μm) were obtained from Anspec (Ann Arbor, MI, U.S.A.). MF-1 Centrifugal microfilters were obtained from Alltech Assoc. (Deerfield, IL, U.S.A.). All other reagents were of analytical-reagent grade.

Preparation of samples for chromatography

Human serum (25 μl) was diluted to a final volume of 500 μl in a solution containing ascorbic acid (pH 7) and dimethyl sulfoxide (DMSO); the final concentrations of ascorbic acid and DMSO were 0.6% and 1%, respectively. For serum samples containing low concentrations of bilirubin (less than 100 mg/l), 100 μl of serum were measured and diluted as above. For samples with bilirubin concentrations greater than 250 mg/l, the serum was diluted 1:2 with 5% human serum albumin before taking the 25-μl aliquot for dilution as above. The diluted serum was then filtered through a 0.45-μm cellulose acetate filter using centrifugal microfilters and a bench-top centrifuge to hasten passage of fluid across the filter. The filter was washed twice by filtering two aliquots (0.25 ml) of solution containing DMSO (1%) and ascorbic acid (0.6%) through it. The filtrate, final volume of 1 ml, was saved for chromatography. The final dilution of the original serum sample was either 1:40 or 1:100 depending on whether 25 or 100 μl were used.

Chromatography

The separation of serum bilirubin was achieved by injecting 100 μl of dilute serum onto a 15 cm × 4.6 mm I.D. HPLC column packed with butyl-bonded 5-μm silica (Hypersil WP-300 Butyl) with 300-Å pores. Columns were packed in-house using the upward slurry technique. Hypersil WP-300 Butyl was obtained from Shandon Southern Products (Sewickley, PA, U.S.A.). Separation was achieved by gradient elution, using an initial mobile phase of 0.5% trifluoroacetic acid (TFAA), pH 2.0, with a linear increase in isopropanol over 18.5 min to a final mobile phase of TFAA—isopropanol (60:40). The flow-rate was maintained at 1 ml/min and the separation was carried out at 45 ± 1°C. The separation of bilirubin species was completed within 20 min and repeated injections were made at 30-min intervals. The chromatography was performed using a Varian 5060 (Walnut Creek, CA, U.S.A.) liquid chromatograph.

The separated bilirubin species were detected by measuring absorbance at 436 nm using a LC-55B variable-wavelength spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The peak areas were quantified by an on-line computer system (Varian Vista 402 system). The baseline during each analysis was determined by programming the integrator to subtract the profile obtained during a blank gradient prior to chromatography of serum samples.

Measurement of serum bilirubin

Routine quantitation of bilirubin was carried out on an Altaire automatic analyzer (Electro Nucleonics, Fairfield, NJ, U.S.A.). The method, a modification of the Jendrassik and Grof method [24], uses DMSO as an accelerating agent. The product formed from the reaction with diazotized sulfanilic acid was quantified by measuring the absorbance at 540 nm.

RESULTS AND DISCUSSION

Chromatography of serum and bile bilirubin

The chromatography of human serum resulted in the fractionation of bilirubin into four major fractions (Fig. 1A). The earliest eluting peak was the diconjugated bilirubin (γ-bilirubin) with a retention time of approximately 10 min. The next eluting species was monoconjugated bilirubin (β-bilirubin) which had a retention time of approximately 12 min. The identity of the conjugated bilirubins was established by using the retention time of the bilirubin species present in dog bile (Fig. 1B). In bile, the predominant bilirubin species is the diconjugated bilirubin with the monoconjugated species being the minor fraction [3, 4].

The glucuronide species were followed by the elution of biliprotein (δ-bilirubin) and unconjugated bilirubin (α-bilirubin) with retention times of approximately 14 and 16 min, respectively. The identity of the biliprotein (δ-fraction) in human serum was verified by monitoring the elution profile at

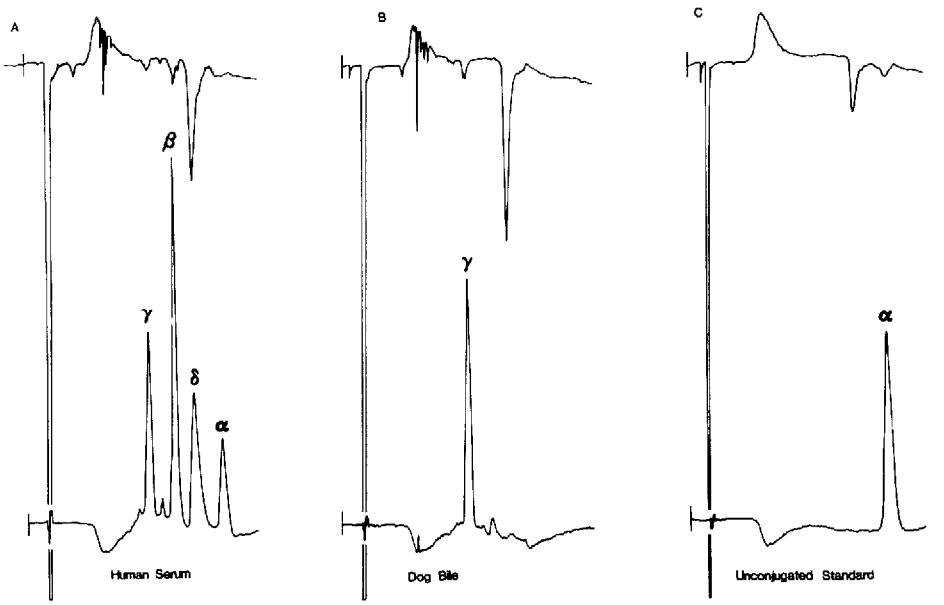

Fig. 1. HPLC separation of bilirubin species in (A) human serum; (B) dog bile; and (C) unconjugated standard bilirubin. Separation was achieved by gradient elution, using an initial mobile phase of 0.5% TFAA (pH 1.0) and a final mobile phase of 0.5% TFAA—isopropanol (60:40). The top tracing shows the absorbance at 280 nm and the bottom tracing is the absorbance at 436 nm. The retention times of the different species were γ = 10.4 min, β = 12.6 min, δ = 14.4 min, and α = 16.7 min.

436 and 280 nm. The retention time of albumin, as measured by its absorbance at 280 nm, coincided with the elution of a bilirubin species with a retention time of approximately 14 min (δ-bilirubin). The slowest eluting peak was the unconjugated bilirubin, which is the only bilirubin species available as a standard (Fig. 1C).

The elution profile of the bilirubin species under the present chromatographic procedure is different from that previously reported by Lauff et al. [13]. The relative retention of the δ-bilirubin compared to the conjugated species is longer under the present conditions, whereas under the conditions described by Lauff et al. [13] the δ-bilirubin was the earliest eluting species. This difference in elution profile could be due either to the shorter hydrocarbon chain length or to greater accessibility of surface silanols.

Effect of ionic strength

The chromatography of the bilirubin species is influenced by the concentration of trifluoroacetic acid. Decreasing the concentration of TFAA resulted in a change in the elution profile of the bilirubin species (Fig. 2). Furthermore, the separation between the peaks, particularly those of the β- and δ-fractions at the lower concentration of TFAA was not as well defined as when 0.5% TFAA was used (Fig. 1).

Effect of temperature

Chromatography at room temperature (Fig. 3A) resulted in broad peaks with

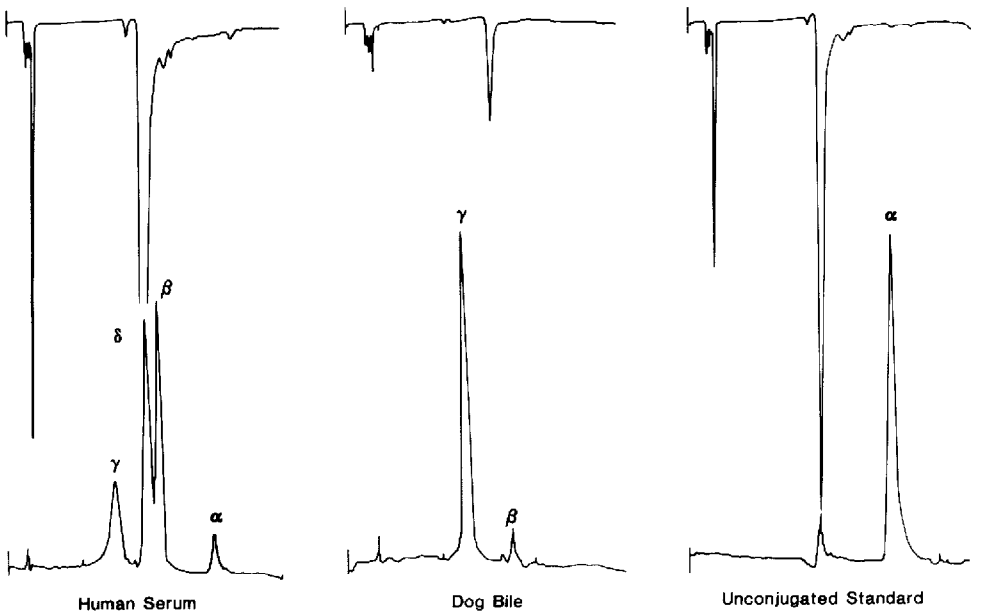

Fig. 2. The effect of ionic strength on the chromatography of bilirubin species. (A) Human serum, (B) dog bile and (C) unconjugated standard. HPLC separation of bilirubin species using an initial mobile phase of 0.1% TFAA (pH 2.0) and a final mobile phase of 0.1% TFAA—isopropanol (50:50). The top tracing shows the absorbance of 280 nm, and the bottom tracing shows the absorbance at 436 nm. The retention times of the different species were γ = 8.0 min, δ = 10.6 min, β = 11.4 min, and α = 15.4 min.

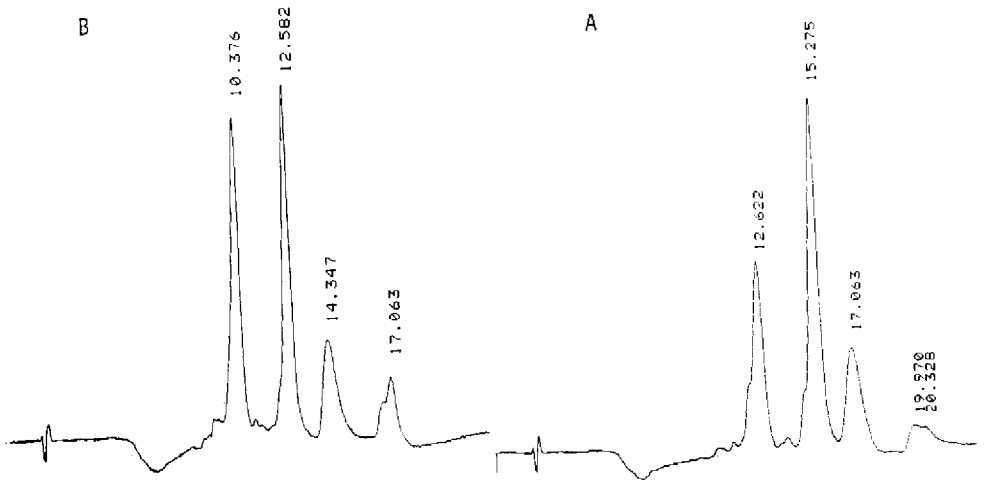

Fig. 3. The effect of temperature on the chromatography of bilirubin species. (A) Room temperature; (B) 46°C. Equal amounts of diluted serum were injected onto the column and separation was performed at the indicated temperature. Separation conditions were identical to those employed in Fig. 1.

shoulders which suggested the possible presence of more than one compound associated with some of the peaks. Increasing the temperature to 46°C resulted in sharper peaks (Fig. 3B). It has been suggested that conjugated and unconjugated bilirubin in the serum exist as a complex mixture of isomers [25]. A number of HPLC procedures have been developed that are capable of resolving these conformational isomers [18, 20, 25]. It appears that our chromatographic conditions are capable of consolidating these conformational isomers of the different bilirubin species into single but sometimes broad peaks.

Linearity studies

The area response to injected unconjugated bilirubin standards was linear up to 300 ng of bilirubin injected onto the column. Furthermore, our procedure ensured that no losses of unconjugated bilirubin occurred during the filtration and dilution process. This recovery was influenced by the presence of DMSO. In the presence of a final concentration of 1% DMSO, no yellow-colored material was left on the filters, and peak area analysis showed total recovery of the sample. Increasing the concentration of DMSO up to 20% did not affect the recovery, but affected the chromatography as broader peaks were obtained at concentrations of 10 and 20% DMSO.

Effects of sample dilution

The quantification of total bilirubin expressed as the sum of area counts associated with each of the four species with increasing dilution of serum sample is shown in Fig. 4. An icteric serum sample was initially diluted 1:2, 1:4, and 1:8 fold with 5% human serum albumin solution. Aliquots (25 μl) from each of the diluted samples and an undiluted sample were treated separately and further diluted 1:40 fold in a solution of ascorbic acid and DMSO as described in the Experimental. section. These four samples with

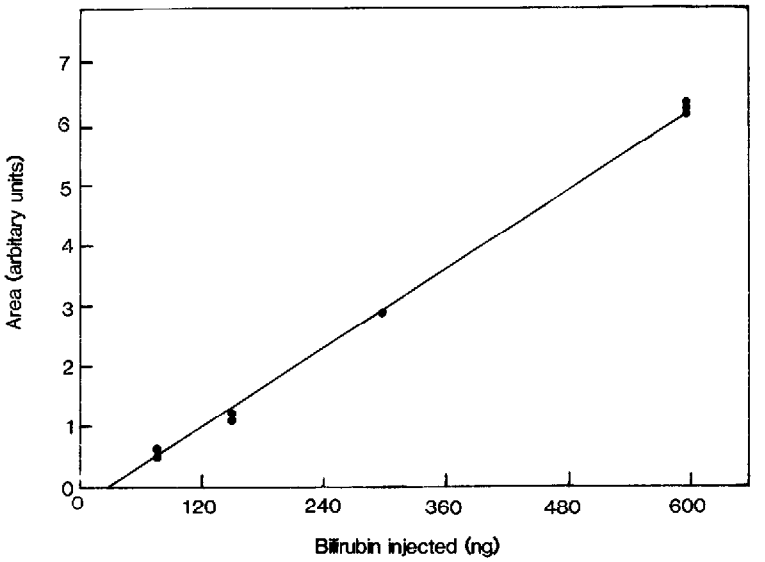

Fig. 4. The effect of dilution of serum sample on HPLC area counts. A human serum sample was diluted initially with 5% human serum albumin solution and finally in a solution containing DMSO and ascorbate. Samples (100 μl) were injected onto the column. For the undiluted sample where no initial dilution with human serum albumin was performed, the total amount of bilirubin injected onto the column was 600 ng. The total area was obtained by summation of area under each of the four species of bilirubin.

dilutions ranging from 1:40 to 1:320 times the original icteric serum sample were analyzed by injecting 100-μl aliquots onto the HPLC column.

As shown in Fig. 4, there was a proportional decrease in total area counts with increasing dilution of the sample. Replicate injections of the same sample showed good precision, validating the fact that neither significant losses nor degradation of sample occurs during the procedure. The negative intercept of the line on the y axis (Fig. 4) can be explained by the fact that with large dilutions of the serum sample such as 1:320 fold dilution, the peaks become very small such that the integration becomes a problem as larger errors are introduced in integrating the diminished signal.

In the procedure of Lauff et al. [13], due to the extensive pretreatment of serum samples at 37°C for 20 min with sodium sulfate, it has been suggested that some hydrolysis of di- and mono-esters of conjugated bilirubin occurs [25]. This, however, does not appear to be a problem with our procedure. The analysis of the area counts associated with each one of the four bilirubin species in this dilution study showed a similar profile to Fig. 4 with a proportional and linear decrease in area counts with increasing dilution.

Precision studies

Table I summarizes the between-day and within-day precision of analysis of bilirubin by the HPLC procedure. The within-day precision was calculated from the analysis of a human serum sample that was divided into ten aliquots and each aliquot was treated and analyzed as a separate sample on the same day. The between-day precision was calculated from the analysis of samples of

TABLE I

PRECISION STUDIES

	Bilirubin species				
	α	β	γ	δ	Total
Within-day					
Serum mean area (counts)	21 801	187 021	98 839	67 027	374 687
C.V. (%) (n = 10)	13.1	4.5	6.7	8.4	5.3
Between-day					
Serum mean area (counts)	19 571	99 586	110 382	19 091	248 631
C.V. (%) (n = 6)	6.1	3.5	4.4	6.9	2.6
National Bureau of Standards					
bilirubin mean area (counts)	358 075				358 075
C.V. (%) (n = 7)	5.6				5.6

human serum and standard unconjugated bilirubin that were aliquoted and stored frozen at −70°C and analyzed on successive days. Excellent precision was obtained from both the within-day and between-day analyses, with a coefficient of variation (C.V.) for total area counts of approximately 5%.

The precision for the smaller peaks, such as α- and δ-bilirubin, appeared to be lower than that of β-bilirubin which had the greatest peak area for the

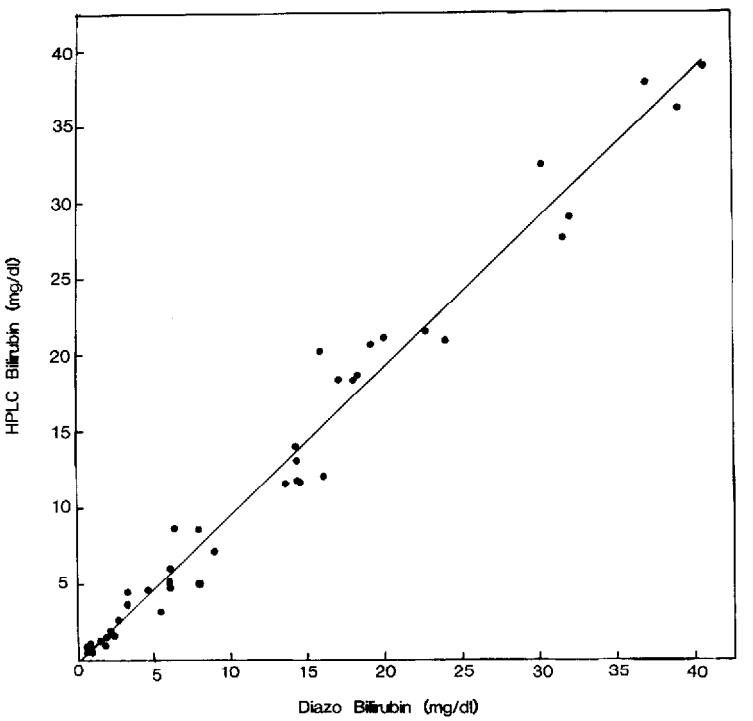

Fig. 5. Correlation of HPLC total bilirubin versus total bilirubin measured by the diazo reaction; r = 0.99.

particular serum samples analyzed. It appears that with low area counts, the variability in peak area is magnified. To overcome this problem, precision for serum samples with low bilirubin (less than 100 mg/l) can be improved by making a smaller dilution of the serum, usually 1:10 and obtaining larger peak area counts.

The between-day precision study also shows that storage of human serum samples at −70°C for a period of one week did not result in the alteration of the composition of the different bilirubin species.

Correlation of HPLC with diazo techniques for bilirubin analysis

Fig. 5 shows the correlation of the total bilirubin quantified by HPLC and the measured value of total serum bilirubin using a modified Jendrassik and Grof diazo reaction [24]. The measurement of the serum bilirubin by the diazo technique was performed using the clinical chemistry autoanalyzer. Linear regression analysis of data yielded the equation $y = 0.97 \pm 0.02x - 0.19 \pm 0.36$, for $n = 46$ samples with $r = 0.99$ and $Sy \cdot x = 1.63$.

The calculations of serum bilirubin concentrations by HPLC were carried out using unconjugated bilirubin standards from which a relationship between peak area and amount of bilirubin injected onto the column were established. This relationship allows the calculation of the concentration of each one of the four species of bilirubin in serum. The basic assumption in this conversion of peak areas to concentration is that at 436 nm, the spectral characteristics of all four bilirubin species are identical [8].

CONCLUSIONS

The reversed-phase HPLC procedure of Lauff et al. [13, 22] has such intrinsic limitations due to the complexity of its pre-chromatographic procedures that it is not readily adaptable to routine clinical analysis. The procedure presented in this paper is simple and accurate, its most attractive feature being the ability to achieve good separation by simply injecting diluted serum samples directly onto the HPLC column. The measurement of absolute amounts and relative proportions of the different bilirubin species by a simplified HPLC procedure could be diagnostically and clinically useful in patients with hyperbilirubinemia. This procedure is readily adaptable for use in routine clinical analysis.

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