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SEPARATION OF BILIRUBIN SPECIES IN SERUM AND BILE BY HIGH-PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance, reversed-phase liquid chromatographic (HPLC) procedure has been developed for the separation of at least three major bilirubin fractions in bile and four fractions in human serum. This procedure was unlike most others, in that serum was not totally deproteinized prior to injection onto the HPLC column; instead, serum was treated with an excess of sodium sulfate solution to precipitate primarily proteins larger than albumin. Injection of the filtered and diluted supernatant onto a reversed-phase column then resulted in the separation of the bilirubin species in a 24-min gradient elution run. Both the initial aqueous acidic mobile phase and the final isopropyl alcohol-based mobile phase contained 5% methoxyethanol (v/v) to facilitate elution of albumin still present in the treated sample. Bilirubin species eluting from the column were detected by absorbance at 450 nm.

Results of a number of chromatographic separations of pathological sera indicated a wide variation in the relative proportions of the four bilirubin fractions observed. A correlation of the sum of the areas of the bilirubin peaks observed by HPLC was found with the total bilirubin value obtained by a standard reference procedure.

INTRODUCTION

The formation of diazo derivatives of bilirubin is the basis for most clinical methods of analyzing bilirubin in serum and bile [1—3]. In general, the diazo methods yield a single value for the total concentration of all the bilirubin species present in a sample and, in certain modifications, an additional lesser value which is considered to be proportional only to the concentration of the more water-soluble bilirubin esters or conjugates. Yet, it has been well established for some time that bilirubin exists in bile as at least three major chemical forms [4—6]: free, monoester, and diester with glucuronic acid as the predominant ester group. Besides these three, in human serum there is a fourth form of bilirubin which is more tightly associated with protein [7—12]. In

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addition, minor amounts of other esters have been found in biological fluids [4, 6, 13, 14]. Thus, the use of a single colorimetric measurement to characterize the sum of several chemical species may be clinically useful but yields little accurate information about the relative proportions of each of the individual species, which may also be of clinical significance.

Chromatographic methods would appear to be ideal for studying the distribution, as well as the clinical significance, of the individual bilirubin species in serum. Paper chromatography has been applied extensively to this problem and has been reviewed by With [15]. Thin-layer methods have also been used [5, 16—19]. In general, these methods do separate several bilirubin species; however, quantification and identification of the separated fractions found in biological fluids are problematical because of the instability of the pigments.

The open-column, reversed-phase chromatographic procedure of Cole and co-workers [20, 21] and Billing [22, 33] is the basis for most early work with column chromatography. Unfortunately, this procedure is not entirely satisfactory because with pathological serum samples, a substantial amount of the yellow color due to bilirubin is lost with the precipitated proteins [24—28]. Kuenzle and co-workers [7—12] modified this procedure by chromatographing serum without first deproteinizing. As a result, they observed four bilirubin fractions: unconjugated bilirubin (α), bilirubin monoconjugate (β), bilirubin diconjugate (γ), and a fourth fraction (δ) strongly associated with protein. This last fraction is distinct from the complex of unconjugated bilirubin (α) and albumin known to exist in serum and aqueous solution [29—32]. Perhaps because of the large volume of serum required and difficulties in quantifying the separated pigments, this work seems to have been neglected in the more recent literature.

In the last few years, several high-performance liquid chromatographic (HPLC) methods have been reported for the separation of bilirubin species in various biological fluids [33—36]. These methods either have not been applied to serum or have required extensive deproteinization prior to chromatography. Difficulties with protein elution from modern, silica-based chromatographic materials are well known [37—40]. In general, most large proteins appear to associate irreversibly with the column, either because of adsorption on free silanol sites or because of a strong partitioning of the hydrophobic side chains of the proteins into the hydrophobic bonded phase. Since proteins are present in serum at 6—8% by weight and since high-performance columns can be destroyed in a short time by this association, common practice is to remove the proteins prior to the chromatographic separation of serum components. This procedure makes it impossible to observe bilirubin species which are attached, either in a very strong complex or by a covalent bond, to protein.

To overcome this limitation of HPLC for the analysis of bilirubin species in serum, we used a solvent system first described by Mönch and Dehnen [37] for the elution of proteins with molecular weights as high as $4 \cdot 10^5$ from a reverse -phase column. This system allowed albumin and that fraction of bilirubin (δ) most tightly associated with it to be separated from other bilirubin species present in serum. Unfortunately, serum contains an appreciable fraction of protein with molecular weight $> 10^6$ daltons [41], and some would not pass through the column, again causing irreversible loss of efficiency in a

short time. To circumvent this effect, prior to the chromatography, we have resorted to a classical precipitation of the higher-molecular-weight proteins in serum by dilution with a sodium sulfate solution [42]. With this separation for serum, some yellow color was still lost with the precipitated higher-molecular-weight proteins. However, the resulting chromatographic separation was reproducible, and the column was extremely stable, a single column having been used for more than 250 injections of serum without serious degradation of resolution or efficiency.

EXPERIMENTAL

Materials

Ascorbic acid, 2-methoxyethanol, caffeine, sodium benzoate, and phosphoric acid were all reagent grade or better, obtained from Kodak Laboratory Chemicals (Rochester, NY, U.S.A.). Caffeine—benzoate solutions were formulated according to Tietz [43]. Sodium phosphate, mono- and dibasic, and anhydrous sodium sulfate were obtained from MCB Reagents (East Rutherford, NJ, U.S.A.). Sodium sulfate solutions were prepared by dissolving 27.7 g of the anhydrous salt in ca. 80 ml of hot distilled water. The pH was then adjusted to 7.0 ± 0.2 with dilute sulfuric acid or dilute sodium hydroxide. The solution was diluted to volume in a 100-ml volumetric flask and kept in a 37°C water bath. Each day a 10% (w/v) aqueous ascorbic acid solution was prepared in a 50-ml volumetric flask with 1 ml of 2 *M* phosphate buffer (pH 6.7) added. This solution was adjusted to pH 5.8 ± 0.2 with sodium hydroxide.

Human serum albumin (fraction V) and unconjugated bilirubin were obtained from Sigma (St. Louis, MO, U.S.A.). A master standard solution of unconjugated bilirubin in 5% albumin solution was made in a manner similar to that of Tietz [44]. Serial dilutions of this standard (5—150 mg/l) were made using 5% human serum albumin in distilled water (w/v).

From these albumin—bilirubin solutions, standards for injection onto the HPLC column were prepared by diluting 0.25 ml of the appropriate solution in a 10-ml volumetric flask with 7 ml of sodium sulfate solution, 0.50 ml of the 10% ascorbic acid solution, and distilled water. Because of instability in room light, all sera, bile and standard solutions of bilirubin were handled under yellow light. Under these conditions, the standards in sodium sulfate solution were stable at room temperature for ca. 15 h ($< 3\%$ loss).

The initial mobile phase for the HPLC separation was prepared by adding 2 *M* phosphate solution (sodium salt, pH 6.7, heated to prevent precipitation) and 2-methoxyethanol to a large Erlenmeyer flask to give, upon dilution with distilled water, a solution 0.05 *M* in phosphate and 5% in 2-methoxyethanol (v/v). The pH of the solution was then adjusted by means of a combination glass electrode and pH meter to 2.0 ± 0.1 by the addition of phosphoric acid. The second mobile phase was 5% in 2-methoxyethanol and 95% in HPLC grade isopropyl alcohol (v/v) obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Phosphoric acid was then added to this solution in the ratio of 25 ml to 1 l of solution. Before use, both mobile phases were thoroughly degassed by stirring under a stream of helium.

Glass-fiber filters were obtained from Gelman (Ann Arbor, MI, U.S.A.)

(Type A-E, 25 mm, No. 61630) and 0.45-μm filters were obtained from Millipore (Bedford, MA, U.S.A.) (Type HA, 25 mm). The HPLC column was stainless steel, 25 cm × 4.6 mm I.D., prepacked with 10-μm silica to which octylsilane had been bonded (LiChrosorb RP-8). This was purchased from Brownlee Labs. (RP-10A) via Rheodyne (Berkeley, CA, U.S.A.). For some separations, an RP-8 guard column, also from Brownlee Labs., was used ahead of the analytical column.

Apparatus

Either of two filtration units obtained from Amicon Corporation (Model MMC or Model 12) was used with the Gelman filter placed over a Millipore filter in the apparatus to remove precipitated proteins from sodium sulfate-treated serum samples. Both units were pressurized with helium.

The chromatography system consisted of a Waters Intelligent Sample Processor (WISP 710B, Waters Assoc., Milford, MA, U.S.A.) to inject samples, a Waters Model 660 programmer to control the gradient and two Waters Model 6000 pumps that had been upgraded to 6000A standards by the substitution of a 6000A reference valve and multisolvent inlet ports for those originally fitted. The chromatographic column was maintained at 41°C in an oven obtained from a DuPont 830 liquid chromatograph. After elution from the column, the eluent passed through a variable-wavelength spectrophotometer (Perkin-Elmer LC-55) set at 450 nm and then through a second spectrophotometer set at 280 nm (HM Holochrome UV Monitor, Gilson Medical Electronics). The outputs of both detectors were monitored with strip-chart recorders while peak areas were obtained simultaneously by means of an on-line computer system.

Procedure

Bilirubin—albumin—sodium sulfate standard solutions were prepared as described above and injected directly into the chromatography system. Bile samples were first diluted 1:10 with distilled water. Then a 0.25-ml aliquot of this diluted bile was added to a 25-ml volumetric flask containing 0.5 ml of ascorbic acid solution and 0.5 ml of 5% albumin solution. Sodium sulfate solution (14 ml) was then added, and the solution was diluted to the mark with distilled water.

Serum was diluted with sodium sulfate solution at 37°C in the ratio of 3.5 ml to 0.25 ml of serum to precipitate high-molecular-weight proteins. The diluted serum was then heated at 37°C in sealed vials, with occasional shaking, for several minutes before filtration through the Amicon filtration unit. The effluent from the Amicon filtration unit was fed directly into a 10-ml volumetric flask containing 0.5 ml of ascorbic acid solution. A second 3.5-ml aliquot of sodium sulfate solution was then passed through the precipitate and combined with that in the flask. The contents were diluted to volume with distilled water for injection into the chromatographic system.

For the chromatographic separation, 500-μl aliquots of the diluted and sulfate-treated sample were injected onto the chromatography column, which was maintained at 41°C. The serum components were eluted over 16 min at a flow-rate of 1.4 ml/min by a linear gradient from 100% initial mobile phase to 80%

final mobile phase—20% initial mobile phase. The mobile phase composition was then held constant for 8 min, by which time all components had eluted from the column. Before injection of the next sample, 100% initial mobile phase was allowed to flow for a 7-min equilibration period.

RESULTS AND DISCUSSION

Chromatography system

The phosphoric acid—methoxyethanol-based chromatography eluent system was adopted from the work of Mönch and Dehnen [37] after a series of preliminary experiments indicated that albumin would not elute reproducibly from a reversed-phase column when a more common acetate-buffered system (pH 4.5) was used. More important, with the acetate system a substantial amount of the yellow material present in pathological sera did not elute from the reversed-phase column. This suggested that some bilirubin species might be strongly associated with albumin. In fact, Kuenzle and co-workers [7—12] demonstrated almost 20 years ago that in pathological serum there was a bilirubin fraction (δ) which was very tightly associated with protein.

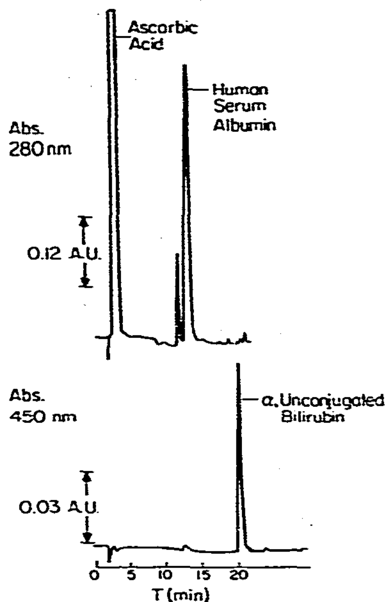
The present chromatography system has enabled the elution and separation of both albumin and unconjugated bilirubin, as shown in the chromatogram for a standard sample in Fig. 1. With fresh columns it was sometimes necessary to condition the column with several injections of a bilirubin standard solution before running samples and standards for a calibration curve. During this initial equilibration period, the peak height of the albumin peak (detected at 280 nm) would often decrease by as much as 10% of its initial value and then stabilize at a constant height. This effect may have been caused by residual adsorptive sites which, once coated with protein, did not interfere further in the analysis.

The small absorbance on the 450-nm trace in Fig. 1 at the retention for albumin was mostly due to the slight inherent yellow color of human serum albumin used to prepare the standard solution and not to unconjugated bilirubin. This peak was minimally enhanced in fresh standards, if at all, when compared with an injection of the same concentration of albumin with no unconjugated bilirubin present. Thus, in this chromatography system, the interaction of the column with bilirubin and albumin was strong enough to break up the complex known to exist between unconjugated bilirubin and albumin in aqueous solution.

In contrast, a typical pathological serum sample separated into four well-defined yellow fractions (Fig. 2). In this case, there was a substantial amount of yellow material which eluted at the same retention as that for albumin. Since interaction with the column did not break this association, it had to be stronger than those existing between albumin and the other bilirubin species in serum. In accord with notation adopted by Kuenzle and co-workers [7—12], we have labeled the peaks (α) unconjugated bilirubin, (β) monoconjugated bilirubin, (γ) diconjugated bilirubin, and (δ) protein-bound bilirubin.

Prechromatography treatment with sodium sulfate

The use of sodium sulfate as a protein precipitant was necessary for a reproducible chromatographic separation and long life of the column. When sodium



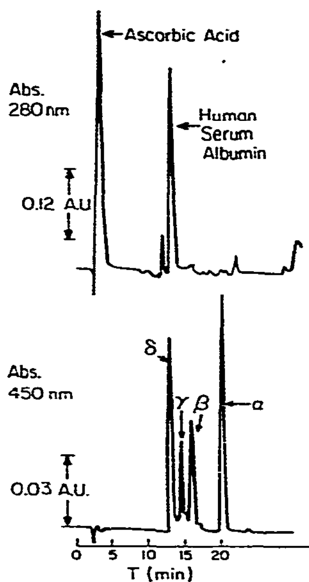


Fig. 1. HPLC separation of unconjugated bilirubin (α) in human serum albumin solution.

Fig. 2. HPLC separation of pathological serum.

sulfate was not added to samples, the initial separation of serum on fresh HPLC columns was similar to that given in Fig. 2, i.e., four well-defined bilirubin fractions. However, as few as eight 50-μl injections of undiluted serum could cause a substantial loss of resolution. In particular, peak broadening and tailing became progressively more severe, and the absolute area of the peaks decreased. These problems were traced to the incomplete elution of proteins with molecular weight higher than that of albumin. Mönch and Dehnen [37] had demonstrated that certain proteins with molecular weights as high as $4 \cdot 10^5$ should elute from the present system, but there are some proteins present in serum with molecular weights greater than 10^6.

For the system to reproducibly separate serum samples, it was necessary to remove these interfering proteins by pretreating sera with a 14-fold volume excess of sodium sulfate at 277 g/l, similar to the method of Yeoman [42]. The precipitate which was formed could not be easily removed by centrifugation and so was separated by filtration. In this procedure, a Gelman fiber filter served as a prefilter to increase the capacity of the filtering system so that blockage of the 0.45-μm filter would not occur. If a sample was cloudy after this treatment, it was filtered through a second 0.45-μm filter. If it was still cloudy after the second filtration, it was discarded, because cloudiness indicated incomplete removal of the interfering proteins, which would lead to rapid column degradation.

This treatment did cause some loss of yellow material which was adsorbed to the precipitated and filtered proteins. These losses may have been partly related to sample storage conditions prior to analysis. Many times, samples

stored frozen (−4°C) for several weeks were used to develop the procedure. Recent experiments have indicated that under these conditions, changes in the chromatographic pattern could occur. Freshly drawn samples are recommended.

Column performance

With this precolumn separation, column lifetime has been dramatically improved. Over a period of a month, more than 400 injections of standards and serum samples diluted with sodium sulfate and separated from higher-molecular-weight proteins were made on the same reversed-phase column. Column resolution was not appreciably degraded during this time and retentions were remarkably stable. In addition, we have found that different columns from the same manufacturer yield nearly identical separations. Unfortunately, we did not find that columns from other manufacturers would afford the same reproducible and long-term stable separation. Since nominally similar columns from different manufacturers have been known to exhibit different selectivities, this result was not surprising. Even with the Brownlee columns, we have found that only fresh columns, i.e., unused for other analyses with different solvent systems, would reliably yield a stable chromatographic system. Extraneous material irreversibly adsorbed to the column bed and/or protein denatured on the column surface from other analyses has, in our experience, led to unsatisfactory separation and/or poor column lifetime.

Standard calibration curve for α-bilirubin

The addition of ascorbic acid at an acidic pH along with albumin minimized oxidation and increased the stability of standard solutions of unconjugated bilirubin when diluted with sodium sulfate for injection onto the chromatography column. However, acidic pH values for the master albumin—bilirubin standard solutions should be avoided because precipitation and/or aggregation of the concentrated bilirubin standards could occur, leading to poor calibration curves. In our experiments, the sodium sulfate diluted standards (pH $\approx$ 6.0), automatically injected interspersed with serum samples in overnight runs, yielded linear calibration curves similar to that shown in Fig. 3. All standards were prepared and diluted for injection at the same time and then remained in the automatic injector in either subdued incandescent light or yellow filtered light for up to 17 h before injection. As indicated in Fig. 3, this procedure produced a linear calibration of area versus concentration with a minimal negative intercept corresponding to about 1—2 mg/l of bilirubin. Peak heights were not generally usable for quantification because of band broadening at the higher concentrations.

Identification of bilirubin components

Fig. 4 shows chromatograms of pathological human serum, adult human bile, dog bile, and rabbit bile. The bile samples, as noted in the Experimental section, were first diluted with human serum albumin and sodium sulfate to closely imitate the matrix from which serum samples were injected, as well as to stabilize the conjugates against degradation. For the bile samples, no appreciable peak was found at the retention for the δ-component, only a minimal

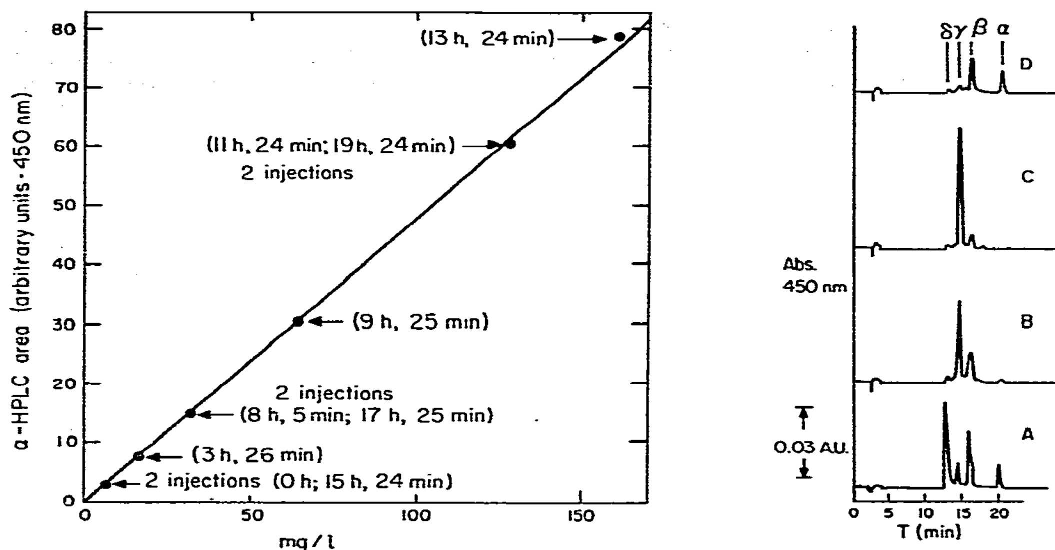


Fig. 3. Calibration curve for unconjugated bilirubin (α). Concentrations are for standard solutions in 5% albumin before dilution with sodium sulfate, as noted in the text. In each case 500 μl of the diluted standard were injected. In parentheses are the relative times in hours for the injection of each standard. The line was fitted by a linear least-squares program (Texas Instruments 58C) with correlation coefficient = 0.9995 and intercept equivalent to −0.9 mg/l unconjugated bilirubin.

Fig. 4. HPLC separation of bile and serum: (A) human serum, (B) human bile, (C) dog bile, (D) rabbit bile.

absorbance due mostly to the yellow color of the human serum albumin added to the samples. If the δ-component is formed only with albumin or protein, this result should be expected, because bile does not contain much protein and thus is less likely to contain protein-bound bilirubin species.

The δ-component, which is both yellow and diazo positive, has been discussed only inferentially in the literature [28, 36, 45], except for the work of Kuenzle and co-workers [7—12]. In both our work and the earlier work, the major distinguishing feature of this material was its tight binding to protein. The material has been isolated in impure form from pathological sera by treatment with caffeine—benzoate reagent followed by diafiltration. The details of this isolation as well as the results of various physical and chemical tests on the properties of the δ-fraction can be found elsewhere [46]. In brief, the material is composed of one or more bilirubin derivatives, very tightly, if not covalently, bound to a protein, most likely albumin.

The γ-fraction was identified as a diconjugate of bilirubin by comparison of its retention with that of the major component in isolates of bilirubin glucuronide from human bile, by the procedure of Wu et al. [47]. In addition, we found (Fig. 4) that the γ-fraction predominated in samples of adult human bile and in the sample of dog bile. Both of these results agree with the work of others [5].

The β-fraction of bilirubin has not been isolated, but much circumstantial evidence indicated that it was a monoconjugate of bilirubin. First (Fig. 4), it predominated in the bile of rabbits which had been treated by ligation of their bile ducts. That a monoconjugate of bilirubin predominates in lower species was observed previously [18]. In addition, we found that this fraction, β, as well as γ, diconjugated bilirubin, decreased in concentration during the keeping of pathological serum samples at room temperature in the dark for periods of up to 72 h. In the same time period, the concentration of unconjugated bilirubin, α, increased. The large loss with time of γ, the slight decrease in β, and the large increase in α were consistent with hydrolysis of the esters. In that case, β was logically a monoconjugate.

Precision and accuracy

The large injection volume (500 μl) was chosen as a compromise to obtain adequate sensitivity for detecting bilirubin at low, nominally normal values, while not overloading the column with albumin, which can shorten column lifetime. Because of the amount of sodium sulfate needed to completely precipitate the higher-molecular-weight proteins, the injection volume chosen allowed detection of bilirubin at 1—2 mg/l, depending upon which species were present. The precision of the measurements was about the same order of magnitude as illustrated in Table I. In this table, the precision of the average area obtained for each of the bilirubin peaks for triplicate injections of the same aliquot of serum is given along with the average area. For the Table, ca. 2200 area units were equivalent to 10 mg/l of unconjugated bilirubin. Thus, it is evident that the precision and accuracy are limited to about ±1.0 mg/l. Since levels less than 10 mg/l are considered benign or normal, this sensitivity and precision should be adequate for most purposes.

TABLE I

HPLC PRECISION STUDY

Three replicate injections.

Serum No.	δ	γ	β	α	Total area*
18	20066 ± 362	4873 ± 59	12467 ± 250	6100 ± 116	43507 ± 435
9	7087 ± 32	1985 ± 33	6347 ± 121	11200 ± 529	26618 ± 612
8	9013 ± 76	1004 ± 122	7013 ± 161	6260 ± 238	23291 ± 675
1	25663 ± 51	4652 ± 47	10549 ± 158	5226 ± 99	46091 ± 323
7	4967 ± 40	1393 ± 110	6233 ± 50	8233 ± 354	20827 ± 416
4	1977 ± 166	3948 ± 146	262 ± 227	17067 ± 563	23253 ± 233
45	36367 ± 315	2760 ± 58	3467 ± 42	12933 ± 65	55527 ± 55
17	1876 ± 186	1553 ± 5	6833 ± 14	16767 ± 50	27029 ± 243

*Peak area at 450 nm given in arbitrary units (ca. 2200 area units = 10 mg/l bilirubin).

Sample stability

Although the sample preparation conditions given in the procedure did stabilize unconjugated bilirubin for 15—20 h, such was not the case for the con-

jugated forms, β and γ. Although the conditions chosen minimized changes in these components, some hydrolysis of γ to β and β to α was observed over 15 h and appeared to be sample or serum dependent. In most cases, this amounted to a change in peak area for individual peaks of less than 10%, with a lesser overall change in the total peak area because the areas are compensating, i.e., as the conjugates hydrolyze, unconjugated bilirubin (α) increases. The effect, then, appeared to be the result of gradual hydrolysis of esters in the sample, rather than oxidation, as the sample awaited injection.

Separation of sera

Fig. 5 shows chromatograms of three pathological sera. These demonstrate the wide variations in concentration of the four bilirubin fractions that have been observed in more than 200 different pathological sera chromatographed by this procedure. In general, without a prior knowledge of the disease state of the patient from whom the sera were drawn, no consistent pattern of the four species even at the same level of total bilirubin has been observed. Any of the four peaks has been found to be the greatest in area in a particular sample, with the exception of that due to the diconjugate (γ). At nominally normal values of total bilirubin ($\leq$ 10 mg/l), bilirubin has been present almost entirely as the unconjugated (α) form with perhaps a marginal amount of the δ-component present. These observations are in general agreement with the more complete investigations of Kuenzle and co-workers [7—12].

The present procedure is only qualitative, or semi-quantitative at best, because some yellow material is still retained with the proteins precipitated by the sodium sulfate treatment. Also, during processing of samples with total bilirubin levels >130 mg/l, a rather large and variable fraction of the total bilirubin, particularly the β- and γ-fractions, was lost with the precipitate. In some cases, as much as 60% was lost. However, with sera elevated to <130 mg/l, less yellow material was lost with the precipitated proteins and the recovery was

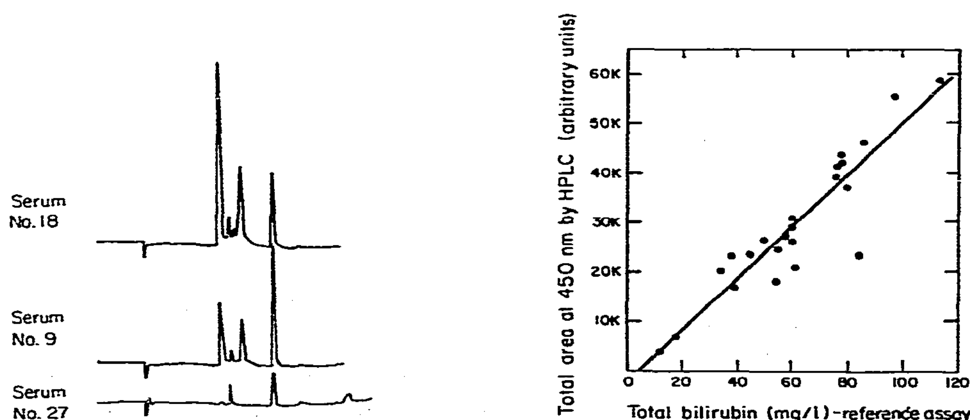


Fig. 5. Comparison of pathological sera.

Fig. 6. Correlation plot of HPLC area (450 nm) versus total bilirubin: r = 0.915 for all points by linear least squares; r = 0.960 if low point at 84 mg/l neglected.

less variable. This is indicated in Fig. 6, in which the total area of all bilirubin peaks separated by HPLC in a number of serum samples is plotted versus the total level of bilirubin as determined by the Jendrassik—Grof reference diazo procedure as modified by Doumas et al. [3]. There is a good correlation but not a predictive one because of the considerable amount of scatter. The scatter still seemed to be caused primarily by irregular losses in the precipitation step. Scatter is also expected because of the assumption, implicit in Fig. 6, that the extinction coefficients of all bilirubin species at 450 nm in the HPLC eluent are equal. This is most likely not true.

CONCLUSIONS

Four bilirubin fractions in serum and three in bile have been separated rapidly on a reproducible and stable reversed-phase chromatography system. The major advantages over the earlier, open-column procedure are the smaller sample size required (here 250 μl but potentially as little as 50 μl), the stability of the HPLC reversed-phase column and the relative speed of the analysis. The procedure yields semi-quantitative information on the concentration of the four species in serum and has demonstrated that they vary widely in different pathological sera.

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