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Optimization of a High-Performance Liquid Chromatography Method to Quantify Bilirubin and Separate It From Its Photoproducts

Effect of Column Length, pH, Mobile Phase Composition, and Flow Rate

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Abstract

A rapid reversed-phase (RP) high-performance liquid chromatography method for the isolation of bilirubin from its photoproducts (e.g., biliverdin) is reported. The method is based on isocratic elution using methanol:water as the mobile phase. A 2^4 full-factorial experimental design approach was adopted. For the optimization, the best separation was obtained using a flow rate of 1.50 mL/min, a mobile phase of 99:1 methanol:water (v/v) at pH 3.60, and a 150 × 4.6 mm id RP (C_{18}) column containing 5-µm particles. These conditions produced the fastest total retention time of 3.38 ± 0.055 min, and other chromatographic parameters were acceptable. Under the optimum conditions, a linear calibration curve for bilirubin was obtained over the 1.0–40.0 µg/L concentration range studied. The limit of quantification was 0.79 g/L and the limit of detection was 0.24 µg/L. Bilirubin in solution was monitored by ultraviolet detection at 450 nm.

Index Entries: Bilirubin; biliverdin; experimental design; reversed-phase chromatography; isocratic elution.

Introduction

Bilirubin is a molecule of great value for the diagnosis of liver diseases or a blocked bile duct *(1)*. Consequently, the isolation of bilirubin and its

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photoproducts for identification and preparative purposes is a continuous area of interest in clinical and biochemical research *(2)*. Bilirubin metabolism starts with the breakdown of red blood cells. The hemoglobin is broken down to heme and globin *(3)*. Heme is degraded by oxidation to produce IXa biliverdin *(4)*, which then undergoes reduction at the methylene group to form bilirubin Ixa *(3–5)*. Bilirubin is then carried by albumin in the blood to the liver *(5)*. In the liver, most of the bilirubin is conjugated to a glucuronide before it is excreted in the bile *(6)*. Conjugated bilirubin is called direct bilirubin and the unconjugated form is called indirect bilirubin. The total serum contains direct and indirect bilirubin *(6,7)*. Under physiologic conditions, bilirubin is present in small amounts in blood, but it is unconjugated in healthy serum *(5)*. Under pathologic conditions (when liver diseases develop), abnormal concentrations of bilirubin can be found in the serum owing to inefficient hepatic uptake, especially in diseases such as neonatal jaundice *(8)*. Bilirubin is excreted into bile after conjugation by the liver. Identification of bilirubin concentration is a vital issue for understanding the normal path of liver malfunctions *(2,9)*. Bilirubin and biliverdin isomers are useful in the characterization of biological species *(10)* and as structural probes in studies of the biochemical mechanisms of conversion and transport of heme and bile pigments *(10–12)*.

The simplest method for measurement of bilirubin involves the use of direct absorptiometry, in which the yellow color of bilirubin allows for absorption measurements to be made in the blue-green region of the visible spectrum *(2,13)*. The conjugated bilirubin species in human bile have long been analyzed using thin-layer chromatography (TLC) *(14)*. TLC of bilirubin is generally performed on silica *(10)*. Over the last 20 years, several high-performance liquid chromatography (HPLC) methods have been reported for the isolation of bilirubin and biliverdin *(15–17)*. Ihara et al. *(17)* recommended HPLC analysis as a standard method for bilirubin measurement. These methods either have not been rapid or the mobile phase and the sample preparation have required special care *(18)*. HPLC not only provides exclusion of oxygen and light, but also offers higher sensitivity and speed of separation. Extensive research on the gradient elution HPLC separation of bilirubin and its photoproducts has been conducted. One limitation of gradient elution is the cost. The instruments used are more expensive than those of isocratic HPLC systems and, hence, less available in developing countries. Jianxin *(19)* developed an isocratic reversed-phase (RP)-HPLC method for the analysis of unconjugated bilirubin in serum, but the bilirubin eluted after about 13 min.

The origins of the present study are found in an earlier preliminary study *(20)*, in which the RP isocratic elution of bilirubin using a mobile phase based on methanol, water, and acetic acid was found to be feasible. The aim of this new study was to develop the earlier idea further and, ultimately, to find a simple and rapid HPLC procedure to isolate bilirubin from its photoproducts (e.g., biliverdin). To aid this endeavor, an experimental design approach was adopted. More specifically, a two-level, four-

factor (2^4) full-factorial design was used *(21)*. This type of design is both popular and simple. It requires relatively few runs and can indicate major trends.

Materials and Methods

Chemicals and Equipment

Reagent-grade NaOH was purchased from Scharlau (Barcelona, Spain). Methanol (HLC-SOL) was procured from Cica-Reagent (Tokyo, Japan). ExcelaR™ analytical reagent-grade acetic acid was obtained from Qualigens (Mumbai, India). Bilirubin was supplied by Fluka Chemie (Biochemica, Buchs, Switzerland). Millipore water was used throughout the study.

For the measuring of pH, a 320/SET meter was purchased from WTW (Weilheim, Germany), and a Jac ultrasonic bath was obtained from Labkorea (Incheon, Korea). A Millipore filtration unit was supplied by Waters (Milford, MA), and syringe filters were procured from Millipore (Type HA, 25-mm diameter).

The HPLC system contained a P580 high-precision pump from Dionex (Germering, Germany), which fulfilled the requirement for a measured delivery rate of the mobile phase. The injector was a Rheodyne (Cotati) 8125 valve fitted with a 20-µL loop. Two Waters C_{18} columns were used. One had a 250 × 4.6 mm id and the other a 150 × 4.6 mm id. Both columns contained 5-µm particles and were maintained at room temperature. After elution from the column, the eluent passed through a Dionex UVD170S diode array detector set at 450 nm for bilirubin. The output of the detector was monitored with a DTK Pentium III computer system. The mobile phase for the HPLC separation was prepared by mixing methanol with water at a known composition, then adjusting the pH to the desired value with acetic acid as described by Joseph and Palasota *(22)*. It was degassed in an ultrasonic bath, then passed through a Millipore filtration apparatus (type 0.22 µm). For chromatographic separation, 20-µL samples and standards were injected onto the chromatography column. Bilirubin and its photoproducts were eluted at a flow rate of either 1.00 or 1.50 mL/min.

Sample Preparation and Irradiation

For the optimization, a 40.0 µg/L aqueous bilirubin solution was made by weighing accurately 1 mg of bilirubin and dissolving in 25.00 mL of aqueous 0.1 *M* NaOH in the absence of light (only a small red lamp). For the production of the calibration curve, a series of more diluted samples were prepared as standards from the 40.0 µg/L solution. A fresh stock solution of 40.0 µg/L of bilirubin was prepared each time an experiment was conducted.

For the isolation of bilirubin from its photoproducts (e.g., biliverdin), 40.0 µg/L samples were prepared and irradiated for 1 h on the same day. Bilirubin solutions were placed in quartz cuvets, which were exposed at a

fixed geometry to the sunlight so that the light fell almost perpendicular to the center of the cuvets. The formation of photoproducts (e.g., biliverdin) was monitored chromatographically at 450 and 375 nm.

Results and Discussion

Standard solutions of pure bilirubin were injected to test the linearity of the calibration curve of peak height against bilirubin concentration in micrograms per liter. The curve was linear ($R = 0.9978$) from 1.0 to 40.0 μg/L of bilirubin, and the equation of the straight line was $y = 0.4148x + 0.8495$. The calibration curve was produced in duplicate with six data points per curve. The coefficient of variation (CV) for duplicate peak heights at a particular concentration was typically 4%. The limit of detection was 0.24 μg/L and the limit of quantification was 0.79 μg/L.

To develop the isocratic HPLC method, a 2^4 full-factorial experimental design approach was adopted. The four factors studied were length of the analytical column, pH, composition, and mobile phase flow rate. Each factor was tried at two levels; for example, mobile phase composition was 97:3 and 99:1 methanol:water (v/v).

Effect of Column Length

Two C_{18} columns of different dimensions were used: a 150 × 4.6 mm id column and a 250 × 4.6 mm id column. As expected, the shorter column produced the quickest bilirubin total retention times regardless of flow rate, mobile phase composition, and pH (Table 1). The use of the 2^4 full-factorial design showed that the column had the greatest effect on retention time of the four variables considered; of the four main effect estimates calculated 9.315 was the largest (Table 2).

Effect of pH

The effect of mobile phase pH was considered, because during some preliminary work it was found that at pH 6.50, no peak for bilirubin was observed. It was therefore suspected that pH affects the chromatography of bilirubin. To investigate this further, two pH values were considered: 2.90 and 3.60. Consequently, a peak for bilirubin was observed at pH 2.90 and pH 3.60. An explanation is that at acidic pH values (2.90 and 3.60), bilirubin should be protonated, but at the near-neutral pH of 6.50, protonation should be considerably reduced, affecting detectability. The use of the 2^4 full-factorial design showed that mobile phase pH had the least effect on total retention time of the four main effects considered; of the four main effect estimates obtained, –0.423 was the smallest (Table 2). In conclusion, either of the acidic pH values was acceptable for the separation of bilirubin. The quickest total retention times, however, were associated with pH 3.60 rather than pH 2.90 when other experimental conditions were constant (Table 1). Comparison of runs 14 and 16 illustrates this point. At pH 2.90 (run 14), the total retention time was 10.05 min, but at pH 3.60 (run 16) it was

Table 1
Summary of Total Retention Times
Obtained for 2^4 Full-Factorial Experimental Design

Run	Flow rate (mL/min)	pH	Methanol (%)	Column (mm × mm)	t_R (min)
1	1.00	2.90	97	150 × 4.6	6.27
2	1.50	2.90	97	150 × 4.6	4.14
3	1.00	3.60	97	150 × 4.6	5.93
4	1.50	3.60	97	150 × 4.6	4.30
5	1.00	2.90	99	150 × 4.6	4.95
6	1.50	2.90	99	150 × 4.6	3.79
7	1.00	3.60	99	150 × 4.6	5.43
8	1.50	3.60	99	150 × 4.6	3.41
9	1.00	2.90	97	250 × 4.6	20.18
10	1.50	2.90	97	250 × 4.6	13.09
11	1.00	3.60	97	250 × 4.6	19.02
12	1.50	3.60	97	250 × 4.6	12.09
13	1.00	2.90	99	250 × 4.6	14.70
14	1.50	2.90	99	250 × 4.6	10.05
15	1.00	3.60	99	250 × 4.6	14.33
16	1.50	3.60	99	250 × 4.6	9.28

Table 2
Summary of Calculated Estimates
of Main and Interactive Effects

Effect	Estimate
Main effects	
Flow rate, *F*	–3.83
pH, *P*	–0.423
% methanol, *M*	–2.38
Column, *C*	9.315
Two-factor interactions	
FP	–0.075
FM	0.6125
PM	0.163
FC	–2.10
PC	–0.403
MC	–1.62

9.28 min. Similar outcomes were observed for runs 1 and 3, runs 6 and 8, and runs 13 and 15.

Effect of Mobile Phase Composition

Mobile phase composition was changed from 97:3 to 99:1 methanol: water (v/v) during the study. As expected for a RP system, the separation

of bilirubin took longer when the water content of the mobile phase was increased (making the mobile phase more polar) from 1 to 3%. Ninety-nine percent methanol in the mobile phase produced the shortest total retention times compared with 97% methanol when the other experimental conditions were constant (Table 1). For example, the retention time achieved for run 1 (97% methanol) was more than for run 5 (99% methanol). The experimental design approach showed that the methanol:water composition of the mobile phase had a greater effect on total retention time than mobile phase pH (Table 2).

Effect of Flow Rate

Finally, mobile phase flow rate was considered. Two flow rates were used: 1.00 and 1.50 mL/min. Higher flow rates were impractical, because the instrument could not cope with the resultant higher pressure. As expected, the faster flow rate gave the shortest total retention times (Table 1). As an illustration, a total retention time for bilirubin of 4.14 min was achieved at 1.50 mL/min (run 2), but 6.27 min was obtained at 1.00 mL/min (run 1). The subtler experimental design approach, however, revealed that flow rate had a bigger effect on bilirubin retention time than mobile phase composition and pH, but was less important than the column (Table 2).

Figure 1 graphically represents the 2^4 factorial design. Two cubes are shown with annotated total retention times. The first one (Fig. 1A) represents a column length of 150 mm and the second one (Fig. 1B) is associated with a column length of 250 mm. This model essentially highlights the differences between the two columns and clearly demonstrates the efficacy of the shorter one.

Two-Factor Interactions

Of the six possible two-factor interactions, the interactive effect of the flow rate and column was the most important; *FC* gave the highest estimate (–2.10) of the two-factor interactions (Table 2). This was not surprising, because at lower flow rates the bilirubin would spend more time on the column with an increased chance of sorption. The interaction between the mobile phase composition and the column was almost as important (estimate: –1.62). As the percentage of water in the mobile phase increases, the bilirubin remains on the column longer. Consequently, there is more chance of attraction between the bilirubin and the column stationary phase. The least important two-factor interaction was between the flow rate and the pH (–0.075). This outcome was not surprising, because flow rate has no bearing on pH whatsoever.

Three- and four-factor interactions are possible, but they were assumed to be negligible *(21)* and not pursued further. The 16-run design was duplicated and similar results were obtained.

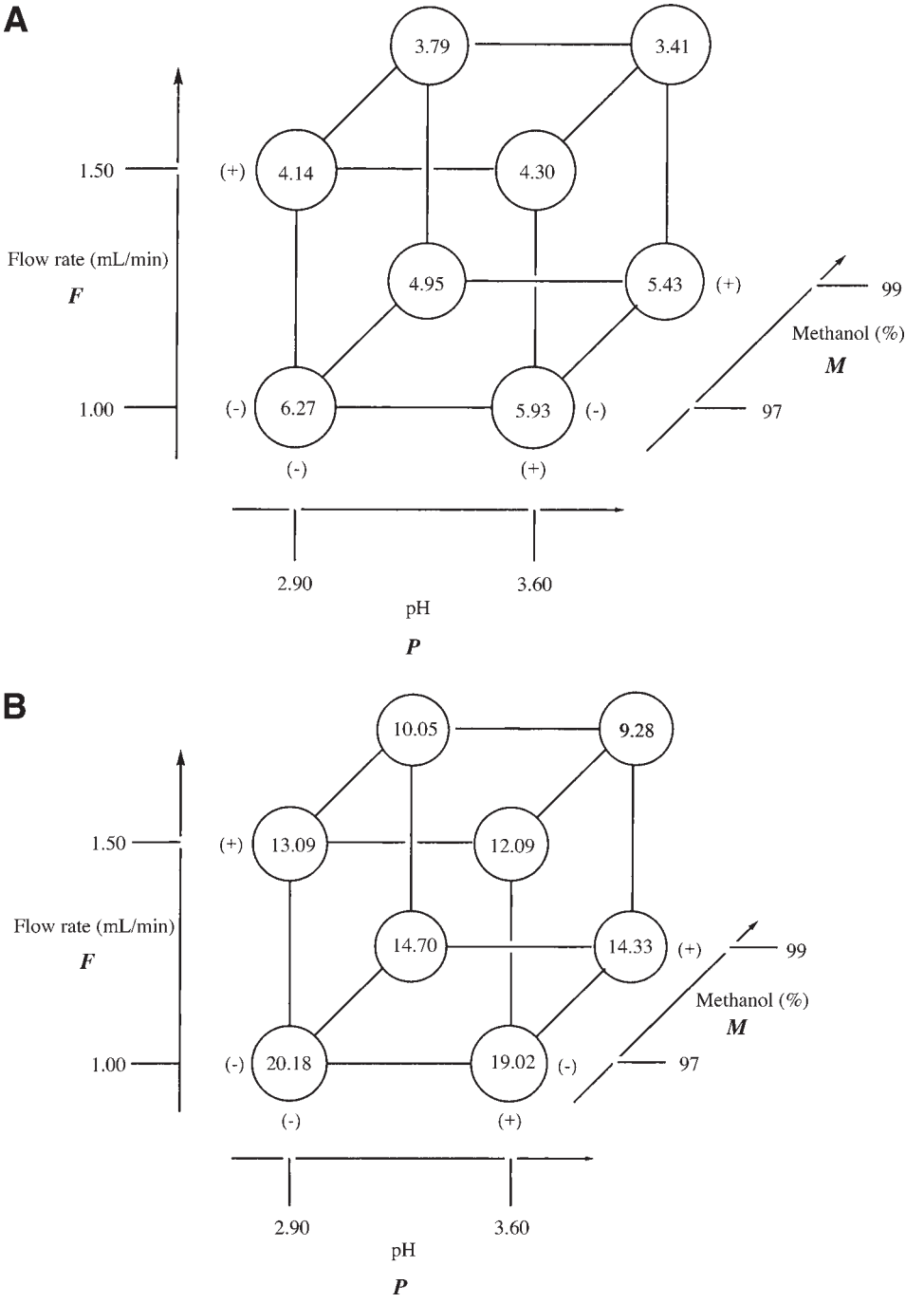

Fig. 1. Model featuring 2^4 factorial design representing column length of **(A)** 150 and **(B)** 250 mm. The respective total retention times are annotated on the two cubes.

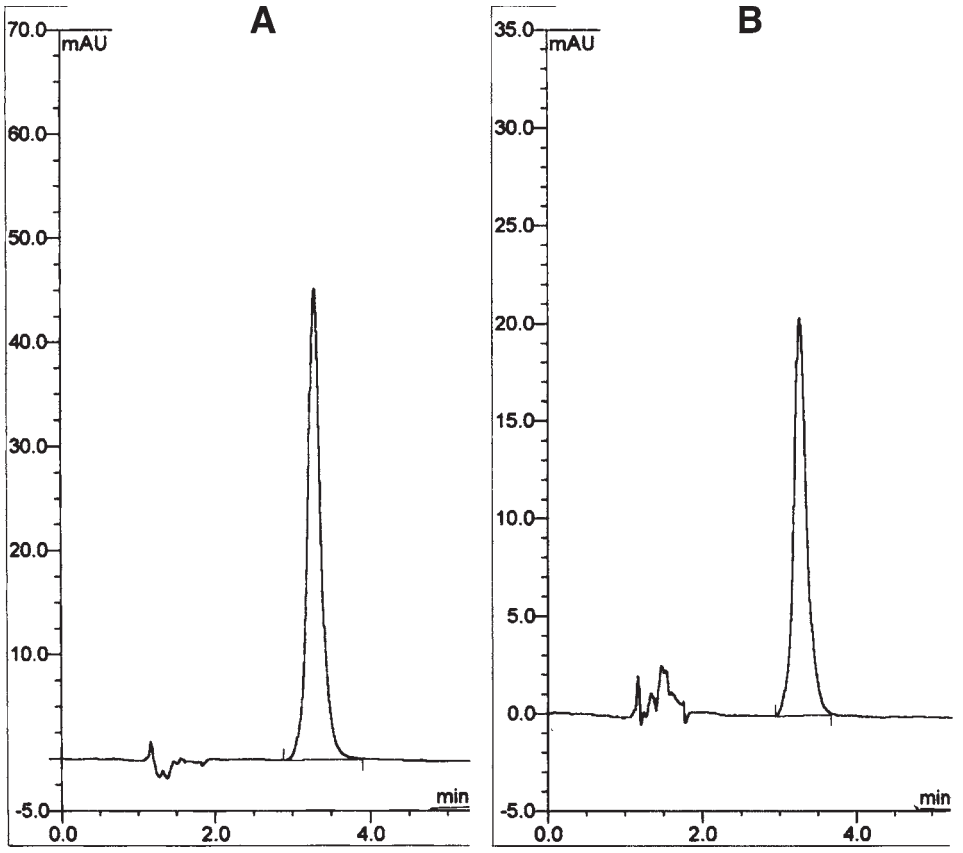

Fig. 2. Chromatogram of bilirubin obtained at 450 nm using run 8 experimental conditions **(A)** before irradiation with sunlight and **(B)** following irradiation for 1 h.

Optimum Conditions

Clearly, the set of experimental conditions used in run 8 (Table 1) gave the best (shortest) total retention time for bilirubin of 3.41 min (Fig. 2A). This was much faster than Jianxin's *(19)* isocratic method and the 15–20 min obtained by Kosaka et al. *(23)* using gradient elution. Total retention time, however, is not the only chromatographic parameter of importance; retention factor (k), number of plates (N), peak asymmetry (A_S), and resolution (R_S) are also of significance. Consequently, run 8 was repeated a further six times and k, N, and A_S data were obtained (Table 3). The precision of the total retention time data was acceptable; the CV was 1.6%. For k, the 1.32 obtained was within the 1–10 range given in the literature *(24)*. The average efficiency of the separation was 2053, which is adequate for routine work. Peak asymmetry was good; the 1.1 obtained was within the 0.9–1.2 quoted in the HPLC literature *(25)*. The bilirubin peak was well resolved ($R_S > 1.5$) from the photoproducts ($t_R < 2$ min) formed after irradiation of bilirubin for 1 h (Fig. 2B). The formation of the photoproducts is better illustrated at

Table 3
Summary of Mean, SD, and CV Data
of Four Chromatographic Parameters

Parameter	Mean	SD	CV (%)
t_R (min)	3.38	0.055	1.6
k	1.32	0.041	3.1
N	2053	8.8	0.43
A_S	1.1	0.082	7.5

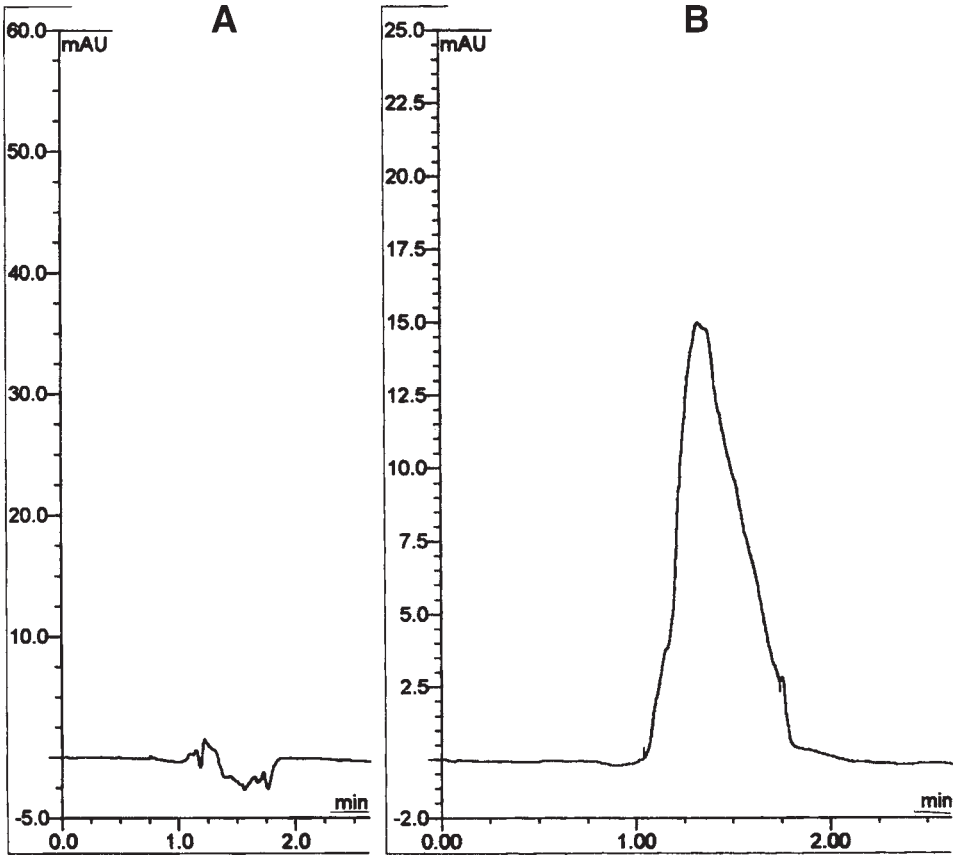

Fig. 3. Chromatogram of photoproducts obtained at 375 nm using run 8 experimental conditions **(A)** before irradiation with sunlight and **(B)** following irradiation for 1 h.

375 nm (Fig. 3). Little or no absorbance was seen before irradiation (Fig. 3A), but after 1 h of exposure to light, a much larger absorbance was observed at about 1.30 min (Fig. 3B). In summary, the best conditions tried were as follows: flow rate of 1.50 mL/min, mobile phase composition and pH of 99:1 methanol:water (v/v) at 3.60, and a 150 x 4.6 mm id C_{18} column.

A rapid method for the isolation of bilirubin from its photoproducts using isocratic RP-HPLC was developed. A simple 2^4 full-factorial experimental design was adopted to optimize the experimental conditions. More sophisticated approaches could have been tried, but their use would have been of little or no advantage; it would be difficult to improve on an average total retention time of 3.38 min with other chromatographic parameters acceptable. The degradation of bilirubin concentration could be monitored by this new method. The next step is to apply the optimized method to determine bilirubin extracted from serum.

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