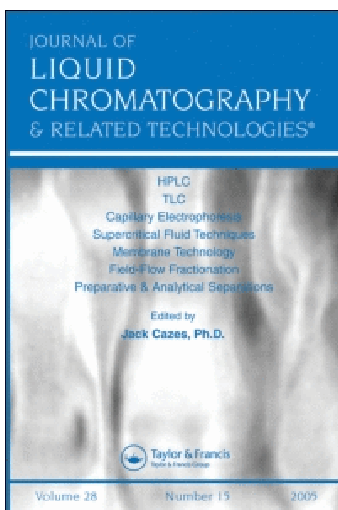
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Micellar High-Performance Liquid Chromatographic Separation of Serum Bilirubin Species with Direct Sample Injection

M. Lin[a]; N. Wu[bc]; J. H. Aiken[ad]
[a] Department of Chemistry State, University of New York at Binghamton Binghamton, New York [b] Department of Chemistry, University of Illinois at Urbana-Champaign Urbana, Illinois [c] A. L. Laboratories, Baltimore, MD [d] Purdue Research Center, Yonkers

To cite this Article Lin, M. , Wu, N. and Aiken, J. H.(1995) 'Micellar High-Performance Liquid Chromatographic Separation of Serum Bilirubin Species with Direct Sample Injection', Journal of Liquid Chromatography & Related Technologies, 18: 6, 1219 — 1229

To link to this Article: DOI: 10.1080/10826079508009286

URL: http://dx.doi.org/10.1080/10826079508009286

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(6), 1219-1229 (1995)

MICELLAR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SERUM BILIRUBIN SPECIES WITH DIRECT SAMPLE INJECTION

M. LIN[1], N. WU[2*], AND J. H. AIKEN[1**]

[1]*Department of Chemistry*
State University of New York at Binghamton
Binghamton, New York 13902
[2]*Department of Chemistry*
University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

Downloaded At: 07:37 25 January 2011

ABSTRACT

A rapid reversed-phase high performance liquid chromatographic method is described using a C_8 column and a mobile phase containing sodium dodecyl sulfate (SDS) as a modifier to separate the four bilirubin species present in serum: unconjugated, monoconjugated, diconjugated and biliprotein. The results show that using SDS as a mobile phase modifier improves separation efficiency and increases sample solubility. This simple HPLC procedure allows direct sample injection and makes it possible to quantitatively determine bilirubin species in biological fluids.

INTRODUCTION

The degradation of hemoproteins in humans and most mammals leads to the formation of bilirubin (1, 2). In normal serum, bilirubin is almost completely unconjugated as it is transported from the reticuloendothelial system to the liver, where conjugation of one (mono-conjugated) or

* Current Address: A. L. Laboratories, 333 Cassell Drive, Baltimore, MD 21224
** Current Address: Purdue Research Center, 99 Saw Mill River Road, Yonkers, NY 10701

Copyright © 1995 by Marcel Dekker, Inc.

both (di-conjugated) propionic acid side-chains with glycones occurs. In 1966, Kuenzle, et al. (3,4) first discovered that sera from adults with jaundice contained a fourth bilirubin species firmly bound to protein which was termed biliprotein.

The measurement of bilirubin and its metabolic forms has been recently reviewed by Doumas and Wu (5). In spite of extensive research that has been published on the assay of bilirubin, there is a lack of analytical methods which are capable of accurate, fast determination and quantitation of the metabolic forms of bilirubin in serum.

Currently, most clinical laboratories utilize methods based on coupling bilirubin with a suitable diazonium salt to form colored diazo derivative for the fractionation of "direct" reacting (β- and γ-fractions) and "indirect" reacting (α-fraction) bilirubins. However, the diazo reaction method is not very selective and is prone to interference problems. The δ-fraction and approximately 10-15% of the α-fraction can be direct reacting; therefore, the "direct" diazo reaction can overestimate the level of conjugated bilirubin.

Downloaded At: 07:37 25 January 2011

Since the early 1980s, HPLC has been utilized for quantitative fractionization of the four bilirubin species (6-8). The first HPLC method is reported by Lauff and co-workers (6), in this method, the serum is treated with saturated sodium sulfate to precipitate most globulins, but not albumin, and bilirubins are resolved on a reversed-phase column, eluting in order of decreasing polarity. The pretreatment of the serum may entail a variable loss of the biliprotein fraction.

Although high-performance liquid chromatography (HPLC) and anion-exchange chromatography (IEC) techniques have permitted the simultaneous resolution and quantitation of the bilirubin species present in the serum, the complexity of these HPLC or IEC methods have prevented their application for routine clinical use.

It is well known that reverse-phase HPLC columns are modified by the addition of alkyl sulfates commonly used as ion-pair reagents or micelle-forming agents. This modification has been shown to "protect" reverse-phase columns from the deleterious effects of repeated injection

of untreated protein-containing samples (9). Unprotected reverse-phase columns suffer severe efficiency loss, followed by plugging of the column frits or the packing material itself with proteinaceous material, usually in short order.

This effect of surface modification, along with the solubilization of serum proteins has been one of the most important aspects of micellar liquid chromatography (MLC) (10). In micellar solutions, there is an unchanging concentration of free surfactant (given by the critical micelle concentration) in the mobile phase. Since it is the free surfactant molecules which modify the reverse-phase column packing in MLC, gradients have been shown to be possible over wide surfactant concentration gradients (11). The purpose of this study is to investigate the feasibility of the using SDS micelles to enhance the selectivity of the reverse-phase packing material and improve the solubility of unconjugated bilirubin and serum proteins at physiological pH.

Downloaded At: 07:37 25 January 2011

EXPERIMENTAL

Materials

HPLC grade methanol, propanol, sodium phosphate monobasic, and phosphoric acid were from Fisher (Springfield, NJ, USA). Human serum albumin (fraction V), normal human serum and SDS were from Sigma (St. Louis, MO, USA). SDS was recrystallized from methanol before use.

Bilirubin Standards

Unconjugated bilirubin containing 9% XIIIα; 80% IXα and 11% IIIα (12) were from Porphyrin Products (Logan, UT, USA). Diconjugated bilirubin (bilirubin ditaurite·Na) was obtained from U.S. Biochemical (Cleveland, OH, USA). Unconjugated bilirubin was used as received since its extinction coefficient was in agreement with accepted value for pure pigment and its separation using high-performance thin-layer chromatograph (HPTLC) and micellar electrokinetic chromatography showed no detectable impurity. However, diconjugated bilirubin

was purified using HPTLC according to previously described procedures (7) since the electropherogram showed approximately 5% impurity. Monoconjugated bilirubin was extracted from rabbit bile using Eberlein's method (13) and further purified on HPTLC plates. Covalent complex of bilirubin and albumin (biliprotein) was synthesized from unconjugated bilirubin and Woodward's reagent K (*N*-ethylphenylisoxazolium-3'-sulfonate) according to the method of Kuenzle *et al.* (14).

Rabbit Bile and Human Serum Samples

Fresh bile from young rabbits was purchased from PEL-Freez Biologicals (Rogers, AR, USA) and pathological serum samples were obtained from three patients in Moses Taylor Hospital (Scranton, PA, USA). Bile and serum samples were kept frozen and stored in the dark before the experiments. To prepare spiked model serum samples, appropriate amounts of a mixture of the four bilirubin standards were dissolved in 20 mM phosphate buffer (pH = 7.0) solutions containing 6% human serum albumin. Serum samples were 1 to 3-fold (v/v) diluted with the mobile phase and then directly injected onto the column.

Downloaded At: 07:37 25 January 2011

Chromatographic Conditions

The HPLC system consisted of a Varian 5500 liquid chromatograph (Houston, TX, USA), a Model 7125 sample injector with 10 μl injection loop (Reodyne, Cotati, CA, USA), and a Spectra-100 UV-Vis detector (Spectra Physics, San Jose, CA, USA) equipped with 10 μl flow cell. The absorption wavelength was set at 450 nm for detecting bilirubin species. Chromatograms were recorded on a Hewlett-Packard Model 3390A integrator (Avondale, PA, USA). The analytical HPLC column was a 4 μm Waters Nova-Pak C_8 (3.9 x 150 mm) column (Waters Associates, Milford, MA, USA). This column was protected by a 30-mm refillable pellicular C_8 guard column (Alltech, Deerfield, IL, USA). Mobile phase A was prepared by adding appropriate amounts of SDS to 15% methanol : 85% 50 mM NaH_2PO_4, pH 7 to give 0 to 50 mM micellar solutions. Mobile phase B was 95% methanol : 5% H_2O. Solutions were degassed by sonication.

A linear gradient was applied from 100% eluent A to 30% eluent B in 5 min, followed by holding at 30% eluent B for 10 min, then returned to initial conditions in 2 min and finally re-equilibrated for 5 min. A total mobile phase flow rate of 1 ml/min was used at all times and retention times were measured from point of injection to the peak maxima on the chromatogram.

Recovery Studies

Appropriate volumes of standard solutions were added to normal human serum. These sample solutions of bilirubins in serum were then thermally equilibrated for 2 hours at 37^0C in the dark.

RESULTS AND DISCUSSION

It has been demonstrated that even in the presence of significant amounts of methanol, SDS will still allow the solubilization and elution of serum proteins with minimal damage to the chromatographic system (9). It is also possible to have gradients both with respect to SDS concentration and methanol concentration, and still allow reproducible separation of analytes of interest in untreated human serum samples (15).

Many of the interactions of analytes with micelles are based primarily on hydrophobic interactions between the analyte and the hydrophobic core of the micelle. Electrostatics also play a role, as oppositely charged analytes and micelles typically have higher degrees of association that do like-charged species. It has been shown that conjugated bilirubins have slightly more affinity for SDS micelles than free bilirubin (16). Protein-bound bilirubin would have the greatest interaction with SDS micelles due to the dissipation of the negative change of bilirubin by the protein and the solubilizing interaction of protein and SDS. The elution order of the bilirubin species as shown in Figure 1A is in agreement with these concepts. It was not observed that SDS affected the protein-bilirubin interaction, although it may prevent any further association of free-bilirubin with protein by competitive binding or slight denaturation.

Downloaded At: 07:37 25 January 2011

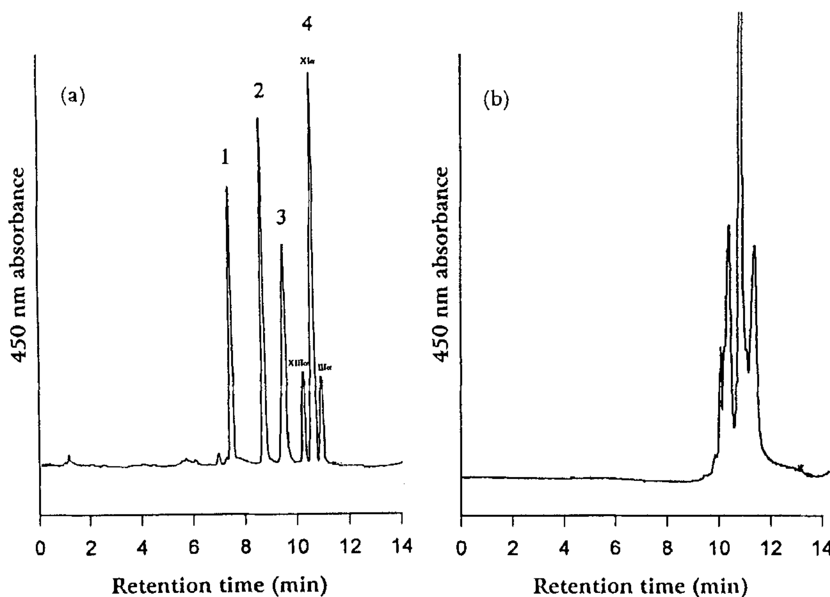


Figure 1. Chromatograms of four bilirubin standards in presence of 3% human serum albumin. Peak Idntification: 1 = 6.1 μM biliprotein; 2 = 5.2 μM diconjugated bilirubin; 3 = 3.8 μM monoconjugated bilirubin and 4 = 11.3 μM unconjugated bilirubin. (a) eluent A contains 25 mM SDS and (b) 5 mM SDS.

Downloaded At: 07:37 25 January 2011

The separation is affected by the amount of surfactant in the mobile phase. The capacity factors of each of form of bilirubin increased with decreased concentration of SDS from 25 to 10 mM as demonstrated in Figure 1B. Figure 2 shows this effect more clearly with only free bilirubin, at an SDS concentration of 2mM, resolution of the unconjugated bilirubin isomers is completely lost.

Limits of Detection (LOD) and Linearity

For each individual bilirubin species investigated, a linear relationship was found between the amount of bilirubin injected and the corresponding peak area on the chromatogram.

Recovery

Recovery was based on a single point comparison of peak areas of bilirubin standards prepared in mobile phase with those prepared in 3% human serum albumin. Average recovery

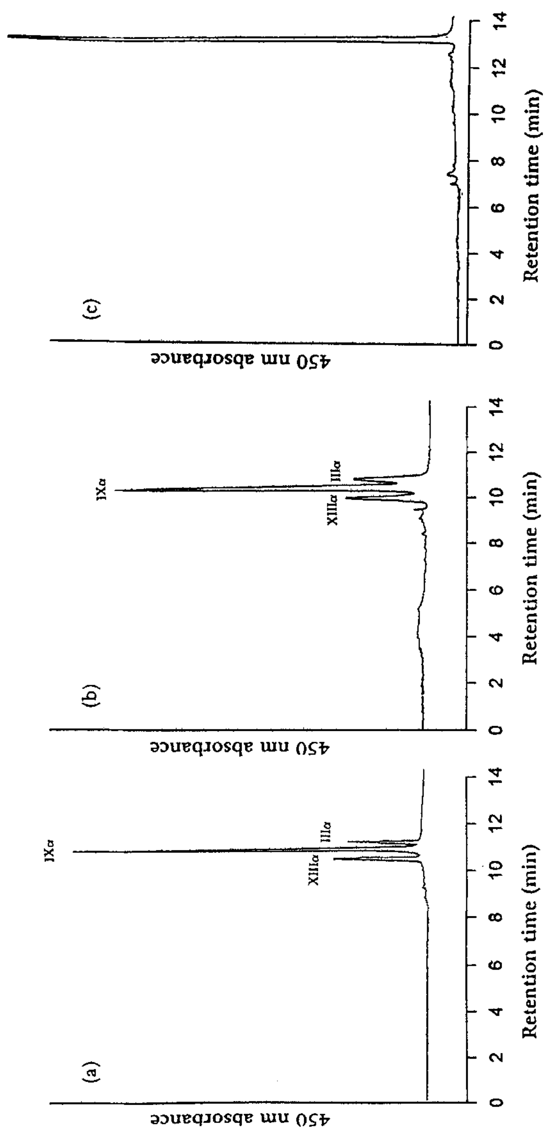


Figure 2. Chromatograms of 12 μM unconjugated bilirubin. Eluent A contains (a) 25 mM SDS, (b) 10 mM SDS and (c) 2 mM SDS.

Downloaded At: 07:37 25 January 2011

TABLE I

Detection Limits and Linearity

Bilirubin	Detection limit (nM)	Linearity Upper limit (μM)	*r*
unconjugated	33	0.85	0.996
monoconjugated	35	1.32	0.995
diconjugated	39	1.41	0.998
biliprotein	43	0.83	0.993

LOD based on S/N = 3 according to peak heights.

Linear regression constants determined from LODs up to the upper limits.

Downloaded At: 07:37 25 January 2011

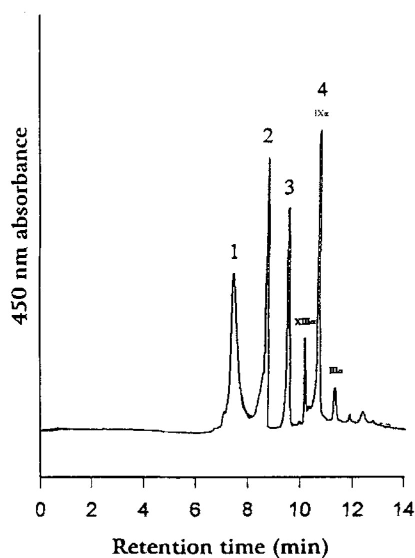


Figure 3. Chromatogram of four bilirubin standards spiked into normal human serum and after 2 hours of 37^{0}C incubation.

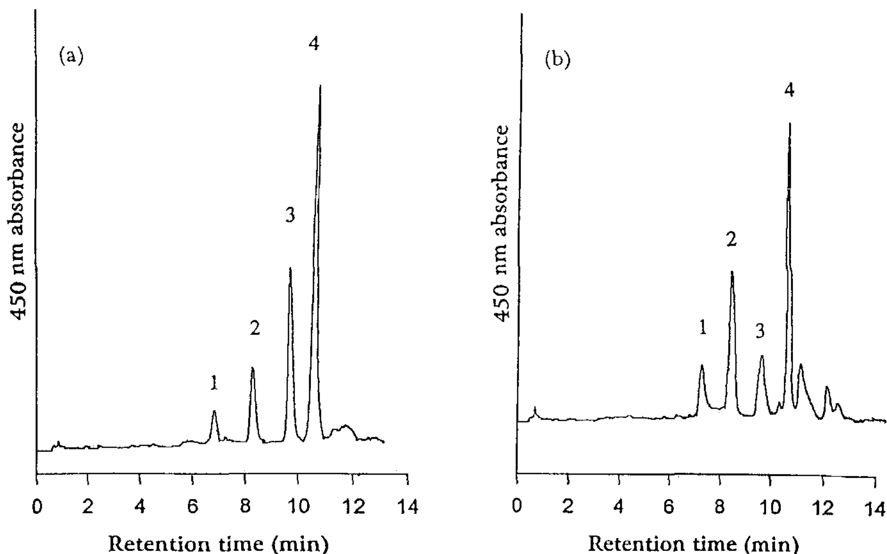


Figure 4. Chromatograms of patient serum samples from individuals with obstructive jaudice. See text for details.

Downloaded At: 07:37 25 January 2011

values for duplicate sample preparation was 96% or the mixture of unconjugated bilirubin, 98% for monoconjugated bilirubin, 92% or diconjugated bilirubin and 89% for biliprotein.

Reproducibility

At approximately 25 μmol of total bilirubins per liter, the relative standard deviation (RSD) of peak areas were less than 3% for within 24 hours, and $< 6\%$ for day-to-day analysis over a period of 7 days. At 0.5 to 1.0 μmol total bilirubins per liter, RSD $< 5\%$ and $< 8\%$ were found for within 24 hours and day-to-day (over 7 days) analysis, respectively.

Stability

Serum samples frozen at -60^{0}C are stable for at least 1 month and bilirubin residues obtained from extracts remain stable for at least a week when stored under argon at -20^{0}C.

Using mobile phase as mentioned earlier, with eluent A containing 25 mM SDS, the separation of bilirubin standard spiked into normal human serum is shown in Figure 3. Although

there is a slight loss of efficiency, probably due to interaction of bilirubin with other serum components, peaks are still well resolved. In cases of obstructive jaundice, where there is an obstruction of the normal hepatic bile flow, serum bilirubin is known to be elevated. Serum samples from patients with this affliction were chromatographed using this system. In Figure 4, the four principle types of bilirubin are easily detected and quantitated in these samples. In Figure 4a, a total bilirubin concentration of 110 μM/L (7.4 μM biliprotein, 12 μM diconjugated bilirubin; 29.3 μM monoconjugated and 62.1 uncojugated bilirubinun) and 4b total bilirubin 102 μM/L (13.5, 26, 9 and 53.5 μM, respectively).

In conclusion, we have demonstrated a simple HPLC procedure which makes possible the quantitative determination of the major bilirubin species in serum. This method makes use of the solubilizing power of sodium dodecyl sulfate micelles to allow direct sample injection without modification of the state of conjugation of bilirubin and minimal damage to the analytical column.

Downloaded At: 07:37 25 January 2011

ACKNOWLEDGEMENTS

We thank Mrs. Janice O. Schmitt at Moses Taylor Hospital (Scranton, PA) for supplying us the pathological human serum samples used in this study and Professor Richard A. Hartwick for the use of HPLC equipment.

REFERENCES

1. J. R. Chowdhury, A. W. Wolkoff and I. M. Arias, in C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle Editors., The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 6th edition, 1989, p. 1367.

2. T. W. Wu, G. A. Levy, S. Yin, J. X. Au, P. D. Greig, S. M. Strosberg, M. Ettles, M. Abecassis, R.A. Superina, B. Langer, L. M. Blendis, M. J. Philips and B. R. Taylor, Clin. Chem., 36 9 (1990)

3. C. C. Kuenzle, M. Sommerhalder, J. R. Ruttner and C. Maier, J. Lab. Clin. Med., 67 282 (1966)

4. C. C. Kuenzle and J. R. Rutter, J. Lab. Clin. Med., 67 294 (1966)

5. B. T. Doumas and T. W. Wu, Critical Rev. in Clin. Lab. Sci., 28 415 (1991)

6. J. J. Lauff, M. E. Kasper, T. W. Wu and R. T. Ambrose, Clin. Chem., 28 629 (1982)

7. N. Blanckaert, Biochem. J., 185 115 (1980)

8. N. Blanckaert, P. M. Kabra, F. A. Farina, B. E. Stafford and R. A. Weisiger, J. Lab. Clin. Med., 96 198 (1980)

9. R. Grohs, F. V. Warren and B. Bidlingmeyer, Anal. Chem., 63 384 (1991)

10. F. DeLuccia, M. Arunganart, L. J. Cline Love, Anal. Chem., 57 1564 (1985)

11. J. S. Landy and J. G. Dorsey, J. Chrom. Sci., 22 68 (1984)

12. C. A. Goresky and E. R. Gordon, Anal. Biochem., 183 269 (1989)

13. W. R. Eberlein, Pediatrics, 25 878 (1960)

14. C. C. Kuenzle, N. G. Cumarasamy and K. J. Wilson, J. Biochem., 251 801 (1976)

15. J. H. Aiken, J. A. Terzian and C. W. Huie, J. Chromatogr., 584 181 (1992)

16. J. H. Aiken and C. W. Huie, Anal. Lett., 24 167 (1991)

Received: October 18, 1994
Accepted: November 2, 1994

Downloaded At: 07:37 25 January 2011