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Enhanced separation and detection of serum bilirubin species by capillary electrophoresis using a mixed anionic surfactant–protein buffer system with laser-induced fluorescence detection

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Abstract

The four major bilirubin species in serum are separated by capillary electrophoresis and detected using laser-induced fluorescence detection. The optimum buffer system consists of 40 m*M* sodium dodecyl sulfate (SDS)–0.012 m*M* bovine serum albumin (BSA). The use of the SDS–BSA mixture in the mobile phase allows for the separation of four major bilirubin species at physiological pH with untreated capillaries. The results show that the use of BSA as a run buffer modifier in SDS solution improves separation efficiency and increases sample solubility via pH changes of the run buffer. The limits of detection for the bilirubin species using laser-induced fluorescence are between 30 and 150 n*M*, depending on the bilirubin species; not only is this approximately two orders of magnitude lower than with visible-light absorption methods, it allows the bilirubin species in normal sera to be quantitatively measured without sample pretreatment.

1. Introduction

Bilirubin, a metabolic breakdown product of blood heme, is a highly significant biological molecule which plays an important role in the understanding, diagnosis, and treatment of a variety of diseases associated with liver dysfunction [1]. Abnormal concentrations of bilirubin and its metabolic forms can be found in serum due to inefficient hepatic uptake or conjugation of bilirubin in diseases such as neonatal jaundice. Under normal conditions, bilirubin in serum exists almost completely (>90%) in its unconjugated form (α-fraction) which is a marker of efficient disposal of bilirubin conjugates from liver to bile. A fraction of the bilirubin is conjugated in the liver by esterification and excreted in bile as bilirubin monoester (β-fraction) or diester (γ-fraction), with glucuronic acid as the predominant ester group. The excess amounts of conjugated bilirubins appearing in serum indicate that certain liver diseases have developed, *e.g.* biliary obstruction. In addition to the three major chemical forms of bilirubin (α-, β- and γ- fractions), there also exists a fourth bilirubin form called the δ-fraction, which is bilirubin covalently bound to albumin in human serum [2].

The measurement of bilirubin and its metabolic forms has been recently reviewed by Doumas

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and Wu [3]. 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 of bilirubin and its metabolic forms in serum.

Currently, most clinical laboratories utilize methods based on coupling bilirubin with a suitable diazonium salt to form colored diazo derivatives 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 it is prone to interference problems because 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. 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 normal and pathological serum, the complexity of these HPLC or IEC methods have prevented their application for routine clinical use [4–6].

Wu *et al.* recently reported the application of a capillary electrophoresis (CE) method for the determination of serum bilirubin species using the anionic surfactant sodium dodecyl sulfate (SDS) in the run buffer to solubilize serum proteins with direct sample injection [7]. However, the detection limits of the visible light absorbance detector did not allow the determination of bilirubin species in normal serum. In this paper, the separation of the serum bilirubin species is enhanced using a mixture of anionic surfactant and protein.

Even more significantly, the use of laser-induced fluorescence (LIF) detection is described. Currently, LIF is the most sensitive detection method available for CE, with detection limits for several research instruments of under 1000 molecules under ideal situations [8,9]. However, relatively few biologically important molecules fluorescence, and so large research efforts are made to attaching fluorescent probes to the analytes of interest [9–12]. As bilirubin species are naturally fluorescent, fluorescence offers the potential for a sensitive and selective detection scheme without the need to modify the analyte. The limits of detection for all test bilirubin species are improved approximately two orders of magnitude over previous absorbance work using a commercially available CE–LIF system. The submicromolar detection limits of the LIF system allow quantitation of all major bilirubin species in normal serum.

2. Experimental

2.1. Apparatus

All CE experiments were performed on commercially available CE instruments: the visible absorbance measurements were made using a SpectraPhoresis 1000 (Spectra-Physics, San Jose, CA, USA) set at 450 nm. A P/ACE system 2100 (Beckman Instruments, Fullerton, CA, USA) was used for laser-induced fluorescence detection. The laser and filters used in the P/ACE were changed to allow the fluorescence detection of bilirubin species. Approximately 10 mW of the 457.9 nm emission line from an air-cooled argon ion laser (Omnichrome, Chino, CA, USA) was focused onto the 140-μm input fiber supplied with the P/ACE using a 25-mm focal length fused-silica lens (Newport Research Corporation, Fountain Valley, CA, USA). The 457 nm line was chosen as it is near the excitation maxima of the bilirubin species. The two emission filters used inside the P/ACE detector head were a 457-nm notch filter (Model Notch Plus, Kaiser Optical Systems, Ann Arbor, MI, USA) and the Beckman Instrument's stock 520-nm bandpass fluorescence filter. The electropherograms were obtained from the P/ACE and all data processing accomplished using System Gold software supplied with the P/ACE Model 2100.

2.2. CE conditions

The parameters employed for operation of the SpectraPhoresis 1000 instrument were as follows: the detector was set at 450 nm with a rise time of 0.3 s, injection was set at 10 V in the electrokinetic mode with a 6.0 s injection time, column

temperature was maintained at 20°C, voltage was at 18 kV and positive polarity. The run time was 15 min with a 65 mA current using 40 m*M* SDS and 10 m*M* borax buffer solutions. The capillary was washed between runs using 0.1 *M* NaOH for 2 min, with the borax buffer for 2 min, and then with the run buffer for another 2 min. For operation of the P/ACE system 2100 instrument the parameters were similar except that detection used LIF, and the washing cycle was reduced to 0.5 min for each step.

2.3. Capillary conditions

Untreated 57 cm × 75 μm I.D. fused-silica capillary tubes (Polymicro Technologies, Phoenix, AZ, USA) were used for all separations. New capillaries were treated by purging with 0.5 *M* NaOH for approximately 0.5 h and then filling the capillary with the run buffer for at least 10 h before use.

2.4. Chemicals and buffers

SDS, essentially fatty acid-free human and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA) and all other chemicals (except the bilirubin standards) were of analytical grade and purchased from Aldrich (Milwaukee, WI, USA) or Fisher (Springfield, NJ, USA). To prepare the run buffer, appropriate amounts of SDS were dissolved in 0.01 *M* sodium tetraborate (borax). Next H_3PO_4 buffer was added to obtain the appropriate pH values, and methanol, acetonitrile and BSA were added to obtain solutions of the desired concentrations. All solutions were filtered through a 0.2-μm membrane before use.

2.5. Bilirubin standards

Unconjugated bilirubin (bilirubin IXa) and diconjugated bilirubin (disodium bilirubin ditaurate) were obtained from U.S. Biochemical (Cleveland, OH, USA). Bilirubin ditaurate has similar spectroscopic, solubility and chromatographic properties as native glucuronides, and similar diazo reactivities [13,14]. For these reasons, and because it is commercially available, it is commonly used as a conjugated bilirubin standard. The individual monoconjugated and biliprotein standards were prepared and purified according to previously described procedures using these starting materials [7]. To prepare spiked model serum samples, appropriate amounts of a mixture of the four bilirubin standards were dissolved in 0.02 *M* phosphate buffer solutions containing 6% human serum albumin. Before direct injection of the model serum sample, a 1:1 (v/v) dilution with the run buffer was made for each sample.

2.6. Human serum sample

Normal serum samples were obtained from healthy adult donors and samples were kept frozen and stored in the dark before use. A 1:1 dilution with the run buffer was made for each sample just prior to injection.

3. Results and discussion

3.1. CE separation using a mixed SDS–BSA buffer system

Although all bilirubin standards are resolved in *ca.* 10 min using an alkaline SDS solution with acetonitrile and methanol as organic run buffer modifiers on a bare silica capillary (Fig. 1a), a greater difference in the mobilities of the bilirubin species is achieved using a mobile phase containing BSA and SDS at pH 7.4 (Fig. 1b). (For a description of the retention characteristic of micellar electrokinetic chromatography, the interested reader is referred to ref. 7). The peak shapes and widths are equivalent to those obtained with the SDS run buffer, but the selectivity improved. In addition, the solubility of the solutes increases as a result of the separation exhibiting an optimum pH approximately 1.6 units lower in the SDS–BSA buffer than in the SDS buffer. This may be significant because the nature of the bilirubin species present in biological samples can be better preserved at the physiological pH. The unconjugated bilirubin

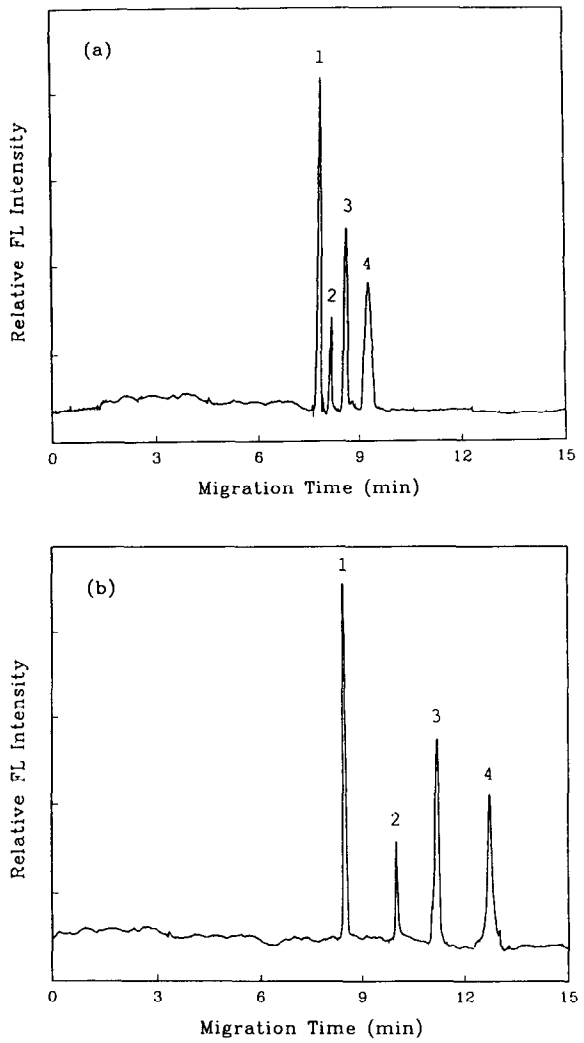

Fig. 1. (a) Electropherograms of four bilirubin standards in 10 m*M* (pH 9.0) buffer solution with 5% methanol, 5% acetonitrile and 40 m*M* SDS. (b) Electropherogram when the buffer consists of 40 m*M* SDS–0.012 m*M* BSA at pH = 7.4. Peaks: 1 = 6.9 μM unconjugated bilirubin, 2 = 1.0 μM monoconjugated bilirubin, 3 = 2.1 μM diconjugated bilirubin, and 4 = 0.6 μM biliprotein. Fluorescence detection: λ_{ex} = 457 nm.

molecule is highly non-polar and water insoluble in aqueous solution at pH values below 8.0 due to the intramolecular hydrogen bonding which twists the bilirubin molecule in such a way that all the polar head groups are buried inside the hydrophobic core of the molecule [15,16]. However, the SDS–BSA complex(es) provides sufficient solubility and selectivity for the separation of the four bilirubin standards. Because SDS is an efficient detergent for denaturing BSA, partially destroying its tertiary structure or folding structure [17], the tertiary structure of the protein may not be an important factor in the retention mechanism and BSA may act primarily as a supporting medium. The performance of separation is strongly influenced by pH.

The optimum pH is determined to be in the range of 7.2 to 7.6 and is dictated by the nature of the protein; the optimum pH is similar to that found in a previous study using BSA affinity CE [18]. The optimum concentration of BSA was in the range 0.010–0.015 m*M* with the 40 m*M* SDS solution, and the efficiency of the separation decreased with increasing concentrations of BSA. This may be due to excess protein adsorbed to the wall of the capillary.

3.2. LIF detection and quantitation

Capillary electrophoresis is a highly efficient technique for the fractionation of bilirubins. In previous work with absorption detection, LODs of approximately 6 μM were achieved. Therefore the visible-light absorption method is only suitable for the determination of the concentrations of bilirubin species in pathological sera that contain elevated bilirubin concentrations. Fluorescence methods offer a higher sensitivity as compared to absorption methods for the determination of molecules that have significant fluorescence quantum yields. The unconjugated bilirubin has a low fluorescence quantum yield in aqueous solution due to the efficient radiationless decay paths that return the excited molecule rapidly to its ground state [19]. Fortunately, the maximum fluorescence signal is significantly enhanced in the presence of micelles and albumin [16,20–22]. Table 1 shows that the average limit of detection is approximately two orders of magnitude lower in CE–LIF than in CE-absorption. Table 1 also lists the linearity data obtained for the bilirubin standards in model serum. The

Table 1
Analytical figures of merit on the determination of bilirubin species using visible absorption (Vis) and laser-induced fluorescence (LIF) methods

Bilirubin	Vis			LIF		
	LOD[a] (μM)	Linearity[b]		LOD[a] (nM)	Linearity[b]	
		Upper limits (μM)	Regression constant		Upper limits (μM)	Regression constant
Unconjugated	6.1	170	0.996	152	13.7	0.998
Monoconjugated	6.2	265	0.997	93.6	8.1	0.996
Diconjugated	5.9	280	0.995	86.8	7.5	0.997
Biliprotein	6.4	165	0.998	35.7	3.2	0.995

[a]Limits of detection (LOD) based on the peak height yielding three times the rms baseline noise.
[b]Linear regression constants determined from detection limits up to the amounts listed for the upper limits.

LODs are calculated using the peak height that produces 3 times the rms baseline noise. The LODs are reduced slightly in human serum compared to the model serum due to an increase in baseline noise. The LODs using the LIF method are in the 30–150 nM range which is adequate for the diagnosis of unconjugated and conjugated hyperbilirubinemia. Table 2 presents the relative standard deviations (R.S.D.s) calculated for migration times and peak areas of the four bilirubin standards spiked in model serum using SDS and the SDS–BSA buffer system. These data show that the precision of this method using an SDS–BSA buffer system is comparable to that obtained using the SDS–MEKC method.

3.3. Serum samples

Fig. 2 shows the electropherogram of a serum sample obtained from a healthy adult. The migration times of peaks 1–4 in the electropherogram show good correlation with those of bilirubin standards in model serum shown in Fig. 1b. As shown in a previous study [7], these results demonstrate that the bilirubin ditaurate standards have similar electrophoretic mobilities as the bilirubin diglucuronides. The electropho-

Table 2
Reproducibility of migration times and peak areas

Bilirubin	R.S.D. (%)			
	Migration time		Peak area	
	SDS	SDS–BSA	SDS	SDS–BSA
Unconjugated	0.24	0.29	3.12	2.98
Monoconjugated	0.23	0.24	2.76	3.24
Diconjugated	0.21	0.27	2.68	3.31
Biliprotein	0.35	0.32	2.96	2.87

Determined by sequential injections over *ca.* 1.5 h time period for $n = 5$.

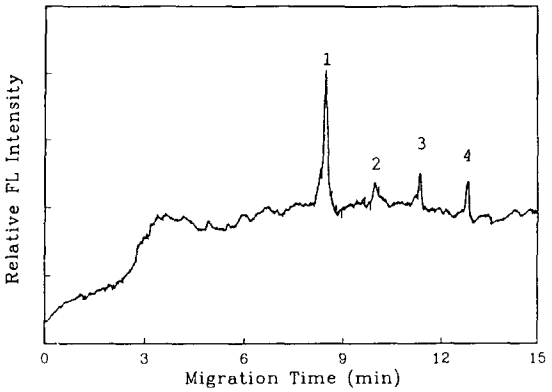

Fig. 2. CE separation of normal serum sample (1:1, v/v). Buffer and detection conditions are the same as in Fig. 1b.

retic mobility of the standard bilirubin ditaurate is within 2% of the mobility of the naturally occurring bilirubin diglucuronide.

The monoconjugated bilirubin is present as two isomers–the glucuronide group can attach to the C_8 or C_{12} carbon. Although many previous HPLC studies have not resolved these isomers [2,3,4,23], Li *et al.* report that the isomers are separated under acidic conditions, but the resolution of the isomers is difficult at pH > 6 [24]. As the resolution of these isomers has not been shown to be clinically important, no effort has been made to separate them in the present study.

Using calibration plots obtained from measurements of the peak areas of unconjugated bilirubin, diconjugated bilirubin and biliprotein standards, the concentrations of peaks 1, 2, 3 and 4 in Fig. 2 are calculated to be 3.6, 0.1, 0.2 and 0.1 μM, respectively. Importantly, these values in normal human serum are not detectable by visible-light absorption methods.

4. Conclusions

Due to the use of small samples, direct sample injection, instrumentation simplicity, and fast analysis, the use of capillary electrophoresis is a promising technique for laboratories requiring a simple, rapid and reliable method for fractionation and quantitation of bilirubin species in sera. Because of the relatively few fluorescent molecules that are found in human serum, this assay is greatly simplified by LIF. The detectability is significantly improved with LIF detection compared to detection by absorption; this increases the area where the CE method can be applied to both pathological and normal sera. The electrophoretic performance of the system can be improved by modification of the SDS run buffer with BSA. More research is necessary to understand the mechanisms by which the separation performance is effected by the anionic surfactant–protein system.

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6. References

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