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Separation of serum bilirubin species by micellar electrokinetic chromatography with direct sample injection

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ABSTRACT

Four major bilirubin species in serum were separated by micellar electrokinetic chromatography with 25 m*M* sodium dodecyl sulfate (SDS) and 20 m*M* sodium tetraborate–boric acid buffer at pH 8.5. Due to the solubilization of the serum proteins by the SDS micelles, serum samples were injected directly into a 50 cm × 75 μm I.D. fused-silica capillary and complete separation of the four bilirubin species was accomplished within *ca.* 10 min without extensive sample pretreatment. Detection was performed by absorbance at 450 nm and average limit of detection was in the 6.0 μ*M* concentration range. The usefulness of this method was demonstrated for the separation and detection of a number of bilirubin species present in pathological human serum samples.

INTRODUCTION

Measurement of bilirubin, a catabolic product of hemoglobin and heme proteins, is invaluable in the diagnosis of a variety of liver diseases [1]. Bilirubin is normally conjugated in the liver by esterification and excreted in bile as bilirubin monoester (β-fraction) and diester (γ-fraction), with glucuronic acid as the predominant ester group. In sera of healthy individuals, bilirubin exists almost completely (> 95%) in the unconjugated form (α-fraction), indicating efficient disposal of bilirubin conjugates from liver to bile. However, when certain liver diseases develop, *e.g.*, biliary obstruction, excess amounts of conjugated bilirubins appear in serum. On the other hand, abnormal concentrations of unconjugated bilirubin can be found in serum due to inefficient hepatic uptake or conjugation of bilirubin in diseases such as neonatal jaundice. In addition to the three major chemical forms of bilirubin (α, β- and γ-fractions), there exists a fourth form of bilirubin that is covalently bound to albumin (δ-fraction) in human serum [2]. Interestingly, it has been recently reported that concentration profiles of delta (δ) and conjugated bilirubins in serum could be used for identifying rejection in patients undergoing orthotropic liver transplant [3].

Most clinical laboratories utilize methods based on modifications of the traditional Jendrassik-Gróf diazo reaction for fractionation of "direct" reacting (β- and γ-fractions) and "indirect" reacting (α-fraction) bilirubins. However, since 10–15% of the α-fraction may be direct reacting, the direct diazo reaction could overestimate the level of conjugated bilirubin. Moreover, the albumin-bound fraction (δ) of serum also ex-

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hibits a direct diazo reaction, resulting in a possible overestimation of β- and γ-fractions [1].

To overcome some of these limitations of diazo methods, a number of high-performance liquid chromatographic (HPLC) techniques have been developed in recent years that are capable of accurate quantitation of individual bilirubin species in human serum [4–9]. However, HPLC methods for bilirubin analyses are currently too expensive and, since these methods always require some types of sample preparation procedures to remove serum proteins prior to chromatographic analyses, they are too elaborate and time-consuming for use in most routine clinical laboratories.

Capillary zone electrophoresis (CZE) has proved to be a highly efficient technique for the separation and determination of ionized substances with short analysis time and at low cost. By the introduction of surfactants above their critical micelle concentrations (CMC) in the run buffer while maintaining the same equipment design of CZE, *i.e.*, in an open-tubular capillary of very small internal diameter, a new type of separation method based on micellar partitioning of the solute and electrophoretic migration of the micelle — micellar electrokinetic chromatography (MEKC) — has been shown to be a powerful technique for the separation of electrically neutral molecules. Interestingly, the selectivity and peak shapes were found to be even better for the MEKC separation of some ionic substances as compared to CZE. Using MEKC, for example, separation of porphyrins [10], phenols [11], some amino acid derivatives [12,13], nucleosides [14] and drugs [15] have been reported. Importantly it has been demonstrated that plasma or serum proteins, which must be at least partially removed prior to HPLC analyses, were solubilized by the surfactants employed in MEKC, thus allowing for the determination of drugs in plasma by direct sample injection methods [16].

In this paper we describe the application of a MEKC method for the determination of serum bilirubin using the anionic surfactant–sodium dodecyl sulfate (SDS) in the run buffer to solubilize serum proteins. Using this method, a direct sample injection analysis of four bilirubin fractions can be performed without the use of any deproteinization or extraction procedures. Separation performances and detectabilities for the measurement of these individual bilirubin species were investigated using spiked model serum and pathological human serum samples.

EXPERIMENTAL

Apparatus

A commercially available capillary electrophoresis (CE) instrument (Model SpectraPHORESIS 1000, Spectra-Physics, San Jose, CA, USA) was used to perform both CZE and MEKC experiments with absorption detection at 450 or 280 nm. An untreated 50 cm × 75 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) was used for all separations and an integrator (Chromjet, Spectra-Physics) interfaced to the CE instrument was used for data processing.

Chemicals and buffers

SDS, Woodward's reagent K and essentially fatty acid-free human serum albumin were purchased from Sigma (St. Louis, MO, USA) and all other chemicals (except for bilirubin standards) were of analytical grade and purchased from Aldrich (Milwaukee, WI, USA) or Fisher (Springfield, NJ, USA). Buffer solutions used for performing CZE experiments were prepared by mixing appropriate volumes of 0.02 *M* sodium tetraborate (borax) and 0.1 *M* boric acid to give buffer solutions of appropriate pH values. Afterward, methanol was added to the run buffer to prepare working buffer solutions of appropriate concentrations. To prepare run buffer for MEKC experiments, appropriate amounts of SDS were added to the CZE run buffer solutions and filtered through an 0.2-μm membrane before methanol was added to give the buffer solutions of the desired concentration.

Bilirubin standards

Unconjugated bilirubin (bilirubin IXα) and diconjugated bilirubin (bilirubin ditaurite · Na)

were obtained from US Biochemical (Cleveland, OH, USA). Unconjugated bilirubin was used as received since its extinction coefficient was in agreement with the accepted value for pure pigment and its separation using high-performance thin-layer chromatograph (HPTLC) and MEKC showed no detectable impurity. However, diconjugated bilirubin was purified using HPTLC according to previously described procedures [17] since the electropherogram showed ~5% impurity. Monoconjugated bilirubin was extracted from rabbit bile using Eberlein's method [18] and further purified on HPTLC plates. A 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.* [19]. To prepare spiked model serum samples, appropriate amounts of a mixture of the four bilirubin standards were dissolved in 0.02 *M* borax 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.

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 six patients in Moses Taylor Hospital (Scranton, PA, USA). These bile and serum samples were kept frozen and stored in the dark before the experiments. Before direct injection of the samples, a 1:2 or 1:3 (v/v) dilution with the run buffer was made for each sample.

Capillary conditions

New capillaries were treated by purging with 0.5 *M* NaOH for about 1.5 h and then filling capillary with the run buffer for 12 h before use.

CE instrument conditions

The parameters employed for operation of the SpectraPHORESIS 1000 instrument were as follows: the detector was set for 450 or 280 nm with a rise time of 0.3 s, injection was set for vacuum at 2.0 s injection time, column temperature was maintained at 20°C, voltage was at 16 kV and polarity +. The run time was 15 min and the current draw was 45 μA using 25 m*M* SDS and 20 m*M* borax buffer solutions. Between runs washing of the capillary was performed with 0.1 *M* NaOH for 2 min and then with the run buffer for another 2 min.

RESULTS AND DISCUSSION

CZE of bilirubin standards

Fig. 1a and b show the electropherograms of unconjugated and diconjugated bilirubin standards, respectively, obtained by free solution, counter migration CE. It is important to note that the run buffer used for obtaining these two electropherograms contained 5% methanol. Without the methanol, asymmetric peaks were found for both unconjugated and disconjugated bilirubins. Interestingly, similar observations have also been reported for the CZE separation of hydrophobic porphyrin standards [10], *i.e.*, coproporphyrin and mesoporphyrin (cyclic tetrapyrroles), which possess similar structures as bilirubin ("linear" tetrapyrroles). It was suggested

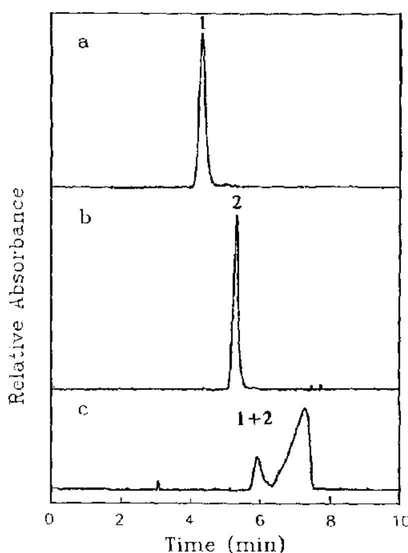

Fig. 1. CZE of (a) 35 μ*M* unconjugated bilirubin, (b) 39 μ*M* diconjugated bilirubin and (c) a mixture of 35 μ*M* unconjugated bilirubin (peak 1) and 39 μ*M* diconjugated bilirubin (peak 2). Buffer: 20 m*M* borax buffer of pH 8.5 with 5% methanol. Absorbance detection at 450 nm.

that poor solubilities of these hydrophobic porphyrins in the run buffer without the presence of methanol contribute to asymmmetric peak shapes and, furthermore, may mediate the adsorption of these porphyrins onto the capillary wall [10].

The diconjugated bilirubin standard appearing in Fig. 1b is a synthetic ditaurine derivative of unconjugated bilirubin. The chemical and physical properties of this compound, *e.g.*, diazo reactivity and chromatographic behaviors, have been shown to be very similar to those of the bilirubin diglucuronide — the predominant bilirubin diester excreted in human bile [20,21]. More importantly, since the molecular size and charge of this compound are also comparable to those of bilirubin diglucuronide, the electrophoretic mobilities of these two forms of diconjugated bilirubin should be quite similar, suggesting that bilirubin ditaurite should be suitable for use as a diconjugated bilirubin standard in CZE and MEKC analyses of serum bilirubins.

As shown in Fig. 1a and b, the diconjugated bilirubin migrated slightly slower than the unconjugated bilirubin towards the detector under identical electrophoretic conditions. This elution order could be explained in terms of charge and perhaps hydrophobicity and conformational differences between these two molecules. In CZE the electroosmotic flow directs the bulk flow of the buffer solution in the direction of the negative electrode towards the detector while the electrophoretic mobility directs the negatively charged solutes towards the positive electrode away from the detector. Thus, in general, the least negative charged solute will elute first, followed by solutes with increasing negative charge when the velocity of electroosmosis is larger than electrophoretic velocities of the solutes (counter migration mechanism).

In Fig. 1a and b the least negative charge solute appears to be the unconjugated bilirubin, which is not surprising since it is well known that this molecule is highly non-polar and water-insoluble in aqueous solution at pH below 8.0 [22,23]. Its non-polar characteristic has been explained in terms of the intramolecular hydrogen bonding that twist the bilirubin molecule in such a way that all the polar head groups are buried inside the hydrophobic core of the molecule. At pH 8.5, the molecule becomes more polar and water-soluble, indicating that the two carboxylic side groups within the molecule should be at least partially ionized and increasing its electrophoretic mobility. However, the hydrophobicity and conformation of unconjugated bilirubin may have a negative impact on its electrophoretic mobility, possibly counteracting the effect of increasing negative charge on the migration of this molecule towards the positive electrode away from the detector [24]. On the other hand, diconjugated bilirubin (in the form of ditaurite or diglucuronide) is polar and water-soluble over a wide range of pH due to lack of intrahydrogen bonding and the possession of two highly acidic moieties on the ditaurine or diglucuronic functional groups (doubly negative charge at pH 8.5), thus resulting in higher electrophoretic mobility towards the positive electrode but longer migration time towards the detector when compared to unconjugated bilirubin.

Although the difference in migration time between unconjugated bilirubin was not significant as shown in Fig. 1a and b, it appears that the separation of a mixture of approximately equal amounts of these bilirubin species into two well resolved peaks with relatively good efficiency and peak shape using CZE should be feasible. Surprisingly, Fig. 1c shows that this is not the case. The injection of a mixture of unconjugated and conjugated bilirubins into the CE instrument resulted in longer migration times and significant peak broadening and distortion for both bilirubin species. In an attempt to explain this phenomenon, it is helpful to note that rapid and reversible dimerization of bilirubin is known to occur at high concentrations [1]. Therefore, it is possible that under the influence of high electric field, there may exist strong interaction between unconjugated and diconjugated bilirubins in the run buffer, perhaps due to intermolecular hydrogen bonding between these two molecules. This aggregation effect may lead to an increase in the electrophoretic mobilities and decrease in the sol-

ubilities of the bilirubin species, resulting in longer migration times and perhaps in enhanced interactions of these species with the capillary wall, respectively.

MEKC of spiked model serum

Fig. 2 shows the separation of a model serum sample consisting of a mixture of the four bilirubin standards using run buffer containing SDS. It can be seen that the resolution and peak shape obtained for the separation of unconjugated and diconjugated bilirubins (peaks 1 and 3) are significantly improved over those obtained by CZE as shown in Fig. 1c. Also, the migration times of these peaks were found to be identical to those obtained from injecting model serum spiked with the individual bilirubin standard into the CE system. These results suggest that SDS was quite effective in minimizing aggregation that occurred between unconjugated and diconjugated bilirubins and possibly also reducing adsorption of bilirubins onto the capillary wall due to solubilization of the bilirubin molecules with SDS micelles [25] and binding of SDS onto electrostatic and hydrophobic sites on the capillary wall [10].

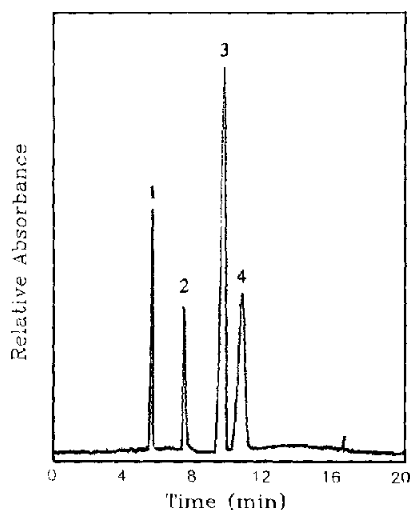

Fig. 2. MEKC separation of four bilirubin standards in the presence of 3% human serum albumin. Buffer: 25 mM SDS in 20 mM borax buffer of pH 8.5 with 3% methanol. Absorbance detection at 450 nm. Peaks: 1 = 41 μM unconjugated bilirubin; 2 = 25 μM monoconjugated bilirubin; 3 = 57 μM diconjugated bilirubin; 4 = 36 μM biliprotein.

The solubilization or interaction of SDS micelles with bilirubin could explain the increase in migration times found for both unconjugated and diconjugated bilirubins due to an increase in the negative charge of these micelle-bound bilirubin species and consequently retarding the counter migration of these species towards the detector. However, a larger change in migration time was obtained for diconjugated bilirubin as shown in Fig. 2. This may be due to the presence of a larger hydrophobic region (additional ditaurine functional groups) for interactions with SDS, thus increasing the negative charge and resulting in higher electrophoretic mobility when compared to unconjugated bilirubin.

As shown in Fig. 2, a relatively large difference in migration times between unconjugated and diconjugated bilirubin was obtained as a result of the use of SDS in the run buffer, allowing for another bilirubin species (peak 2) to be separated in this migration window. This particular bilirubin species was obtained from the bile of rabbit because it has been shown that monoconjugated bilirubin (β-fraction) in bile predominates in lower animals such as rabbit and decomposition profiles of this particular bilirubin fraction, along with γ- and α-fractions, were consistent with hydrolysis of esters [26,27]. Fig. 3 shows an electro-

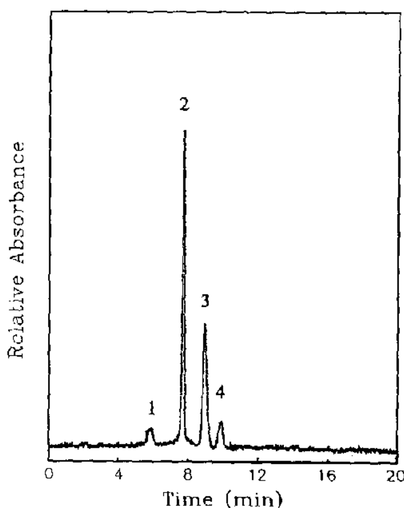

Fig. 3. MEKC separation of rabbit bile (1:3, v/v dilution). Buffer and detection conditions same as Fig. 2. Peaks: 1 = α-fraction; 2 = β-fraction; 3 = γ-fraction; 4 = δ-fraction.

pherogram of a rabbit sample using MEKC. It is clear that the predominant peak (peak 2) appears at a migration time of ~7.5 min and was, therefore, isolated for use as the monoconjugated bilirubin standard as shown in Fig. 2. Peaks 1, 3 and 4 in Fig. 3 were tentatively assigned as the α-, γ- and δ-fractions, respectively, since their migration times correlate with those obtained from the corresponding bilirubin standards as shown in Fig. 2. Also, the identities of these species were previously assigned as such in the reversed-phase HPLC separation of rabbit bile [27].

Peak 4 in Fig. 2 was identified as the bilirubin species covalently bound to human serum albumin (biliprotein), which was synthesized from standard procedures [19]. It can be seen that biliprotein has the highest electrophoretic mobility and eluted the latest among the four bilirubin standards separated by MEKC. This is reasonable since the electrophoretic mobility of biliprotein should be governed primarily by the relatively strong negative charge of albumin resulting from the solubilization with SDS, thus leading to a longer migration time. If this is the case, the migration time of biliprotein should be very similar to that of free human serum albumin bound to SDS alone. Indeed, Fig. 4 shows that the migration time of free human serum albumin in MEKC correlates with that of biliprotein as shown in Fig. 2, suggesting that biliprotein co-eluted with human serum albumin but did not appear to interfere with the separation of biliprotein from diconjugated or other bilirubin species present in the model serum samples.

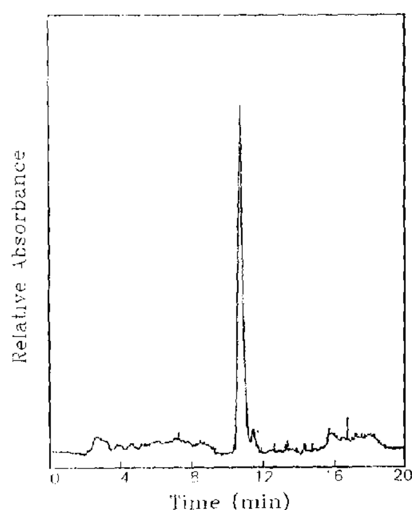

Fig. 4. MEKC of 3% human serum albumin. Buffer conditions same as Fig. 2. Absorbance detection at 280 nm.

The effect of SDS concentration on the migration of the four bilirubins standards is presented in Fig. 5. The migration times increase with increasing SDS concentrations for all the bilirubin species, which is consistent with trends observed for other ionic solutes solubilized or interacting with the SDS micelles [16]. The change in migration time of diconjugated bilirubin and biliprotein at higher SDS concentrations appear to be more pronounced as compared to unconjugated and monoconjugated bilirubins, suggesting the former species may have stronger interactions with the SDS micelles due to perhaps larger hydrophobic regions within these molecules.

Quantitation

Limits of detection and linearity data obtained for the determination of various bilirubin standards in model serum are listed in Table I. Since the normal concentrations of unconjugated bili-

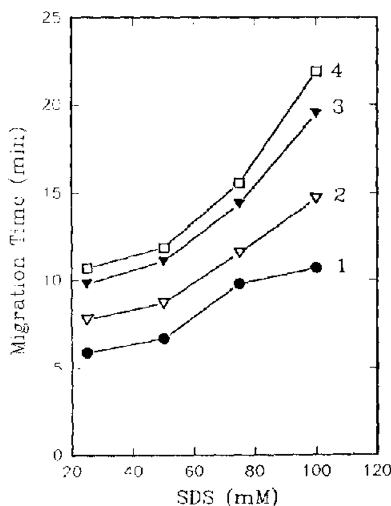

Fig. 5. Effect of SDS concentration on the migration time of four bilirubin standards: 1 = 41 μM unconjugated bilirubin; 2 = 25 μM monoconjugated bilirubin; 3 = 57 μM diconjugated bilirubin; 4 = 36 μM biliprotein. Buffer: 20 mM borax buffer of pH 8.5 with 3% methanol. Absorbance detection at 450 nm.

TABLE I

DETECTION LIMITS AND LINEARITY

Bilirubin	Detection limit[a] (μM)	Linearity	
		Upper limit (μM)	Linear regression constant[b]
Unconjugated	6.0	170	0.995
Diconjugated	5.8	284	0.993
Biliprotein	6.5	162	0.998

[a] Detection limits based on a signal-to-noise ratio of 3 according to peak heights.

[b] Linear regression constants determined from detection limits up to the amounts listed for the upper limits.

rubin in human sera are in the range 6–17 μM and for conjugated bilirubin in the range 0.1–0.2 μM [22], the detectabilities that can be achieved using the present method are adequate for the diagnosis of mild to acute cases of certain types of unconjugated and conjugated hyperbilirubinemia. Table II presents the relative standard deviation (R.S.D.) calculated for migration times and peak areas of the four bilirubin standards spiked in model serum and bilirubin species found in pathological human serum samples. These data show that the precision of this method is comparable to those obtained in other MEKC methods developed for the determinations of analytes in plasma or serum samples [16].

Recovery tests were performed by adding various bilirubin standards (20–100 μg/ml) into healthy adult sera and the individual bilirubin concentrations were determined by emplying MEKC. The average recovery (R) calculated for each bilirubin standard was as follows: unconjugated bilirubin (R = 101%, n = 5); diconjugated bilirubin (R = 98.6%, n = 5); and biliprotein (R = 94.3%, n = 4). These results indicate that good recovery can be obtained for unconjugated and conjugated bilirubins using the present method.

Patient samples

Fig. 6a and b show two MEKC electropherograms of serum samples obtained from patients with bone cancer and jaundice, respectively. Importantly, the migration times of peaks 1–4 in these electropherograms show good correlation with those of unconjugated and conjugated bilirubin standards spiked in model serum as shown in Fig. 2. Using calibration plots obtained from measuring peak areas of unconjugated bilirubin, diconjugated bilirubin and biliprotein standards, the concentrations of peaks 1–4 as appeared in Fig. 6a were calculated to be 46.7, 69.2, 14.5 and 8.1 μM, respectively, and in Fig. 6b the concentrations were 63.1, 132.2, 27.1 and 5.8 μM, respectively (concentrations of peak 2 in Fig. 6a and b were calculated using calibration plots of diconjugated bilirubin). These values are signif-

TABLE II

REPRODUCIBILITY OF MIGRATION TIMES AND PEAK AREAS

Determined by sequential injections over *ca.* 1.5 h time period for n = 4.

Bilirubin	R.S.D. (%)			
	Migration time (n = 4)		Peak area (n = 4)	
	Model serum	Patient serum	Model serum	Patient serum
Unconjugated	0.23	0.16	3.04	4.20
Monoconjugated	0.25	0.28	2.66	3.20
Diconjugated	0.18	0.25	2.48	3.50
Biliprotein	0.32	0.38	2.73	2.80

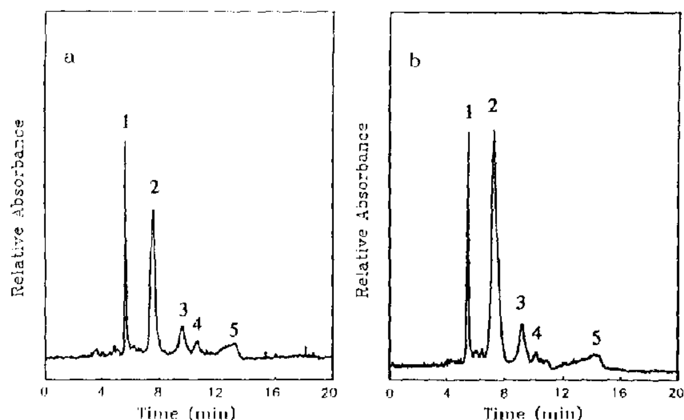

Fig. 6. MEKC separation of patient serum samples: (a) bone cancer (1:2, v/v dilution) and (b) jaundice (1:3, v/v dilution). Buffer and detection conditions same as Fig. 2.

icantly higher than those found in normal human serum [2], indicating that these patients may be suffering from some type of hemolytic disorders and/or hepatic dysfunction. An unknown asymmetric peak (peak 5) appears in both electropherograms at a migration time of ~12 min, which may be due to binding of small amounts of various bilirubin species adsorbed to serum proteins and/or the presence of some other protein-bound pigments which absorb at 450 nm.

In summary, we have demonstrated that MEKC can be successfully used for the separation of major bilirubin species present in serum with good selectivity and reproducibility. The major advantages over conventional HPLC methods are direct sample injection capability, instrumental simplicity, minimal sample usage, and lower cost of MEKC, suggesting it could be a practical method for the fractionation and measurement of serum bilirubin species in routine clinical laboratories. Further improvement in detectability of this method is necessary, which may be accomplished by employing multi-reflection absorption [28] or laser-induced fluorescence detection techniques [29].

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