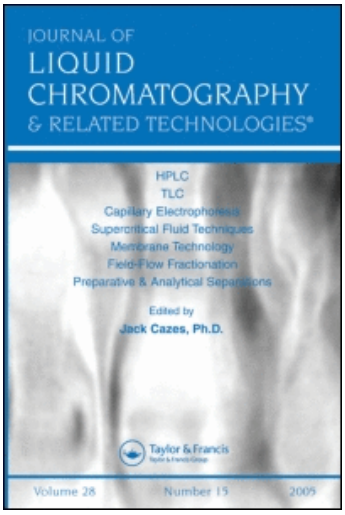

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Recent Developments in Porphyrin Separations Using Capillary Electrophoresis with Native Fluorescence Detection

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RECENT DEVELOPMENTS IN PORPHYRIN SEPARATIONS USING CAPILLARY ELECTROPHORESIS WITH NATIVE FLUORESCENCE DETECTION

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ABSTRACT

A mixed anionic surfactant buffer system is used in the micellar electrokinetic chromatography (MEKC) separation of porphyrin carboxylic acids as well as hematoporphyrin derivatives (HPD) at physiological pH with untreated capillaries. The resolution of type I II, III and IV isomers of coproporphyrin is achieved in bile salt micellar solution. The effects of altering the composition of sodium taurodexyocholate (TDC) and triton QS-15 (QS) on the separation efficiency of the porphyrins are presented. The results show that separation efficiencies are enhanced by using the mixture of bile salt and triton micelles. The limits of detection (LOD) for the porphyrin acids are significantly improved with laser-induced fluorescence (LIF) detection of the native porphyrin fluorescence to the 1 - 10 nM range.

INTRODUCTION

The separation and determination of tetrapyrrole molecules, such as porphyrins, is an interesting and important area for the application of capillary electrophoresis (CE). The determination of total content as well as individual component concentration of porphyrins in biological materials is important for the diagnosis of a family of disease known as porphyria [1, 2]. In addition, their use as therapeutic drugs has increased dramatically in the last decade [3]. The

MEKC separation of urinary porphyrins as well as hematoporphyrin derivatives has been reported previously [4-7]. The porphyrins can be separated by CE using a SDS-CAPS run buffer under rigorous pH conditions; the SDS acts more to compete with the samples for wall sites than as a means of enhancing selectivity of the system [4]. The purposes of this study are twofold: (1) to investigate the feasibility of the combining bile salt with anionic triton (QS) micelles to enhance the selectivity of MEKC and improve the solubility of selected porphyrins and (2), to improve the detectability by using laser-induced fluorescence of their native fluorescence.

Bile salts are biological surfactants possessing substituted steroidal structures which have been postulated to form rigid helical micellar aggregates with the hydrophobic portions of the monomer facing the aqueous solution while the hydrophilic portions turn inward [8, 9]. Bile salt micelles have been successfully applied to the resolution of optical isomers and hydrophobic compounds in HPLC and MEKC [10, 11]. The mixed bile salt and QS micellar system is necessary to improve the capability of bile salt micelle for separating negatively charged or highly polar species [12] and minimizing the phenomena of solute-wall interaction.

Currently, LIF is one of the most sensitive detection methods available for CE and detection limits under 1000 molecules have been reported [13, 14]. However, relatively few biologically important molecules fluorescence, and so large research efforts are involved with attaching fluorescent probes to the analytes of interest [15-17]. In an earlier paper, we reported using the LIF detection method for capillary electrophoretic separation of bilirubin species [18], a group of naturally fluorescent tetrapyrrole molecules with an open-ring porphyrin-like structure. Similarly, porphyrins also are naturally fluorescent. This offers the potential for a sensitive and selective detection scheme without the need to modify the analyte. The detection of the native fluorescence of porphyrins has been demonstrated previously using a Xe arc lamp at the absorption maxima of the porphyrins of 395 - 405 nm [4]. The absorption maximum of the porphyrins is caused by an intense Soret band around 400 nm, however, several smaller absorption bands exist. The second most intense band, the δ band,

absorbs in the 490 - 510 region, depending on porphyrin substituents and solution pH [19]. We report the detection of porphyrins using laser-induced fluorescence detection with the 488-nm Argon-ion line.

MATERIALS AND METHODS

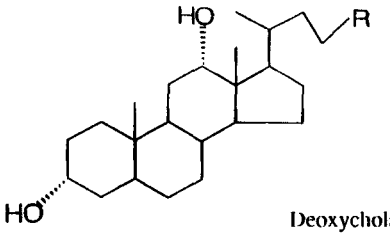
Chemicals Porphobilinogen, Hematoporphyrin, hematoporphyrin derivative, copro- (I and III), penta-, meso-, hexa-, hepta-, and uro- (I and III) porphyrin are from Porphyrin Products (Logan, Utah). A mixture of type I to IV isomers of coproporphyrin are prepared by previously described procedures [20, 21]. TDC (taurodeoxycholic acid, sodium salt), DCA (deoxycholic acid, sodium salt), SDS and triton QS-15 are from Sigma (St. Louis, MO). The structures of the porphyrin carboxylic acids and bile salts are shown in Table I. All other chemicals are of analytical grade from Fisher (Springfield, NJ) or Aldrich (Milwaukee, WI). The run buffer solutions are prepared in doubly deionized water and filtered through a 0.2 μm membrane.

Apparatus All CE experiments are performed on a commercially available LIF/CE instrument (P/ACE system 2100, Beckman Instruments, Fullerton, CA). Approximately 15 mW of the 488 nm emission line from an air-cooled argon ion laser (Omnichrome, Chino, CA) is focused onto the 140 μm input fiber supplied with the P/ACE using a 25 mm f.l. fused silica lens (Newport Research Corporation, Fountain Valley, CA). The emission filter used inside the P/ACE detector head is a 595-nm long-pass fluorescence filter (Omega Optical, Brattleboro, VT). The electropherograms are obtained from the P/ACE and all data processing accomplished using System Gold® software supplied with the P/ACE Model 2100.

CE Instrument Conditions The parameters employed for operation of the P/ACE 2100 instrument follow: the detector is set at LIF mode, injection is set at 8 kV for electrokinetic mode at 4.0 s

TABLE. I

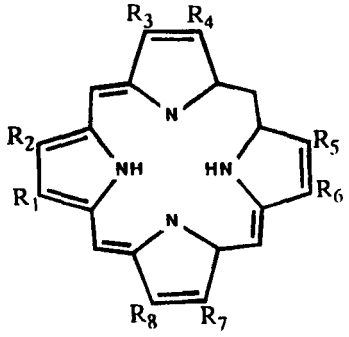
a) Structures of bile salts



Deoxycholate: R = COO^-

Taurodeoxycholate: R = $CONH(C_2H_4)SO_3$

b) Structures of Porphyrin



#	Porphyrin (isomer I)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	Coproporphyrin	M	P	M	P	M	P	M	P
2	Pentaporphyrin	M	P	M	P	A	P	P	M
3	Mesoporphyrin	M	E	M	E	M	P	P	M
4	Hexaporphyrin	M	P	M	P	A	P	P	A
5	Heptaporphyrin	M	P	A	P	A	P	A	P
6	Uroporphyrin	A	P	A	P	A	P	A	P

Substituents: M = $-CH_3$; E = $-C_2H_5$; A = $-CH_2COOH$; P = $-CH_2CH_2COOH$.

injection time, column temperature is maintained at 20° C, voltage is set to 16 kV with a positive polarity. The run time is 25 min and the current draw is 85 μA using 60-mM DCA and 10-mM borax buffer solutions. The capillary is washed between runs with 0.1 M NaOH for 0.5 min, followed by a rinse with the borax buffer for 0.5 min, and then with the run buffer for another 0.5 min.

Capillary Conditions Untreated 55-cm x 75-μm I.D. fused-silica capillary tubes (Polymicro Technologies, Phoenix, AZ) are used for all separations. New capillaries are conditioned by purging with 0.5-M NaOH for about 0.5 h and then filling capillary with the run buffer for 10 h before use.

RESULTS AND DISCUSSION

Figure 1 shows the electropherograms of six porphyrins that have three to eight carboxylic acid side-chains in TDC, QS and a mixture of TDC and QS micellar solutions. As shown in Figure 1(a) and 1(b), attempts to separate the mixture of six porphyrins have been unsuccessful using TDC or QS micelle alone in the running buffer solutions. Because of the hydrophobicity of the bile salt and triton, these micelles have poor retentivity for hydrophilic molecules such as uro-porphyrin. Figure 1(c) shows the separation of the six porphyrins with excellent efficiency using the mobile phase containing triton QS-15 (a sulfonated anionic surfactant) and sodium taurodeoxycholate at pH 7.4 in a bare silica capillary. As shown in Figure 1 (c), three peaks are observed for hexacarboxyl-porphyrin in the mixed micellar solution. Similar results are also obtained by Weinberger et. al [4] who suggests that these additional peaks arise from geometrical isomers of hexacarboxyl-porphyrin produced during the manufacturing process. In an attempt to understand the mechanisms that contribute to the successful separation of various hydrophilic and hydrophobic porphyrins as shown in Figure 1 (c), the retention behavior in the TDC-QS micellar solution needs to be better understood. The major interactions between bile salt and QS are thought to be the Van der Waals

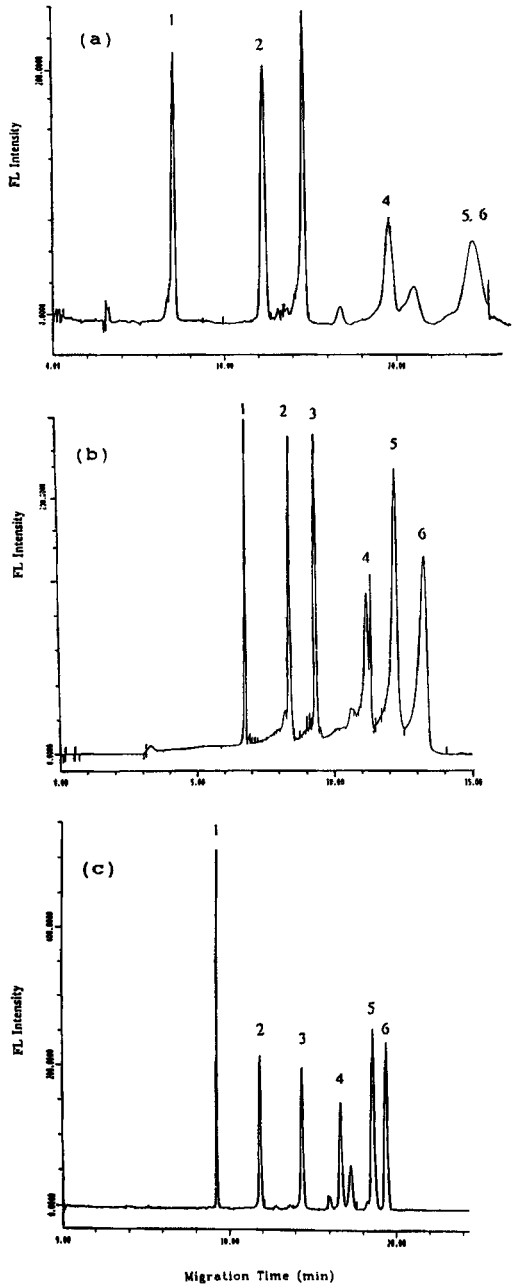


Figure 1. Electropherograms of six porphyrin acid standards (2 μM each) in (a) 60 mM DCA, 15% acetonitrile (v/v), 10 mM sodium borate buffer (pH = 9), (b) 0.5% QS (w/v), 15% acetonitrile, 10 mM sodium borate buffer (pH = 9.0) and (c) 60 mM TDC, 0.2% QS, 15% acetonitrile, 10 mM H_3PO_4 buffer (pH = 7.4). Peaks: 1. copro-, 2. pentacarboxyl-, 3. meso-, 4. hexacarboxyl-, 5.heptacarboxyl- and 6. uro-porphyrin.

forces acting through the hydrophobic face of the bile salt molecule and hydrophobic polyoxyethylene backbone of triton. Although there is little direct evidence, the hydrophobic lateral external surface of the bile salt micelle can be adsorbed onto the QS phase by hydrophobic interactions while maintaining its micellar structure. This suggests that the retention behavior characteristic of electrokinetic chromatography has been maintained in the mixed micelle solution formed in the system. Therefore, it is possible that specific TDC-QS complex(es) may exist in the run buffer and that these provide solubility and selectivity for the separation of the six porphyrin standards with good efficiency and resolution at physiological pH. A possible explanation for the distorted peak shapes for heptacarboxyl-, and uro-porphyrins as shown in Figure 1 (a) and (b) may be attributed to the adsorption of the porphyrins at the capillary wall.

The average of concentration limits of detection (LOD) for the porphyrin acids shown in Figure 1 is ~ 4 nM using the commercially available CE/LIF system. This is very close to the concentration limits of detection reported for HPLC/FL and ~ 50-fold better than previous MEKC LODs [4]. The mass limits of detection are ~ 10 amol, approximately 40-fold lower than previously reported fluorescence method [4]. These improved LODs make detection of nM concentrations of porphyrins in biological sample systems possible; i. e., the total amount of uroporphyrin in normal urine specimens is in a range of 5 - 35 nM [19].

The simultaneous separation of copro-porphyrin type I to IV isomers using MEKC has not been described before. The analysis and separation of pure isomers is important in the fields of porphyrin chemistry and biochemistry. The separation of a mixture of synthetic copro-porphyrin I to IV isomers is achieved with good efficiency using the mobile phase containing 60 mM sodium deoxycholate (DCA) and acetonitrile at pH 9 (Figure 2). The attempt to separate uro-porphyrin isomers of type I to IV is unsuccessful using this method, presumably due to their hydrophilic nature.

Figure 3 shows the electropherograms of the separation for hematoporphyrin derivative (HPD), a clinically important group of molecules for photodynamic therapy [3]. As shown in Figure

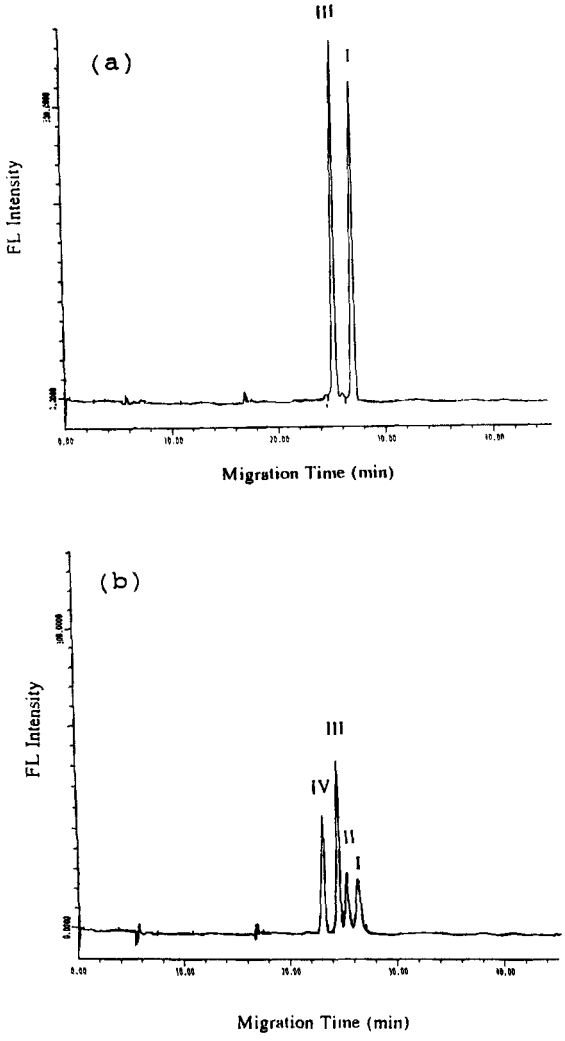


Figure 2. Electropherograms of (a) coproporphyrin III (1.9 μM) and I (1.8 μM) isomers (commercially available standards); (b) synthetic coproporphyrin IV, III, II and I (2.5 μMtotal) isomers in 60 mM DCA-15% acetonitrile-10 mM sodium borate buffer (pH = 9.0).

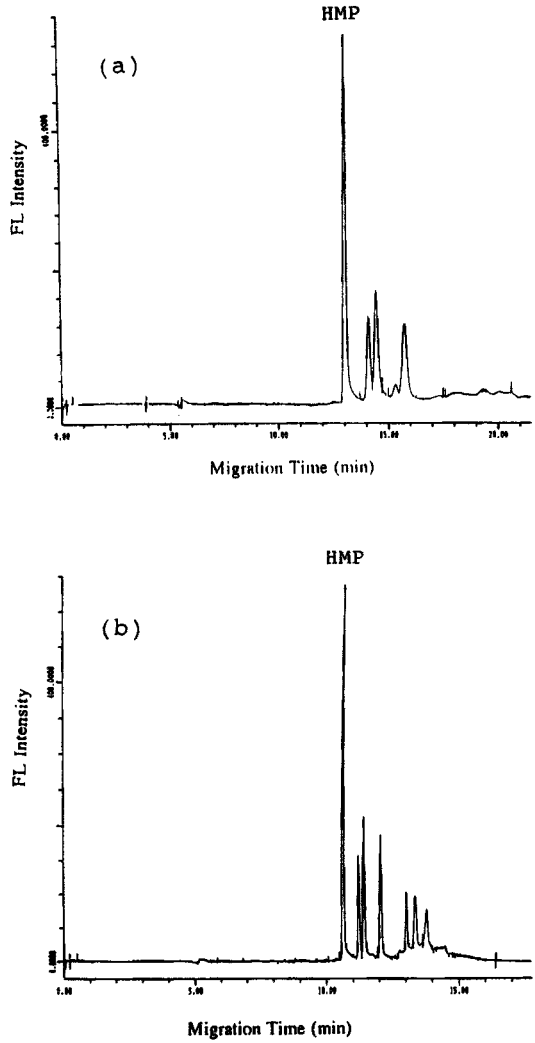


Figure 3. Electropherograms of hematoporphyrin derivative (25 μg / ml) in (a) 50 mM SDS-10% acetonitrile-10 mM sodium borate buffer (pH = 9.0) and (b) 60 mM TDC - 0.2% QS-10% acetonitrile-10 mM H_3PO_4 buffer (pH = 7.4). Peak "HMP" is referred to the migration times of hematoporphyrin standard under the same experimental conditions.

3 (b), there are seven peaks observed using a mobile phase containing a mixture of TDC-QS micelles but only four peaks are observed in an SDS micellar solution (Figure 3 (a)) under similar conditions as previously reported [5]. Although we are unable to identify the additional peaks because the precise compositions and structures of HPD are not known with certainty, the MEKC method has been shown to be a very efficient separation technique to assist in the search for the critical photosensitizing species of hematoporphyrin derivatives.

In summary, we have demonstrated the use of a mixture of anionic surfactant buffer additives in capillary electrophoresis that allows for the separation of various hydrophobic and hydrophilic porphyrins and the hematoporphyrin derivative as well as the simultaneous resolution of several clinically important geometrical porphyrin isomers using untreated capillaries. Using small amounts of an anionic triton micelle as a run buffer modifier in bile salt micellar electrokinetic chromatography can significantly improve separation efficiency and increase sample solubility. More research is necessary to elucidate the mechanisms in which separation performance is improved by various ionic surfactant-surfactant systems; in particular, the separation efficiency and the retention behavior in the TDC - QS solution will be investigated in future studies.

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