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Separation of porphyrins and porphyrin isomers in capillary electrophoresis using mixed ionic surfactant–bovine serum albumin buffer systems

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ABSTRACT

The separation of six porphyrins having two to eight carboxylic acid side-chains by capillary electrophoresis using a combination of ionic surfactant and protein as a novel modifier in the run buffer is reported. Using sodium dodecyl sulfate (SDS) together with bovine serum albumin (BSA) as buffer additives, efficient and reproducible separation of mesoporphyrin, coproporphyrin, uroporphyrin, penta-, hexa- and heptacarboxylporphyrins was achieved at pH 7.4 whereas sodium taurodeoxycholate (bile salt) combined with BSA in the run buffer separated type I and III isomers of coproporphyrin as well. The presence of SDS or bile salt appeared to minimize protein–and/or porphyrins–inner capillary wall (untreated silica) interaction and to enhance solubilization and selectivity of porphyrins due to the formation of ionic surfactant–protein complex(es) in the run buffer.

INTRODUCTION

Capillary zone electrophoresis (CZE) is an efficient technique for the separation of proteins [1]. On the other hand, the feasibility of adding proteins in the run buffer as stereoselective complexing agents to effect chiral separations in CZE has also been demonstrated [2–4]. A major problem associated with protein separations or the use of proteins as buffer modifiers in CZE is the inherent tendency of these macromolecules to interact with the wall of the fused-silica capillary [5], resulting in the loss of separation efficiency and reproducibility. A number of experimental approaches have been devised to minimize this type of interaction, including the use of buffers with a pH either substantially higher [6] or lower [7] than the isoelectric points of the proteins, the addition of high concentration of salts in the run buffer [8] and the covalent attachment of specific coatings to the capillary wall [3].

The introduction of an ionic surfactant in the run buffer at a concentration above the critical micelle concentration (CMC) has given rise to the development of a special form of CZE —micellar electrokinetic capillary chromatography (MECC)— in which separation of neutral as well as ionic compounds can be performed with unique selectivities [9,10]. The most common type of ionic surfactant employed in MECC to date has been the linear *n*-alkyl sulfate–sodium dodecyl sulfate (SDS), and importantly, the presence of micellar concentrations of SDS has recently been demonstrated to facilitate protein separations in coated silica capillary by minimizing protein–capillary wall interaction [11].

Bile salts are biological surfactants possessing substituted steroidal structures which are not only rigid and chiral, but also have been postulated to form helical micellar aggregates with the hydrophobic portions of the monomer facing the aqueous solution while the hydrophilic por-

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tions turn inward [12,13]. When used as an ionic surfactant in MECC, the unique structures and aggregation properties of bile salts have yielded certain distinctive advantages, including the abilities to separate optical isomers [14] as well as highly hydrophobic compounds [15]. Furthermore, it is worth noting that one of the primary physiological functions of bile salts involves the solubilization of compounds such as serum albumin, thus allowing the clearance of these substances by the liver [13]. However, the analytical exploitation of bile salt surfactants in MECC in the presence of proteins has not been reported.

The determination of total contents as well as individual component concentration of porphyrins in biological materials are important for the diagnosis of a family of disease known as porphyrias [16–19]. Weinberger *et al.* [20] have demonstrated that urinary porphyrins having different number of carboxylic acid side-chains can be separated with good resolution using MECC. More recently, the MECC separation of various porphyrins was optimized using an overlapping resolution mapping scheme [21]. However, in both of these studies, the separation of type I and III isomers of coproporphyrin and uroporphyrin, each having four and eight carboxylic acid groups, respectively, was not reported.

The ability to determine coproporphyrin and uroporphyrin type I and III isomers is clinically significant for the differential diagnosis and confirmation of various forms of porphyrias [16,17]. Using bovine serum albumin (BSA) as a stereoselective agent in the run buffer, we have previously demonstrated that a mixture of type I and III isomers of coproporphyrin and uroporphyrin can be resolved in less than *ca.* 30 min using CZE. However, this method requires the use of coated capillary to minimize protein–and/or porphyrin–wall interaction [4], and more importantly, we later found that significant loss of separation efficiency and resolution occurred when other porphyrin compounds having different number of carboxylic acid groups were present in the sample solution. In this paper the feasibility of the use of SDS or bile salt surfactant in conjunction with BSA as novel buffer modifiers in capillary electrophoresis (CE) was investigated for the separation of a mixture of six porphyrins having two to eight carboxylic acid side-chains together with type I and III isomers of coproporphyrin and uroporphyrin at physiological pH using untreated fused-silica capillary.

EXPERIMENTAL

Chemicals

Standards of meso-, copro- (I and III), pentacarboxyl-, hexacarboxyl-, heptacarboxyl- and uro- (I and III) porphyrins were purchased from Porphyrin Products (Logan, UT, USA). Sodium taurodeoxycholate (STDC), SDS, 3-cyclohexylamino-1-propanesulfonic acid (CAPS) and essentially fatty-acid free BSA were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade from Fisher (Springfield, NJ, USA) or Aldrich (Milwaukee, WI, USA). The run buffer solutions were prepared in doubly deionized water and filtered through a 0.4-μm pore size filter membrane.

Apparatus

A commercially available CE instrument (Model Spectra PHORESIS 1000, Spectra-Physics, Freemont, CA, USA) connected to an integrator (Chromjet, Spectra-Physics) was used to obtain the data. The wavelength spectra were obtained from the output signal using the data software supplied with the CE instrument on an IBM Model 70 computer. The MECC experiments were performed in untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with dimensions 55 cm $\times$ 375 μm O.D. $\times$ 75 μm I.D.

Porphyrin standard solutions

Stock solutions of porphyrins were prepared by dissolving appropriate amounts of porphyrin standards in small amount of 0.5 *M* NaOH and then diluted with appropriate volume of 50 m*M* SDS or STDC, followed by pH adjustment to match the corresponding run buffer. Further dilutions were made to prepare the sample solutions. Stock and sample solutions were stored at 4°C and direct light irradiation was avoided before experiments.

Procedures

New capillaries were conditioned with 20 m*M* borate–H_3PO_4 buffer for 12 h before use. The capillary was cleaned between runs with 20 m*M* borate–H_3PO_4 buffer. The sample injection was set for vacuum (*ca.* 78 mmHg below ambient; 1 mmHg = 133.322 Pa) at 2 s injection time and absorbance detection was measured at 400 nm. Column temperature was maintained at 35°C.

RESULTS AND DISCUSSION

Fig. 1a shows the separation of six porphyrin standards having two to eight carboxylic acid groups using a run buffer which contained 100 m*M* SDS and 15% methanol at pH 11.0. The separation behaviors of these porphyrins are consistent with those reported by Weinberger *et al.* [20] for the MECC separation of urinary porphyrins under similar experimental conditions. It has been stated that the major purpose of adding SDS to the run buffer was not to optimize separation selectivity but rather this anionic surfactant was used to bind to any electrostatic and hydrophobic sites on the capillary wall [20,21], thereby reducing (or eliminating) the adsorption of porphyrins with the silica wall. One the other hand, the addition of 15% methanol may enhance the solubility of the more hydrophobic porphyrins, *e.g.*, coproporphyrin, and yield an extended elution range; however, the use of a high percentage of methanol resulted in a long analysis time, probably in large part due to reduction in the electroosmotic flow.

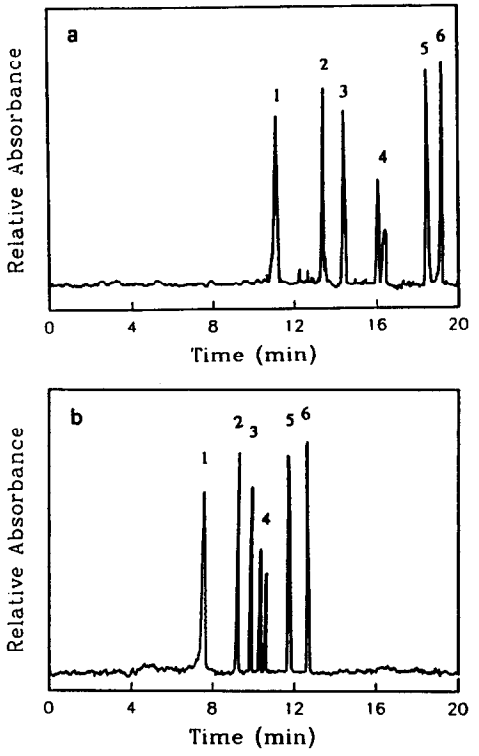

Fig. 1. Electropherograms of six porphyrin standards (10 μM each) in (a) 100 m*M* SDS, 15% (v/v) methanol, 20 m*M* CAPS (pH 11) and (b) 50 m*M* SDS–0.015 m*M* BSA, 10 m*M* borate–H_3PO_4 (pH 7.4). Voltage 18 kV. Peaks: 1 = copro (III and I)-; 2 = pentacarboxyl-; 3 = meso-; 4 = hexacarboxyl-; 5 = heptacarboxyl-; 6 = uroporphyrin.

Fig. 1b shows the separation of the same mixture of porphyrin standards used in Fig. 1a except that the run buffer contained 50 m*M* SDS and 0.015 m*M* BSA as the additives and it was buffered at pH 7.4. As in Fig. 1a, all the individual porphyrin standards were well resolved from each other but the separation was accomplished in a shorter analysis time (*ca.* 13 min) and at a significant lower pH. Various studies have been made regarding the interaction of BSA with anionic surfactants such as SDS and provided evidence suggesting the formation of various ionic surfactant–protein complexes [22,23]. Therefore, it is possible that certain SDS–BSA complex(es) may exist in the run buffer and provide sufficient solubility and selectivity for the separation of the six porphyrin standards with good efficiency and resolution at physiological pH. It is important to note that without the presence of SDS, we found that BSA alone in the run buffer was incapable of separating the six porphyrin standards (an ill-defined broad band was obtained), probably in large part due to the adsorption of BSA and/or porphyrins onto the bare silica wall. If this is the case, the formation of SDS–BSA complex(es) in the run buffer may not only contribute to enhanced solubilization and selectivity, but also to reduction in adverse effects associated with BSA/porphyrin–capillary wall interactions.

As shown in Fig. 1a and b, two peaks were observed for hexacarboxylporphyrin with or without the presence of BSA. Similar results

were also obtained by Weinberger *et al.* [20] who suggested these two peaks arose from geometrical isomers of hexacarboxylporphyrin produced during the manufacturing process. In a previous report [4], we have shown that the use of BSA as a stereoselective agent was successful for the separation of type I and III of coproporphyrin and uroporphyrin using a coated silica capillary. However, using either experimental conditions as shown in Fig. 1a or b, we were unable to resolve type I and III isomers of coproporphyrin or uroporphyrin. It appears that even at extremely alkaline conditions (*i.e.*, pH 11.0) as shown in Fig. 1a, the difference in migration time between the ionized type I and III isomers was too small to allow for separation to occur when using SDS and an organic modifier (*i.e.*, methanol) as the buffer additives. On the other hand, when using the mixed SDS–BSA buffer system as shown in Fig. 1b, BSA could be strongly denatured by SDS, thus the stereoselective binding between BSA and type I or III isomers of coproporphyrin and uroporphyrin may be diminished or eliminated.

Fig. 2a shows the separation of type I and III isomers of coproporphyrin using a run buffer which contained 50 m*M* of the bile salt STDC in presence of 5% butanol and 10% methanol. The alcohols were added to enhance the solubility of the hydrophobic coproporphyrin isomers, thus resulting in sharper and more symmetric peaks. Importantly, the two geometrical isomers of coproporphyrin were baseline resolved as a result of the differential interaction with the bile salt micelles during the separation process. It appears that the type I isomer of coproporphyrin interacted more strongly with the bile salt, resulting in a longer migration time. However, we found that the use of bile salt alone (with or without high percentage of alcohols) in the run buffer for the separation of the six porphyrin standards as shown in Fig. 1a and b was unsuccessful. Interestingly, as shown in Fig. 2b, the addition of 40 m*M* of STDC together with 0.012 m*M* of BSA in the run buffer resulted in not only the successful separation of the six porphyrin carboxylic acids but also the resolution of type I and III isomers of coproporphyrin with good efficiency and relatively short analysis time

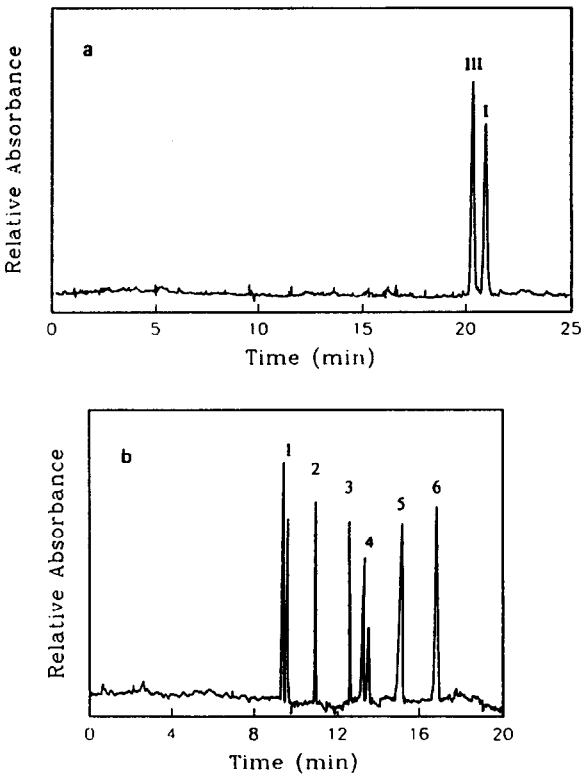

Fig. 2. Electropherograms of (a) coproporphyrin III (12.5 μM) and I (10 μM) in 50 m*M* STDC + 6 m*M* borate–H_3PO_4 (pH 8.0) plus 5% butanol and 10% (v/v) methanol and (b) 10 μM each of the porphyrin standards (peaks 1–6) in 40 m*M* STDC–0.012 m*M* BSA and 6 m*M* borate–H_3PO_4 (pH 7.4). Peaks as in Fig. 1. Voltage 18 kV.

at pH 7.4. It should be noted, however, that the separation of type I and III isomers of uroporphyrin was unsuccessful using this particular mixed STDC–BSA buffer system.

In an attempt to understand the mechanisms which contributed to the successful separation of type I and III isomers of coproporphyrin in the presence of various hydrophilic and hydrophobic porphyrins as shown in Fig. 2b, it is important to remember that as in the case of SDS, bile salt surfactants are also known to form complexes with proteins such as BSA [12]. Fig. 3a and b show the electropherograms of BSA and STDC when injected separately into the CZE system. It can be seen that a relatively sharp peak was obtained for BSA whereas a broad and asymmetric peak was obtained for STDC. A

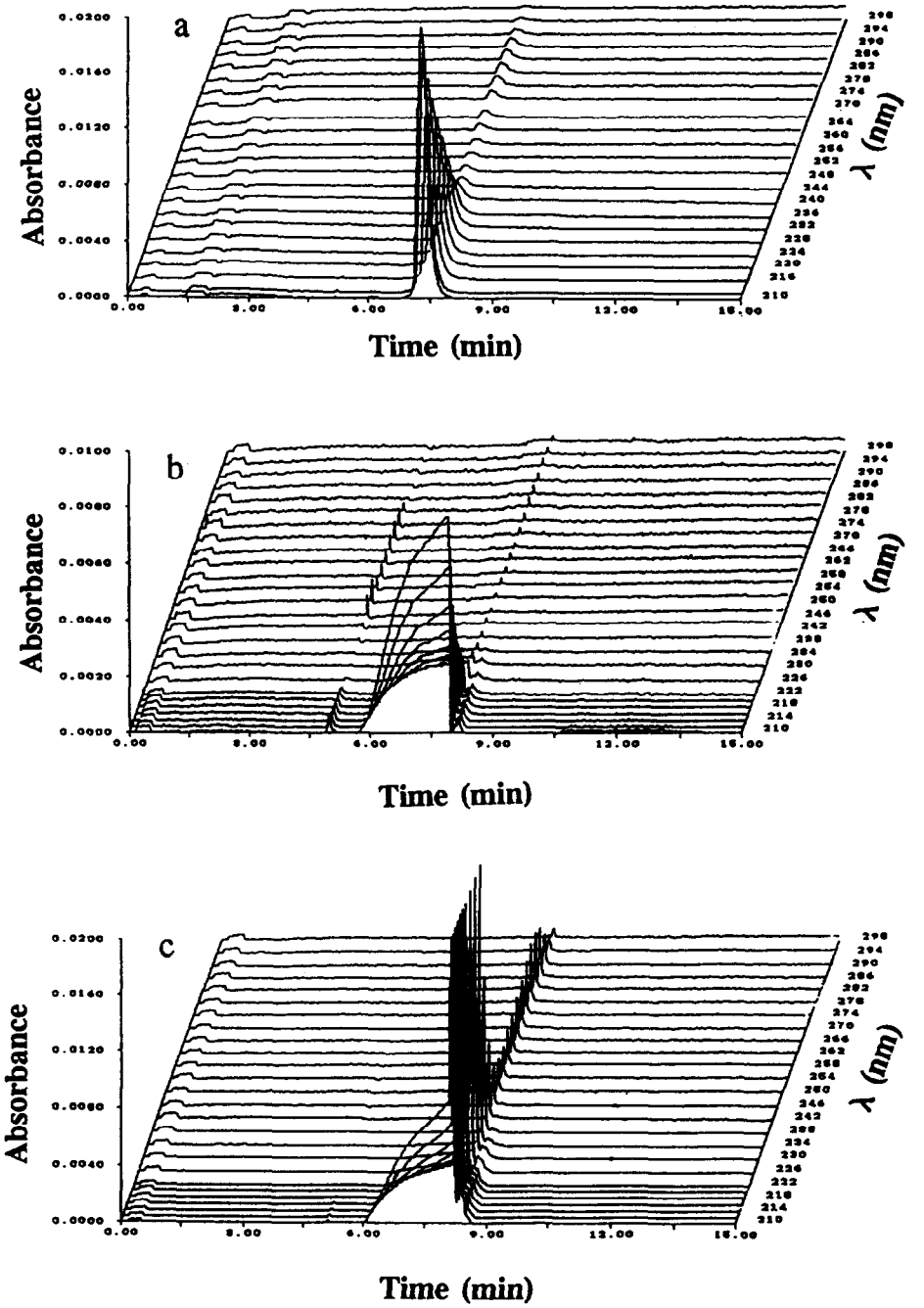

Fig. 3. Electropherograms of (a) 0.015 m*M* BSA, (b) 50 m*M* STDC and (c) a mixture of 50 m*M* STDC–0.015 m*M* BSA in 10 m*M* borate–H_3PO_4 (pH 7.4). Voltage 16.5 kV.

possible explanation for the distorted peak shape as shown in Fig. 3b may be attributed to the adsorption of the bile salt at the capillary wall and/or the inherent polydispersity of the bile salt micelles, *e.g.*, micelles of different aggregation number and size [13]. Fig. 3c shows an electropherogram of an injection sample containing both BSA and STDC. It appears that the peak

maximum of BSA which centers at a migration time of *ca.* 7.5 min as shown in Fig. 3a has shifted and overlapped with portions of the asymmetric bile salt peak located at *ca.* 8.0 min, suggesting that the bile salt may associate with the BSA to form certain ionic surfactant–protein complex(es) when mixed together in the run buffer.

Fig. 4 shows the UV–visible absorption spectra of coproporphyrin III eluted with run buffers containing different additives. By comparing spectra a and b, it can be seen that with the presence of both bile salt and BSA in the buffer, the position of the peak maximum of coproporphyrin III remained relatively constant as compared to buffer solution containing only bile salt. In contrast, spectrum c indicates that a relatively large red shift occurred in the presence of BSA alone, probably as a result of the binding of coproporphyrin III with BSA in the run buffer [4,24]. These spectroscopic results suggest that the combination of bile salt with BSA as shown in spectrum b may have led to changes in the conformation of the protein and resulted in the loss of stereoselective binding of BSA with coproporphyrin III. If such is the case, the successful separation of type I and III of coproporphyrin present in a mixture of porphyrin standards as illustrated in Fig. 2b was probably not due to the differential affinities of coproporphyrin isomers with specific binding sites located in the BSA structure, but rather arose from the differential interaction of coproporphyrin isomers with the bile salt bound to the BSA [bile salt–BSA complex(es)] and/or unbound bile salt micelles present in the run buffer. This postulation may also explain why type I and III isomers of uroporphyrin cannot be separated using the STDC–BSA buffer system since we found that the presence of bile salt alone in the run buffer was unsuccessful while BSA alone was successful for the separation of type I and III isomers of uroporphyrin.

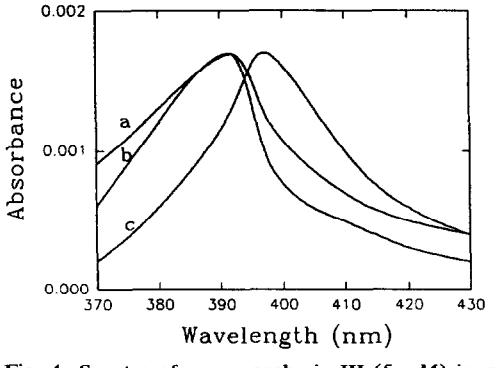

Fig. 4. Spectra of coproporphyrin III (5 μM) in run buffer containing (a) 50 mM STDC, (b) 50 mM STDC–0.015 mM BSA and (c) 0.015 mM BSA in 10 mM borate–H_3PO_4 (pH 7.4). Each spectrum was obtained by scanning the peak maximum from 370 to 430 nm. Voltage 16.5 kV.

To account for the excellent separation of the six porphyrin carboxylic acids and coproporphyrin isomers as shown in Fig. 2b, it is important to note that under optimum experimental conditions, the molar ratio of bile salt to BSA was greater than 3000, suggesting that a relatively large amount of unbound bile salt micelles could exist in the run buffer and interact dynamically with the bile salt–BSA complex(es) and the bare silica capillary wall. Under these conditions, the unbound bile salt micelles together with the bile salt–BSA complex(es) may provide enhanced solubilization and selectivity for the various porphyrins at pH 7.4 as compared to either bile salt or BSA alone. Furthermore, the interaction of anionic bile salt surfactant with BSA and the capillary wall could minimize the adsorption by the "sticky" porphyrin molecules or BSA, resulting in improved peak shape and the reproducibility of separation.

The migration time and peak area precision data obtained for the separation of the six porphyrin standards using either SDS or STDC with BSA in the run buffer are presented in Table I. When compared to the use of BSA alone in the buffer [4], a significant improvement in the migration time reproducibility can be obtained using the ionic surfactant–BSA buffer system. Furthermore, we found that without the addition of SDS or STDC, most untreated capillaries lasted for 10 to 15 runs with the presence of BSA and had to be replaced; however, a bare capillary can be used for at least 50 runs or more when SDS or STDC was added with BSA in the run buffer. The average limits of detection ($S/N = 3$) for the six porphyrin standards under the

TABLE I

RELATIVE STANDARD DEVIATIONS (%) OF MIGRATION TIME AND PEAK AREAS OBTAINED FOR PORPHYRINS AND PORPHYRIN ISOMERS SEPARATION

Measured on sequential injections over *ca.* 2 h period for five replicate injections ($n = 5$).

Porphyrin	STDC–BSA system[a], R.S.D (%, $n = 5$)		SDS–BSA system[b], R.S.D. (%, $n = 5$)	
	Migration time	Peak area	Migration time	Peak area
Copro III	0.29	2.9	0.35	2.1
Copro I	0.30	2.9	0.35	2.1
Meso	0.16	3.2	0.28	3.1
Penta	0.31	2.5	0.11	2.1
Hexa[c]	0.21	3.2	0.15	2.4
Hexa[d]	0.22	3.5	0.14	2.4
Hepta	0.35	3.8	0.25	2.9
Uro	0.38	4.2	0.27	2.9

[a]In 6 m*M* sodium borate–H_3PO_4 buffer (pH 7.4) containing 40 m*M* STDC and 0.012 m*M* BSA.
[b]In 10 m*M* sodium borate–H_3PO_4 buffer (pH 7.4) containing 50 m*M* SDS and 0.015 m*M* BSA.
[c,d]Hexacarboxylporphyrin positional isomers.

experimental conditions as shown in Figs. 1b and 2b were about 1.0 nmol/ml and 1.6 nmol/ml, respectively. These values are similar to those reported by Weinberger *et al.* [20] using absorption detection.

In summary, the use of an anionic surfactant in conjunction with a protein as buffer additives in CE allowed for the separation of various hydrophobic and hydrophilic porphyrins as well as the simultaneous resolution of a pair of clinically significant geometrical porphyrin isomers using untreated capillaries at physiological pH. Rapid, efficient and reproducible separation of porphyrins and porphyrin isomers could be attributed to the abilities of SDS and bile salt to minimize adsorption of protein/porphyrins at the bare silica wall and to the formation of ionic surfactant–protein complexes in the run buffer. More research is necessary to identify the mechanisms in which separation performance can be improved by various ionic surfactant–protein systems; in particular, the possible roles played by ionic surfactant–protein complexes in effecting stereoselective resolution of hydrophobic and hydrophilic isomers need to be addressed in more detail in future studies.

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