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Effects of bile salt structure on chiral separations with mixed micelles of bile salts and polyoxyethylene ethers using micellar electrokinetic capillary chromatography

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Abstract

The chiral resolving abilities of micellar solutions of four different bile salts alone and in mixtures with polyoxyethylene-4-dodecyl ether ($C_{12}E_4$) and methanol were investigated using MECC. The four bile salts investigated were the unconjugated sodium salts of cholic, deoxycholic, chenodeoxycholic and ursodeoxycholic acids. The test solutes included verapamil, norverapamil, gallopamil, bi-2-naphthol, atenolol and BAYK8644. The relative hydrophobicities of the micellar aggregates formed in solutions of binary mixtures of each bile salt with $C_{12}E_4$ were investigated by fluorescence spectroscopy using pyrene as a probe molecule. The observed enantiomeric resolution for the test compounds using these binary mixtures as MECC pseudo-stationary phases was determined. Correlations between micellar hydrophobicity for these solutions and chiral resolution of these test solutes are presented. The addition of $C_{12}E_4$ with or without methanol to solutions of sodium cholate and sodium deoxycholate enhanced the chiral resolution observed for compounds containing a longer hydrocarbon chain separating some of the major functional groups from the chiral center. The pure bile salt solutions generally provided better chiral resolution for the compounds where the major functional groups, such as aromatic rings, were closer to the chiral center.

Keywords: Verapamil; Norverapamil; Gallopamil; Bi-2-naphthol; Atenolol; Bile salts; Polyoxyethylene ethers; Cholic acid; Deoxycholic acid; Chenodeoxycholic acid; Ursodeoxycholic acid

1. Introduction

The addition of polyoxyethylene ethers to bile salt solutions has been reported previously to improve chiral resolution for verapamil and related compounds in micellar electrokinetic capillary chromatography (MECC) [1]. Mixtures of one bile salt, sodium deoxycholate and three polyoxyethylene ethers, polyoxyethylene-4-dodecyl ether ($C_{12}E_4$), polyoxyethylene-6-dodecyl ether ($C_{12}E_6$) and polyoxyethylene-8-decyl ether ($C_{10}E_8$), were investigated previously. The four types of solutions used for the MECC studies included: (1); bile salt; (2); bile salt and polyoxyethylene ether; (3); bile salt and methanol and (4); bile salt, polyoxyethylene ether and methanol. The greatest increase in chiral resolution was observed using bile salt solutions containing $C_{12}E_4$ and methanol. Further studies have been conducted with solutions containing $C_{12}E_4$ in binary mixtures with sodium deoxycholate (NaDC) and three additional bile salt species: sodium cholate (NaC), sodium chenodeoxycholate (NaCDC) and

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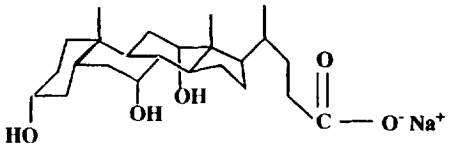

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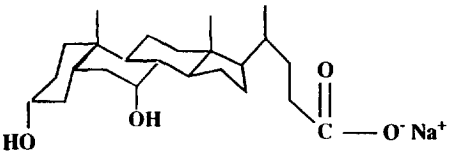

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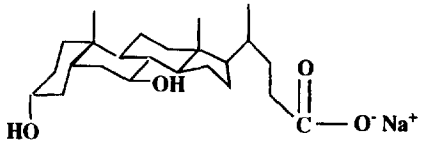

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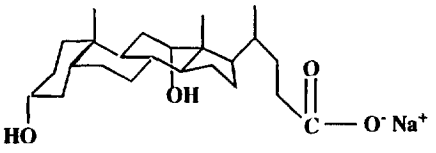


SODIUM DEOXYCHOLATE

Fig. 1. Structures of bile salts.

sodium ursodeoxycholate (NaUDC). The structures of these four bile salts are given in Fig. 1. These mixed surfactant solutions, with and without added methanol, have been evaluated as chiral pseudo-stationary phases in MECC. Fluorescence spectroscopy using pyrene as a probe molecule has provided information on the relative hydrophobicities of these mixed surfactant solutions. Studies of these solutions indicate that correlations exist between micellar hydrophobicity, as determined by the fluorescence studies, and chiral resolution for test analytes. These correlations have the potential for providing a means to predict the appropriate mole fraction of ether, in a mixed micellar solution of bile salt and ether, likely to provide chiral resolution for a given enantiomeric pair.

The importance of the bile salts as naturally occurring surfactants has led to many studies concentrating on determining the physical properties and fundamental constants (critical micelle concentration, micellar aggregation number, solution surface tension and viscosity) of aqueous bile salt solutions. A review of the literature concerning the determination of size and aggregation number of bile salt micelles in aqueous solutions using static light scattering, sedimentation equilibrium, membrane osmometry, sedimentation velocity and translational diffusion techniques presents variability of more than an order of magnitude between some of the reported results [2]. The scatter in the data was related to the precise control of solution conditions such as pH and metal ion concentration, as well as environmental variables such as temperature and pressure.

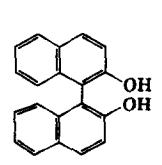
The aggregation behavior of the bile salts under various conditions has been studied using light scattering [3,4], nuclear magnetic resonance, electron spin resonance and X-ray techniques [5–7]. From the results of these studies, a helical model has been proposed in which the hydroxyl groups of the steroid ring backbone are oriented towards the core of the micelle [5–7]. This model may be important for the understanding of chiral recognition with bile salt micelles. The number and orientation of the hydroxyl groups will change the possibilities for chiral interactions with the bile salt micelles. The data presented here show that the number and orientation of the hydroxyl groups does have an effect on chiral separations obtained using MECC for the compounds investigated.

The properties of mixed micelles formed in solu-

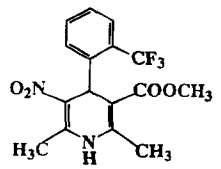
tions of bile salts and polyoxyethylene ethers have been investigated by several techniques [8–12]. The addition of polyoxyethylene-8-decyl ether ($C_{10}E_8$) to NaDC solutions has been shown to alter the critical micellar concentration (CMC), the aggregation number (AN) of the micelles and the ability of the micelles to solubilize cholesterol. For example, a mixed solution of the ether, $C_{10}E_8$, and NaDC containing an ether mole fraction of 0.25, was determined to have a CMC of 1.63 m*M* and an AN of 25. A solution containing only NaDC was determined to have a CMC of 3.16 m*M* and an AN of 18 under the same experimental conditions [12]. Asano and coworkers indicate that the hydrophobicity of the interior of the mixed micelle decreases with increased mole fraction of ether in the bile salt solutions. For example, increasing the mole fraction of ether from 0.00 to 0.43 in mixed solutions of NaDC and $C_{10}E_8$ results in the formation of micelles which decreased in hydrophobicity [11]. Thus, polyoxyethylene ethers can be added to bile salt solutions at various concentrations to provide a series of micellar solutions with decreasing hydrophobicities which can be used as variable pseudo-stationary phases in MECC.

The relative hydrophobicities of aggregates in micellar solutions have been correlated to spectral data using pyrene as a probe molecule [13–17]. The fluorescence emission spectrum of pyrene in aqueous micellar solutions possesses considerable fine structure. Five distinct fluorescence emission peaks are observed between 350 and 400 nm using an excitation wavelength of 330 nm. Hydrophobicity measurements have been reported based on the ratio of the intensities of the first to third fluorescence peaks [13–17]. These reports have described a general method which can be used to determine the hydrophobicities of aggregates in detergent solutions [13–15] and bile salt solutions [16,17].

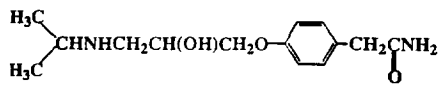
Several test compounds were chosen to evaluate the ability of solutions containing the bile salts and polyoxyethylene ethers to provide enantiomeric separations in MECC. The separation of the *R*- and *S*-enantiomers for the compounds verapamil, norverapamil, gallopamil, atenolol, BAYK8644 and bi-2-naphthol were chosen for the study. The structures for these compounds are given in Fig. 2. Separation



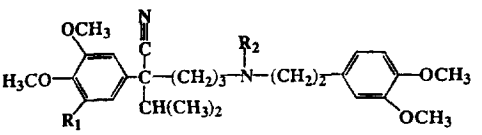
BI-2-NAPHTHOL



BAY K 8644



ATENOLOL



	R_1	R_2
verapamil	-H	$-CH_3$
norverapamil	-H	-H
gallopamil	$-OCH_3$	$-CH_3$

Fig. 2. Structures of solutes.

of the enantiomers of bi-2-naphthol using mixed micellar solutions and mixed micellar solutions with methanol provided additional reference points for comparison to solutions of bile salt and bile salt with methanol in the literature [18]. Enantiomeric separation of the widely administered calcium ion channel blocking drug, verapamil, has been an area of active research in HPLC using several types of chiral stationary phases [19–24] and MECC using cyclodextrins [25]. Enantiomeric resolution has also been observed using some of the same chiral stationary phases for the major metabolite of verapamil, norverapamil [19–21] and a methoxy derivative, gallopamil [20]. Enantiomeric separation of another cardiac drug atenolol, a β-andrenergic blocker, has also been an area of active research in HPLC using several types of chiral stationary phases [26–30] and MECC with cyclodextrins [31–35] and a novel chiral surfactant [36]. The enantiomeric resolution of a compound under development as a cardiac drug,

BAYK8644, has been reported using a cyclodextrin stationary phase in HPLC [37].

2. Experimental

2.1. Apparatus for CE

Studies were conducted using a laboratory assembled capillary electrophoresis instrument. The laboratory assembled instrument consisted of a 30 kV power supply (Model MJ30P400, Glassman High Voltage, Whitehouse Station, NJ, USA) and a variable-wavelength UV-Vis absorbance detector (Model AD-200, SpectroVision, Chelmsford, MA, USA). The absorbance at 210 nm was recorded using both a strip chart recorder (Model BD40, Kipp and Zonen, Netherlands) and an integrator (Model 3390A, Hewlett-Packard, Wilmington, DE, USA). Bare fused-silica capillary columns with a 50 μm I.D. and 350 μm O.D. (Supelco, Bellefonte, PA and Polymicro Technologies, Phoenix, AZ, USA) were used.

2.2. Materials

Sodium cholate (NaC), sodium deoxycholate (NaDC), polyoxyethylene-4-dodecyl ether, bi-2-naphthol enantiomers, pyrene and the *R*- and *S*-enantiomers of atenolol were purchased from Aldrich. (Milwaukee, WI, USA). Sodium chenodeoxycholate (NaCDC) and sodium ursodeoxycholate (NaUDC) were purchased from Calbiochem (La Jolla, CA, USA). The manufacturer stated purities of the four bile salts: NaC, NaDC, NaCDC and NaUDC were reported to be in excess of 98, 99, 97 and 98 percent by mass, respectively. The enantiomers of BAYK8644, verapamil hydrochloride and methoxyverapamil hydrochloride (gallopamil hydrochloride) were purchased from Research Biochemicals (Natick, MA, USA). The enantiomers of norverapamil were kindly provided by Dr. L. Miller (Searle Chemical Sciences Department, Skokie, IL, USA). Reagent grade sodium hydroxide, sodium chloride and HPLC grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized, distilled water was used for the preparation of all solutions.

2.3. Solution preparation

Surfactant solutions were prepared by mixing appropriate volumes of stock solutions containing each type of surfactant. The solutions were prepared containing methanol in the indicated percentages by volume. Polyoxyethylene ether stock solutions were prepared by dissolving the appropriate quantity of the surfactant in water to achieve a final surfactant concentration of 50 m*M*. Stock solutions of each bile salt at a concentration of 100 m*M* were prepared by dissolving the appropriate quantity of the bile salt in water. Mixed surfactant solutions were prepared having a total surfactant concentration of 50 m*M* by combining appropriate volumes of bile salt stock solution, polyoxyethylene ether stock solution, methanol and water. The pH of the resulting solutions was between 8.1 and 8.3. Methanol solutions were prepared containing the test analytes; verapamil, norverapamil, gallopamil, bi-2-naphthol, atenolol and BAYK8644. The injected concentration of each enantiomer was between 0.25 and 1.0 mg/ml.

2.4. Fluorescence studies

Pyrene was used as a probe to determine the relative hydrophobicities of the micelles using a method previously reported for mixtures of bile salts and polyoxyethylene ethers [9–13]. The fluorescence emission spectrum of pyrene in the micellar solutions was measured from 350 nm to 500 nm using an excitation wavelength of 333 nm. The pyrene concentration used for these experiments was 1×10^{-5} *M*. The spectral data were acquired using an SLM Aminco Bowman Series 2 Luminescence Spectrometer (Rochester, NY, USA). The emission bandpass and scan rate were set to 0.5 nm and 0.5 nm/s, respectively.

2.5. CE experimental technique

Prior to performing an experiment, the capillary was rinsed with a 0.1 *M* sodium hydroxide solution for 2 min. The surfactant solution used for the experiment was rinsed through the capillary for an additional 2 min by applying pressure to a vial containing the solution on the anode side of the

capillary. Injection of analytes was performed hydrodynamically by raising the anode end of the column to a height of 15 cm above the cathode for times ranging from 1 to 6 s.

3. Results

3.1. Fluorescence experiments

Fluorescence experiments were performed, using pyrene as the probe molecule, for solutions containing binary mixtures of $C_{12}E_4$ and each of the four bile salts, NaC, NaDC, NaCDC and NaUDC. Measurements of the ratios of the first to third vibronic band intensities of pyrene were calculated based on the fluorescence spectra. This ratio of fluorescence intensities has been correlated to the hydrophobicity of the molecular environment of the pyrene probe [10,13–17]. A plot of the ratio of these vibronic bands versus mole fraction of bile salt in the solution is given in Fig. 3. The total surfactant concentration of these mixed surfactant solutions was maintained at 50 m*M*.

3.2. MECC separations using bile salt micelles

Experiments were performed using 50 m*M* solutions of each of the four bile salts as pseudo-stationary phases for MECC to separate the enantiomers of the test compounds. The chiral resolution obtained

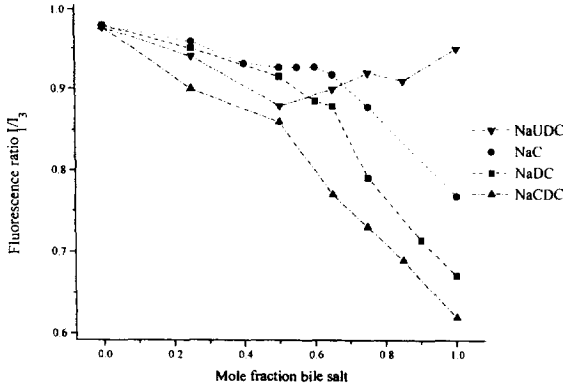


Fig. 3. Fluorescence emission peak ratios, I_1/I_3, of pyrene versus mole fraction of bile salt in solutions containing $C_{12}E_4$, bile salt and binary mixtures of $C_{12}E_4$ and bile salt.

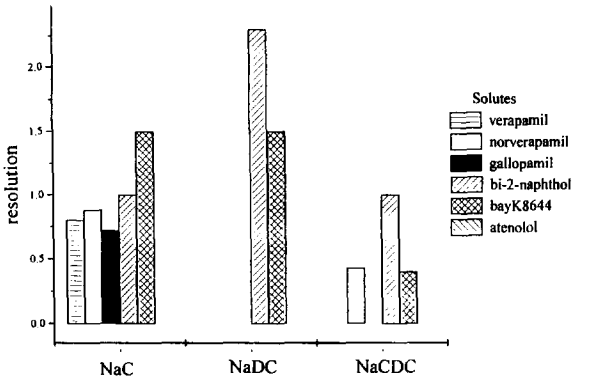


Fig. 4. Resolution of test compounds using 50 m*M* pure bile salt solutions.

for each of the test compounds using solutions of NaC, NaDC and NaCDC are given in Fig. 4. Some chiral resolution was observed for BAYK8644, bi-2-naphthol and verapamil using solutions containing NaUDC, but the resolution obtained in all cases was less than 0.5 and difficult to reproduce.

3.3. MECC separations using mixed micelles of bile salts and $C_{12}E_4$

Experiments were performed using solutions of each of the four bile salts, in binary mixtures with $C_{12}E_4$, as pseudo-stationary phases for MECC. Plots of the chiral resolution versus mole fraction of ether obtained by MECC for each of the test compounds using solutions of bile salt and binary mixtures of bile salt and $C_{12}E_4$ are given for NaC, NaDC and NaCDC in Fig. 5, Fig. 6 and Fig. 7, respectively. Plots of the chiral resolution versus mole fraction of ether obtained by MECC for each of the test compounds using solutions of bile salt and binary mixtures of bile salt and $C_{12}E_4$, all containing 25% methanol by volume, are given for NaDC and NaCDC in Fig. 8 and Fig. 9, respectively.

4. Discussion

4.1. Fluorescence experiments

The ratios of the first to third vibronic band intensities, I_1/I_3, decrease as the mole fraction of the

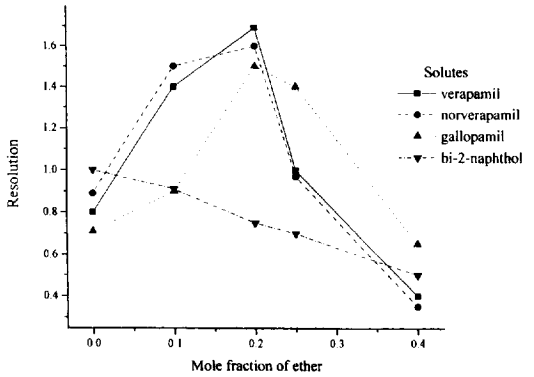


Fig. 5. Resolution of the test compounds versus mole fraction of ether using solutions containing NaC and binary mixtures with $C_{12}E_4$.

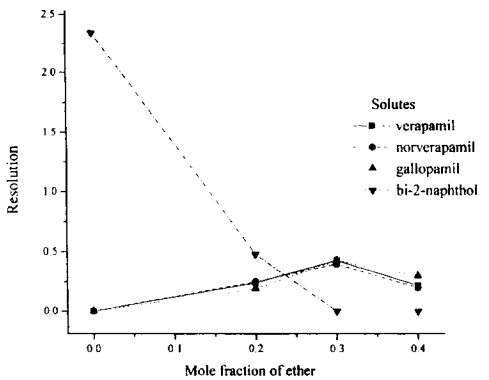


Fig. 6. Resolution of the test compounds versus mole fraction of ether using solutions containing NaDC and binary mixtures with $C_{12}E_4$.

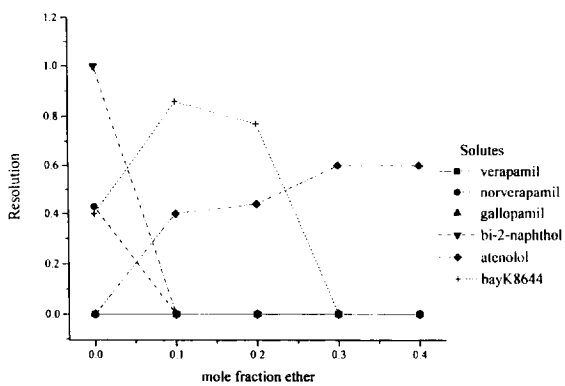


Fig. 7. Resolution of the test compounds versus mole fraction of ether using solutions containing NaCDC and binary mixtures with $C_{12}E_4$.

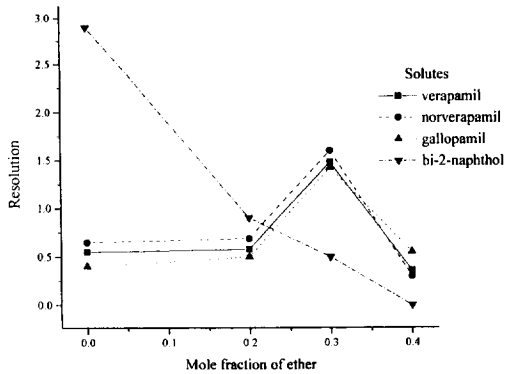


Fig. 8. Resolution of the test compounds versus mole fraction of ether using solutions containing NaDC with 25% methanol and ternary mixtures with $C_{12}E_4$ and 25% methanol.

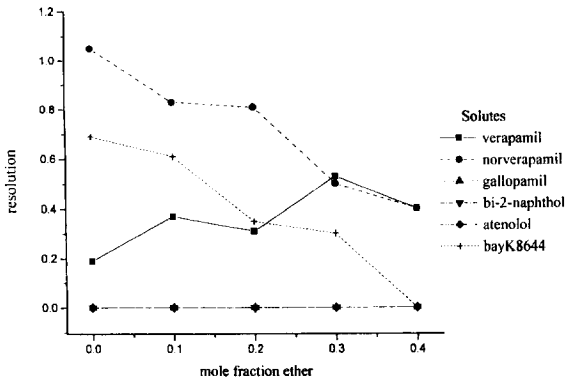


Fig. 9. Resolution of the test compounds versus mole fraction of ether using solutions containing NaCDC with 25% methanol and ternary mixtures with $C_{12}E_4$ and 25% methanol.

bile salt increases for solutions containing $C_{12}E_4$ in mixtures with NaC, NaDC and NaCDC. As can be seen in Fig. 3, two distinct linear regions are observed in the plots of I_1/I_3 versus mole fraction of bile salt for NaC, NaDC and NaCDC. Asano and coworkers observed similar linear regions in plots of I_1/I_3 versus mole fraction of bile salt using solutions containing a similar polyoxyethylene ether, $C_{10}E_8$, in mixtures with NaC and NaDC [11]. The linear regions were assigned to pyrene being in an environment of an ether-rich micelle at lower bile salt concentrations and a bile salt-rich micelle at higher bile salt concentrations. The relationship obtained for the ratio, I_1/I_3, using solutions containing $C_{12}E_4$ in mixtures with NaUDC is much different and does

not show a linear region for a bile salt-rich micelle. It has been reported that NaUDC molecules prefer to associate with themselves rather than form mixed micelles with phosphatidylcholine [38,39]. Thus, the fluorescence data may indicate that NaUDC molecules are not forming mixed micelles with $C_{12}E_4$.

The ratios of I_1/I_3 obtained for the pure bile salt solutions were 0.62, 0.67, 0.77 and 0.95 for NaCDC, NaDC, NaC and NaUDC, respectively. The same order in the ratios of I_1/I_3 was previously reported for solutions of NaDC and NaC at higher concentrations [11,16]. These data suggest that the hydrophobicity of micellar aggregates in solutions of the four bile salts increases in the order of NaUDC≤NaC≤NaDC≤NaCDC. The hydrophobicities of individual bile salts have been correlated to the reversed-phase HPLC retention times of the bile salt species in monomeric solutions [40,41]. The order of hydrophobicity of the monomers inferred from these studies increases in the order NaUDC≤NaC≤NaCDC≤NaDC. Thus, a difference in the order of hydrophobicity is observed for NaDC and NaCDC using the two techniques. Micellar solutions of NaCDC were shown here to contain the most hydrophobic aggregates using pyrene as a probe molecule, but NaDC was shown to be more hydrophobic than NaCDC in monomeric solutions [40,41].

4.2. MECC separations

Enantiomeric separations of a series of test analytes were investigated using solutions of these four bile salts. A graph representing the enantiomeric resolution observed for each of these analytes, using 50 m*M* solutions of each of the four bile salts by MECC, is given in Fig. 4. Clearly, the resolution obtained for the enantiomers of each solute depends on the species of bile salt used. Excellent resolution was observed for the enantiomers of bi-2-naphthol and BAYK8644 with several of the pure bile salt solutions. Limited chiral resolution was observed for enantiomers of verapamil, norverapamil and gallopamil with solutions containing only the bile salts. Chiral resolution was not observed for the enantiomers of atenolol with any of the pure bile salt solutions. Previous studies had shown that adding $C_{12}E_4$ and methanol to solutions of NaDC provided chiral resolution for the enantiomers of verapamil. $C_{12}E_4$ and methanol were evaluated as modifiers in binary and ternary mixtures with each of the four bile salts for the chiral separations of all six test analytes by MECC.

The addition of $C_{12}E_4$ in binary mixtures with NaC provided enhanced chiral resolution of some solutes. Plots of the resolution observed in CE versus mole fraction of ether for solutions containing binary mixtures of NaC and $C_{12}E_4$ are given in Fig. 5. The data show that with the addition of $C_{12}E_4$ to the NaC solutions, the resolution increases for the enantiomers of verapamil, gallopamil and norverapamil up to a $C_{12}E_4$ mole fraction of 0.2. The resolution observed for the enantiomers of bi-2-naphthol steadily decreases with the addition of $C_{12}E_4$. Additional experiments were performed to evaluate methanol as a modifier in binary solutions with NaC and in ternary solutions with NaC and $C_{12}E_4$ for the resolution of the enantiomers of bi-2-naphthol and verapamil. A decrease in the resolution of these enantiomers was observed with the addition of methanol. An increase in peak asymmetry was also observed with added methanol. No increase in resolution was observed for the enantiomers of BAYK8644 using solutions of ternary mixtures of NaC with $C_{12}E_4$ or methanol, or using solutions which are ternary mixtures of NaC, $C_{12}E_4$ and methanol. No resolution was observed for atenolol using solutions of NaC or binary and ternary mixtures with methanol and $C_{12}E_4$.

Solutions of the bile salt, NaDC, in mixtures with $C_{12}E_4$ and methanol were also investigated as chiral pseudo-stationary phases in MECC. The addition of these modifiers in binary and ternary mixtures with NaDC improved chiral resolution for some solutes. Plots of the resolution observed for the enantiomers of some of the test compounds in CE versus mole fraction of ether for solutions containing binary mixtures of NaDC and $C_{12}E_4$ are given in Fig. 6. The enantiomeric resolution increases slightly for verapamil, gallopamil and norverapamil with the addition of $C_{12}E_4$ up to a mole fraction of 0.3. The resolution of bi-2-naphthol enantiomers decreases with the addition of $C_{12}E_4$. Plots of resolution versus mole fraction of ether for solutions containing ternary mixtures of NaDC and $C_{12}E_4$ with 25% methanol are given in Fig. 8. The resolution observed for verapamil, norverapamil and gallopamil in binary

solutions with $C_{12}E_4$ increased further in ternary solutions, with the addition of methanol, at all mole fractions of ether. A resolution of 0.4 was observed for the verapamil enantiomers using a binary solution containing NaDC and $C_{12}E_4$ at an ether mole fraction of 0.3. Resolution of the verapamil enantiomers increased to approximately 1.5 using a ternary solution at an ether mole fraction of 0.3 with 25% methanol.

The highest chiral resolution observed for several compounds with the NaDC and NaC series of mixed micellar solutions corresponded to solutions containing micelles of similar hydrophobicity. For example, the highest enantiomeric resolution was observed for verapamil, norverapamil and gallopamil using binary solutions with $C_{12}E_4$ having bile salt mole fractions of 0.8 and 0.7 with NaC and NaDC, respectively. As can be seen in Fig. 3, the I_1/I_3 ratio for these two solutions is very similar which indicates that the two binary solutions contain micelles of similar hydrophobicity.

Further MECC experiments were conducted using the solutions of the bile salt NaCDC in mixtures with $C_{12}E_4$ and methanol. The addition of these modifiers in binary and ternary mixtures with NaCDC improved chiral resolution for some solutes. Plots of the resolution obtained for the test compounds versus mole fraction of ether for solutions containing binary mixtures of NaCDC and $C_{12}E_4$ are given in Fig. 7. The enantiomeric resolution observed for atenolol increases with the mole fraction of $C_{12}E_4$ added to the NaCDC solution. The resolution for BAYK8644 increases with some mole fractions of $C_{12}E_4$ added to the NaCDC solution, with the highest resolution observed at a mole fraction of 0.1 $C_{12}E_4$. The resolution decreases for the enantiomers of bi-2-naphthol and norverapamil with the addition of $C_{12}E_4$ to the NaCDC solution. The addition of 25% methanol by volume to the solutions of NaCDC and $C_{12}E_4$ resulted in increased chiral resolution for some solutes. A plot of chiral resolution obtained for the test compounds versus mole fraction of ether for solutions which are ternary mixtures of NaCDC and $C_{12}E_4$ with 25% methanol is given in Fig. 9. The enantiomers of atenolol are not resolved using solutions containing methanol. An increase in resolution for the enantiomers of verapamil and norverapamil is observed with the addition of methanol to some binary solutions of NaCDC with $C_{12}E_4$. No enantiomeric resolution is observed for gallopamil using any solution containing NaCDC.

5. Conclusions

The enantiomeric resolution of verapamil, norverapamil and gallopamil by CE using solutions containing bile salts is improved by the addition of $C_{12}E_4$ as a solution modifier for NaDC and NaC. The general trend in the results of these studies using solutions containing NaC or NaDC show increased chiral resolution for these solutes, yet the chiral resolution for bi-2-naphthol and BAYK8644 decreases, with the addition of $C_{12}E_4$ as a solution modifier. Conclusions about the chiral interactions of the solutes studied with aggregates of NaCDC or NaUDC are not as straightforward as with NaC and NaDC. MECC studies using methanol as a modifier in solutions containing NaDC or NaCDC with and without $C_{12}E_4$ show increased chiral resolution for some solutes.

The fluorescence studies have indicated that the order of hydrophobicity of the aggregates formed in these mixed micellar solutions follows the order: NaCDC≥NaDC≥NaC≥NaUDC. For each test solute studied, correlations exists between the best chiral resolution observed and the hydrophobicities of the micellar aggregates, for solutions containing mixtures of $C_{12}E_4$ with NaC and NaDC. Improvement in chiral resolution was observed using $C_{12}E_4$ as solution modifiers for verapamil, norverapamil, gallopamil and atenolol with certain bile salt species. No improvement in chiral resolution of bi-2-naphthol or BAYK8644 was observed using $C_{12}E_4$ and methanol as modifiers for NaC and NaDC solutions. Correlations between the chiral resolving abilities of MECC using pure bile salt solutions and solute structure have been reported [42]. The addition of $C_{12}E_4$ with or without methanol to solutions containing NaC or NaDC enhanced the chiral resolution observed for compounds containing a longer hydrocarbon chain separating some of the major functional groups from the chiral center. The pure bile salt solutions generally provided better chiral resolution for the compounds where the major functional groups, such as aromatic rings, were closer to the

chiral center. These conclusions can be used as an aid to assist in predicting the proper solution parameters to begin methods development in chiral separations using bile salts and bile salts with modifiers by assessing the structural components of the enantiomers of interest.

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