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Investigation of chiral resolution using displacement interactions with polymer networks in capillary affinity zone electrophoresis

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Abstract

The effect of displacers on the resolution of the stereoisomers folinic acid, mandelic acid and N-benzoylalanine at physiological pH using capillary affinity zone electrophoresis is described. Bovine serum albumin (BSA) is used as a run buffer additive to incorporate stereoselectivity into the system, while salicylates exhibiting different affinities for BSA are added to the electrolyte for the displacement studies. The salicylate, α-resorcylic acid, was found to increase retention and stereoselectivity for the samples, while other displacers exhibited a negative effect on chiral resolution. Changes in effective mobility and resolution of stereoisomers using different displacers added to the run buffer did not correlate with reported equilibrium binding constants (K_b) for the displacer–BSA complexes. Protein–wall interactions were minimized using a combination of dextran in the supporting electrolyte and capillaries derivatized with polyacrylamide. Dextran played a secondary role by improving resolution. The results indicate that displacers may be used to optimize chiral separations in affinity capillary electrophoresis. In addition, the use of albumin as a pseudostationary phase may be used to screen drug–displacer interactions with BSA.

Keywords: Enantiomer separation; Displacers; Buffer composition; Affinity electrophoresis; Folinic acid; Mandelic acid; Benzoylalanine

1. Introduction

The separation of stereoisomers is critical in the development of new medicine, as many pharmaceutical drugs have asymmetric centers, with most of them being used clinically in racemic form. Many of these enantiomers exhibit different pharmacological activities and differ in their pharmacokinetic effects. With the improved understanding of the biological action of pharmaceutical drugs with respect to their stereochemistry, investigations concerning the pharmacology and toxicology of individual drug enantiomers and other biological molecules have become increasingly important. The pharmaceutical industry has, therefore, a strong interest in techniques for the resolution of stereoisomers to investigate the optical purity of drugs. High-performance liquid chromatography (HPLC) has emerged as a viable tool for the separation of stereoisomers. A variety of stationary phases have been utilized for this purpose. In particular, protein phases including bovine serum albumin (BSA) [1–3], α-acid glycoprotein (α_1-AGP) [4,5] and ovomucoid [6,7], have been employed successfully in HPLC. However, commercially available protein immobilized columns are expensive and often show poor efficiency [8].

Capillary electrophoresis (CE) represents another approach for achieving separations of stereoisomers

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of drugs and biological solutes. The pioneering work of Terabe et al. [9], utilizing a run buffer modifier to alter selectivity in CE, was demonstrated with micelles to separate neutral molecules. The first use of CE for chiral resolution was reported by Gassmann et al. [10], who used Cu^{2+} in combination with an amino acid as run buffer additives to incorporate a chiral pseudophase into the system. Since then, a number of pseudophases have been used to incorporate stereoselectivity in CE. Most notably, cyclodextrin and its derivatives have been widely used to form inclusion complexes with enantiomers [11–13]. Capillary affinity zone electrophoresis (CAZE) represents another approach for performing chiral separations in CE by using peptides or proteins that exhibit natural chiral sites as pseudostationary phases. Many CAZE studies have been reported recently on successful separations of stereoisomers using BSA [14,15], human serum albumin (HSA) [16,17], α_1-AGP [15] and ovomucid [18] as selectors. The major factor contributing to stereoselectivity in CAZE is the differential interaction of the stereoisomers with the chiral pseudostationary phase. In the case of drug–protein equilibria, interactions may be characterized by equilibrium binding constants. Consequently, it has been demonstrated that drug–protein binding constants may be determined by CAZE [19–21].

Concomitantly administered drugs can influence one another's binding to proteins, by competition for receptor sites. Such competition of drugs that partially displace one another from albumin sites can lead to altered pharmacological action. For example, administration of aspirin influences drug–albumin binding for acetrizoate, flufenamic acid and phenylbutazone, by permanent acetylation of a lysine residue within the peptide A region of albumin [22]. In addition, displacing drugs may bind to albumin at sites that do not bind the drug of interest, leading to allosteric effects and structural changes in the tertiary conformation of albumin. Recent CAZE studies show that the addition of drug displacers to a protein-containing run buffer can reduce enantioselectivity of the protein by competition [21,23]. Lloyd et al. [21] demonstrated that the strength of solute–protein binding for HSA may be modified in this way, leading to measurable changes in the net mobility of the solute. Arai et al. [23] used displacers to alter the enantioselectivity of HSA for racemic ofloxacin and suggested that CAZE may allow screening of drug–displacer interactions with proteins.

In this paper, the effect of displacers on the stereochemical resolution of folinic acid, mandelic acid and N-benzoylalanine at pH 7.0, using BSA as a pseudostationary phase in CAZE is described. The displacers are selected from a representative group of salicylates and include salicylic acid, α-resorcylic acid, β-resorcylic acid, γ-resorcylic acid and gentisic acid. The results indicate that displacers may be used to optimize separations of stereoisomers in CAZE and to screen drug–displacer interactions with BSA. The effect of dextran concentration on the retention of BSA and the resolution of sample stereoisomers is also discussed.

2. Experimental

2.1. Chemicals

Folinic acid (Fig. 1) and 3-(trimethoxysily)propyl methacrylate (97%) were purchased from Aldrich (Milwaukee, WI, USA). DL-Mandelic acid (Fig. 1) and N-benzoyl-DL-alanine (Fig. 1), salicylic acid,

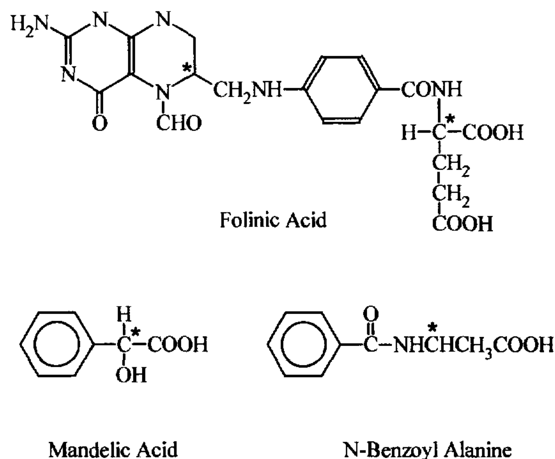

Fig. 1. Molecular structure of stereoisomers analyzed in this investigation. Asterisks indicate the chiral center. There are potentially four different diastereomers for folinic acid, although only two isomers (those involving the six- position on the pteridine ring) are in the sample analyzed in this work.

α-resorcylic acid, β-resorcylic acid, γ-resorcylic acid, gentisic acid, dextran (M_r 2 000 000) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were all obtained from Sigma (St. Louis, MO, USA). Bovine serum albumin fraction V (fatty acid free) and ammonium persulfate were purchased from ICN Biomedicals (Aurora, OH, USA). Acrylamide (electrophoresis grade) was obtained from Bio-Rad (Richmond, CA, USA). Sodium phosphate monobasic, hydrochloric acid, acetic acid and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Equipment

The experiments were performed on a laboratory-constructed integrated instrument using a Glassman Model PS/MK30P02.5 high-voltage power supply (Glassman, Whitehouse Station, NJ, USA) and a high-power-supply local control (Chamonix Industries, Binghamton, NY, USA). The detector was a Spectra-Physics Model 100, set at 230 or 280 nm and was interfaced with a Hewlett-Packard HP3396 Series II integrator (Hewlett-Packard, Palo Alto, CA, USA). Fused-silica capillaries, with the dimensions 360 μm O.D.×75 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) were coated with linear polyacrylamide by the method reported by Hjerten [24]. The effective length of the capillaries was either 50 or 60 cm, with a total length 100 cm. The applied electric field was 265 V/cm.

2.3. Procedure

Samples were prepared at desired concentrations in doubly deionized water. Run buffers were prepared with 10 m*M* sodium phosphate monobasic in doubly deionized water containing appropriate amounts of BSA and/or salicylate, when necessary. Solutions were filtered through 0.45 μm membrane filters and degassed. The buffer pH was adjusted to 7.0 using dilute NaOH, after the addition of modifiers and prior to use. All experiments were performed at room temperature. Samples were electrically injected (4 kV×1 s) at the cathode, unless otherwise noted. Capillaries were rinsed with water for 30 min, followed by run buffer for 20 min prior to use and 20 min with run buffer in between runs.

3. Results and discussion

3.1. Effect of dextran concentration on the migration of BSA

The effective electrophoretic mobility of folinic acid and BSA as a function of dextran concentration was measured under the electrophoretic conditions listed in Section 2.3. These values were adjusted by subtracting the electroosmotic flow ($3\cdot10^{-5}$ cm^2/V s) in the capillary. Both species are negatively charged at pH 7.0 and flow towards the anode. Since the sample and protein migrate in the same direction, a larger difference between their mobilities should improve chiral separation. The effective mobility of the protein was altered significantly more than that of the drug between 0 and 3% (w/v) dextran, while the reverse was observed for dextran concentrations greater than 3%. As the dextran concentration was increased, the overall effective mobilities of all analytes studied in this work decreased. This relationship is expected, since electrophoretic mobility is inversely proportional to buffer viscosity.

However, the rate of decrease for folinic acid and BSA differed. This can be explained by polymer network effects [25]. The molecular mass of BSA (~65 000) is much greater than that of folinic acid. At dextran concentrations below 3%, the polymer network (ca. dextran) exhibits a much larger "size exclusion" effect on the mobility of the larger species. Consequently, the mobility of BSA decreases considerably more than that of folinic acid upon initial addition of dextran to the buffer. At dextran concentrations greater than 3%, the polymer network appears to influence the mobility of folinic acid more than that of the BSA. Fig. 2 shows a plot that represents the difference in effective mobilities of the folinic acid and BSA with varying concentrations of dextran added to the run buffer. A maximum value was achieved using 3% dextran in the run buffer. The data shown in this plot indicates that the use of 3% dextran in solution is optimal for the resolution of stereoisomers of folinic acid, using BSA as a chiral pseudostationary phase.

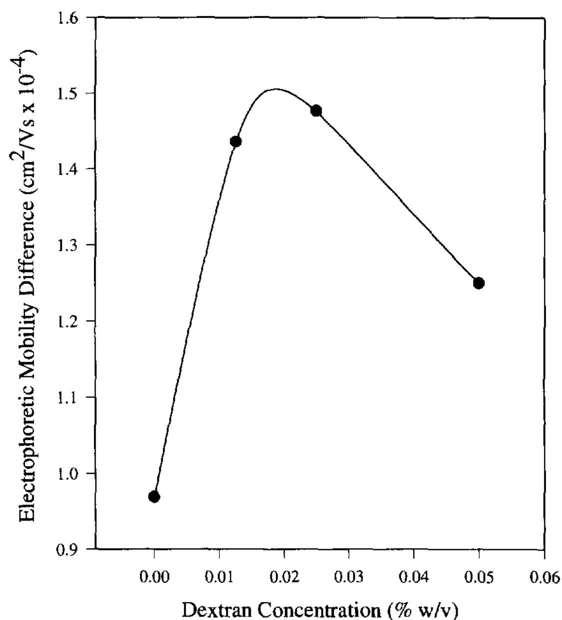

Fig. 2. Differences in the effective electrophoretic mobilities of folinic acid and BSA with varying concentrations of dextran added to the run buffer. Conditions: linear polyacrylamide-coated capillary with a 50 cm effective length, 100 cm total length; 1 s injection at 4 kV, running electric field strength 265 V/cm; 10 mM phosphate buffer, pH 7.0; detection wavelength at 210 nm (BSA) and 280 nm (folinic acid).

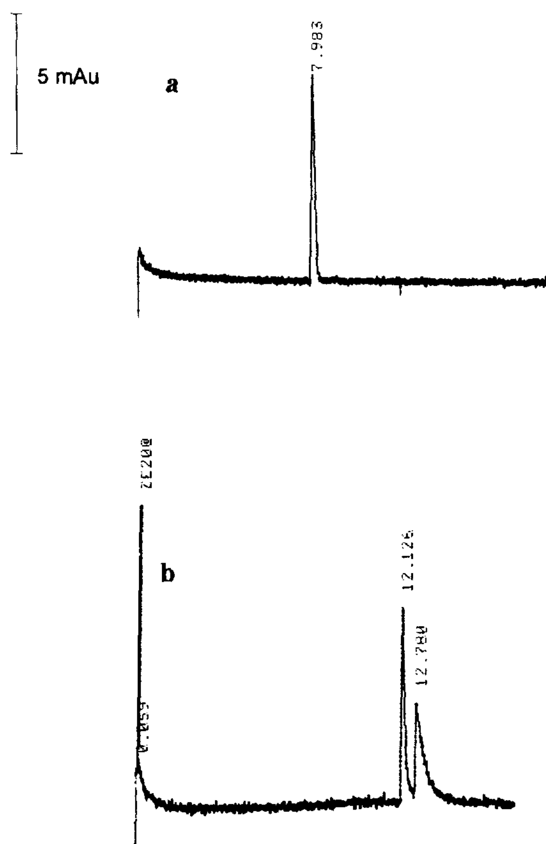

Fig. 3. Electropherogram of folinic acid using phosphate buffer at pH 7.0 and using (a) BSA (34 μM) and (b) dextran (5%, w/v) as run buffer modifiers. Conditions: sample concentration, 1 mg/ml, 1 s injection at 4 kV; capillary dimensions, 360 μm O.D.×75 μm I.D., coated with linear polyacrylamide; running electric field strength, 265 V/cm, pH 7.0, 10 mM phosphate; detection wavelength, 280 nm.

3.2. Chiral separation of folinic acid using dextran and BSA as buffer additives

Fig. 3 shows the separation of folinic acid diastereomers at pH 7.0, with 5% dextran and 32 μM BSA added to the run buffer. A dextran concentration of 5% was chosen in place of the previously determined optimum value of 3% (Fig. 2) for two reasons. First, dextran increases buffer viscosity, which indirectly contributes to an increase in peak efficiency [26]. Second, dextran may suppress protein–wall interactions by competing for sites on the interior surface of the capillary. Capillaries exhibited typical lifespans of 25 h running time.

The concentration of the protein selector in the running buffer plays an important role in the resolution of folinic acid (Fig. 4). An increase in the concentration of BSA in run buffer is analogous to increasing the phase ratio in chromatography, leading to an increase in retention for folinic acid. The effect of added protein to the run buffer has been shown to increase viscosity only minimally and therefore does not contribute to electrophoretic mobility changes [21]. It should be noted that these trials were also performed without dextran added to the run buffer. These results showed that resolution increased with increasing BSA concentration. However, BSA concentrations necessary to achieve resolution greater than 1.0 were larger than 40 μM and protein–wall interactions became pronounced. In addition, stereochemical resolution was found to be inversely proportional to sample injection time, and 1 s injection times were used in the experiments for folinic acid (Fig. 4).

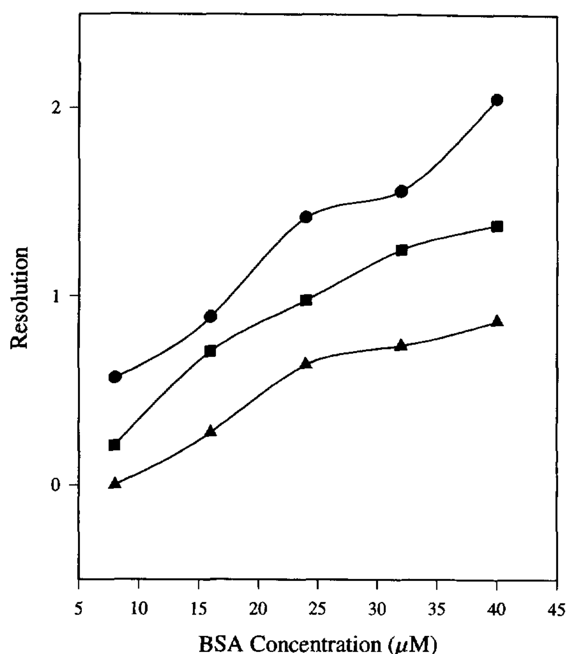

Fig. 4. Effect of protein concentration in run buffer on the stereoselectivity of folinic acid. Conditions: sample concentration 1 mg/ml, 4 kV for 1 s (●), 3 s (■) and 5 s (▲); capillary dimensions, 360 μm O.D.×75 μm I.D., coated with linear polyacrylamide; running electric field strength 265 V/cm, pH 7.0, 10 mM phosphate; detection wavelength, 280 nm.

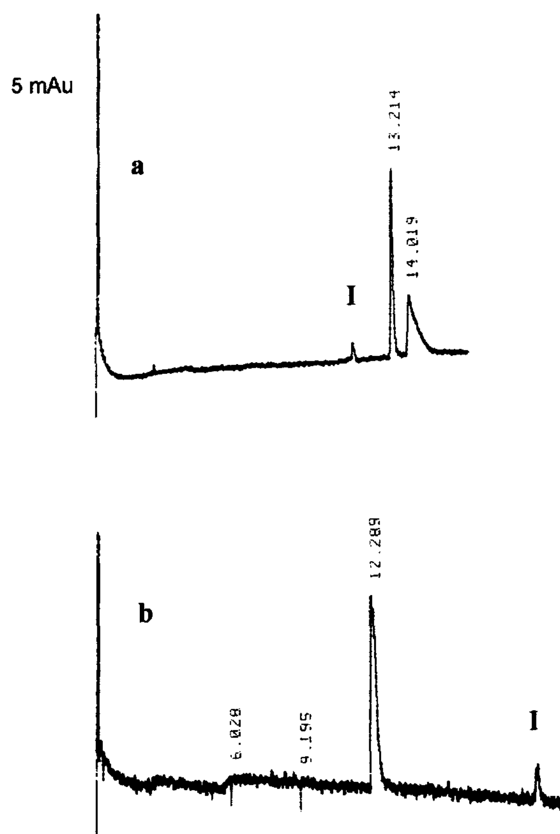

Fig. 5. Electropherograms of folinic acid using BSA (34 μM) in combination with 5.0 mM α-resorcylic acid (a) and 1.0 mM γ-resorcylic acid (b), added to the running buffer. Conditions: sample concentration, 1 mg/ml; 1 s injection at 4 kV; capillary dimensions, 360 μm O.D.×75 μm I.D., coated with linear polyacrylamide; running electric field strength, 265 V/cm, pH 7.0, 10 mM phosphate; detection wavelength, 280 nm. I indicates an interference peak.

3.3. Evaluation of displacer–protein phases on stereoselectivity of BSA: chiral resolution

The effect of the addition of displacer on the stereoselectivity of BSA was studied. In general, chiral resolution and effective mobility of the samples studied decreased as the concentration of displacer increased. However, the use of α-resorcylic acid as a displacer improved the stereoselectivity and reduced sample mobility. Fig. 5 shows electropherograms of folinic acid, with BSA, in combination with α-resorcylic acid (Fig. 5a) and γ-resorcylic acid (Fig. 5b), added to the running buffer. Fig. 6 shows a plot that summarizes the observed effect of concentration of the five different salicylates on the stereoselectivity of folinic acid. The results provide insight into the affinity and stereoselective recognition of BSA for the drugs in the presence of competing displacers. The observed effects are a result of one or both of the following mechanisms [27]: (1) direct competition between folinic acid and the displacer for the same binding site(s) and (2) alteration of the affinity of BSA for the folinic acid isomers via allosteric interactions caused by displacer–protein binding at other sites. Arai et al. [23] used displacement measurements in CAZE and concluded that the simple model of three primary drug binding sites on albumin is an approximation at best. The information shown in Fig. 6 supports their findings. If mechanism 1 played a critical role in the stereochemical resolution of folinic acid, then a decrease in the resolution of

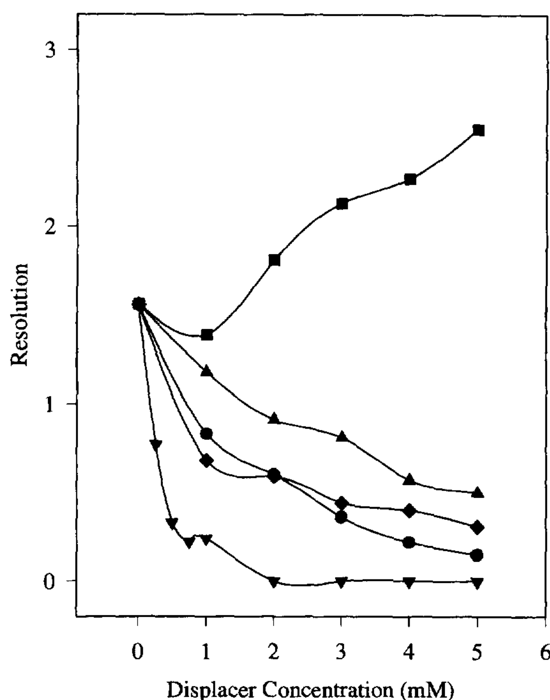

Fig. 6. Effect of displacer concentration in run buffer on the resolution of folinic acid diastereomers using BSA (34 μM) as a chiral selector. Conditions: sample concentration, 1 mg/ml; 1 s injection at 4 kV; capillary dimensions, 360 μm O.D.×75 μm I.D., coated with linear polyacrylamide; running electric field strength, 265 V/cm, pH 7.0, 10 mM phosphate; detection wavelength, 280 nm. Key: ■, α-resorcylic acid; ▲, β-resorcylic acid; ●, salicylic; ◆, gentisic acid and ▼, γ-resorcylic acid.

folinic acid diastereomers would be expected if salicylates were added to the run buffer. However, the addition of γ-resorcylic acid improves the stereoselectivity dramatically. In addition, the effective mobility of folinic acid decreases with increasing concentrations of γ-resorcylic acid, corresponding to an increase in folinic acid–BSA binding affinity. McMenamy [28] pointed out that the binding of organic anions occurs inside a major crevice in albumin. When this crevice opens to accommodate a ligand, the affinity at other ligand sites may be increased or inhibited.

It is important to note that the change in effective mobility and resolution of folinic acid stereoisomers with different displacers added to the run buffer in this work did not correlate with reported equilibrium binding constants (K_b) for the displacer–BSA complexes [29]. For example, the K_b for salicylic acid is $3.0 \cdot 10^4$ (primary site) and $8.0 \cdot 10^2$ (secondary site), whereas the K_b for γ-resorcylic acid is $1.6 \cdot 10^4$ (primary site) and $1.5 \cdot 10^2$ (secondary site). If mechanism 1 dominated, one would expect salicylic acid to reduce retention and stereoselectivity to a greater extent than γ-resorcylic acid. However, the reverse is observed. Consequently, mechanism 2 must play an important role in the observed stereoselectivity changes shown in Fig. 6. This conclusion is supported by the observed increase in retention and stereoselectivity for folinic acid when α-resorcylic acid is used as the displacer.

The results of displacers on the chiral resolution of the samples studied in this investigation are summarized in Table 1. The resolution values for the samples studied in the absence of the displacers were determined to be 1.56, 1.07 and 0.7 for folinic acid, mandelic acid and N-benzoylalanine, respectively. For the compounds studied in this work, α-resorcylic acid improved resolution for two of the isomer mixtures, i.e., folinic acid and mandelic acid. The remaining salicylates acted by decreasing the

Table 1
Summary of the effect of displacers on the resolution of stereoisomers studied

Sample	Resolution				
	Salicylic acid	α-Resorcylic acid	β-Resorcylic acid	γ-Resorcylic acid	Gentisic acid
Folinic acid	0.83	1.39	1.18	0.24	0.68
Mandelic acid	0	0.57	0.60	0	0
N-Benzoylalanine	0	1.12	0.20	0	0.52

Conditions: sample concentration, folinic acid, 1 mg/ml; mandelic acid, 0.2 mg/ml; N-benzoylalanine, 4 mg/ml; 1 s injection at 4 kV; capillary dimensions, 360 μm O.D.×75 μm I.D., coated with linear polyacrylamide; running electric field strength, 265 V/cm; pH 7.0, 10 mM phosphate, 2 mg/ml BSA; displacer concentration, 1 mg/ml; detection wavelength, 280 nm (folinic acid), 220 nm (mandelic acid) and 228 nm (N-benzoylalanine).

stereoselectivity of the protein, with γ-resorcylic acid exhibiting the greatest effect on all of the samples.

Peak efficiency (*N*) was also altered when the displacers were used in the run buffer. Fig. 7 shows a plot of the observed changes in *N* for the first eluting isomer of folinic acid as a function of displacer concentration. There is a general trend for peak efficiency to increase as displacer concentration increases, and this phenomena is consistent with the CE theory that efficiency increases as migration time decreases. However, a significant increase in *N* is observed when α-resorcylic acid is used as the displacer. This increase is inconsistent with CE theory, as folinic acid is more retained when using this salicylate as a displacer, and a corresponding decrease in *N* would be expected. The increase in *N* is apparently related to an increase in the kinetics of mass transport between the solute and protein. This theory is supported by mechanism 2, where the mass transport of folinic acid with the protein selector is influenced by allosteric interactions caused by displacer–protein binding at other sites.

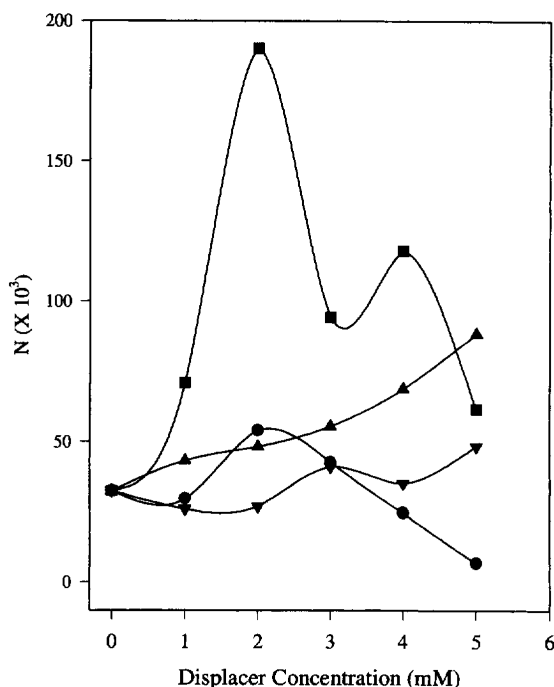

Fig. 7. Effect of displacer concentration in run buffer on chromatographic peak efficiency for the first eluting isomer of folinic acid using BSA (34 μ*M*) as a selector. Conditions: as for those listed in Fig. 6. Key: ■, α-resorcylic acid; ▲, β-resorcylic acid; ▼, gentisic acid and ●, salicylic acid.

3.4. Effect of displacer on the mobility of BSA

A study was done to determine the effect of salicylate type on the effective mobility of the protein selector. The purpose of the study was to determine the extent of change in the electrophoretic mobility of the protein in the presence of the displacers. A significant change in mobility should have a large influence on sample migration and consequently on chiral resolution. In the study, BSA was selected as the sample, and the run buffer consisted of 5% dextran and 0 to 2 m*M* salicylate ion, pH 7.0. The results show that four of the five displacers reduced the net electrophoretic mobility of the protein selector by approximately 10%. The salicylate, α-resorcylic acid, reduces the mobility of BSA by about 25%. The results indicate that changes in the selector mobility upon addition of displacer to the affinity run buffer may contribute to changes in the chiral separation characteristics of CAZE. A decrease in protein mobility should lead to a direct increase in the chiral resolution of the solutes studied. However, α-resorcylic acid was the only displacer that improved the stereoselectivity of BSA. These results indicate that the observed changes in sample mobility and chiral resolution are a function of protein mobility changes and modification of sample-protein binding affinity.

4. Conclusion

In this paper, the effect of displacement interactions on the resolution of the stereoisomers of folinic acid, mandelic acid and N-benzoylalanine, using BSA as a pseudostationary phase in CAZE is described. The results indicate that chiral resolution may be modified using the protein selector in the presence of displacers that are known to bind with the selector. In addition, CAZE may be useful for the investigation of drug–protein interactions. The addition of α-resorcylic acid to the protein-modified buffer resulted in an increase in the retention and resolution of stereoisomers. This can be explained by

the following mechanism; alteration of the affinity of BSA for the samples studied occurs via allosteric interactions caused by displacer–protein binding at other sites. Additionally, the mass transport of the binding mechanism of the sample with the protein selector may also be influenced by allosteric interactions caused by displacer–protein binding at other sites.

The results of this study should be useful in the development and optimization of chiral separations using CAZE. The data suggest that displacers may be used as a means of enhancing retention and stereoselectivity, when using proteins as chiral pseudostationary phases. In addition, this investigation supports recent claims that CAZE may be used to screen drug–displacer interactions with protein additives.

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