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Effect of organic modifiers on retention and enantiomeric separations by capillary electrophoresis with human serum albumin as a chiral selector in solution

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Abstract

We have investigated the effect of methanol, ethanol, 1-propanol, 2-propanol and acetonitrile on the retention and enantiomeric separations of benzoin and propiomazine by capillary electrophoresis, using human serum albumin as a chiral selector. The effects of these modifiers on mobilities of analytes are rather difficult to interpret. Calculation of capacity factors reveals the underlying analyte–protein interactions; pitfalls in making such calculations are pointed out. In the case of benzoin and propiomazine binding to human serum albumin, capacity factors were observed to always decrease upon addition of organic modifiers, although the effects of 1- and 2-propanol suggest a possible specific interaction or modification of the protein conformation.

Keywords: Enantiomer separation; Organic modifiers; Background electrolyte composition; Benzoin; Propiomazine; Albumin

1. Introduction

A variety of protein-based high-performance liquid chromatography (HPLC) phases have been developed, particularly for chiral separations [1]. As well as their analytical role, such columns have been used to probe protein–drug binding interactions [2]. More recently, protein additives to the background electrolyte (BGE) in capillary electrophoresis (CE) have been employed as chiral selectors [3]. Although this approach has some limitations for analytical purposes, the use of proteins in CE has attracted attention for studies of protein–drug interactions both in non-chiral [4] and chiral systems [5]. The small quantities of protein and ligand needed make such an approach attractive and, in the case of chiral analytes, the ability to perform simultaneous separation and measurement of binding strength for each enantiomer is useful.

When using protein chiral stationary phases (CSPs) in HPLC, it is common to use significant proportions (e.g., up to 15% or more) of organic modifiers such as 1-propanol to reduce the retention of many analytes to acceptable values. Often the

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function of the modifier is to reduce hydrophobic interactions between the protein and the analyte [6]. However, at the highest concentrations of modifier used, one must be wary of also affecting binding through changes in the protein's conformation. This explanation has been used to explain a number of anomalous observations using protein CSPs [2,7] and it seems reasonable when the denaturant effect of organic solvents is considered [8]. In CE, proteins are sometimes used without organic modifier, or with a relatively low proportion of modifier in the BGE compared with typical HPLC experience; even under such conditions, capacity factors are often rather low. This can be explained by considering the effective concentration of protein in a HPLC CSP (approaching millimolar levels) compared to those typically used in CE (low to tens of micromolar). When one accounts for this large difference in protein concentration in the two systems, a quantitative link can be made between the capacity factors of an analyte determined in HPLC and in CE [9].

Despite the typically lower amounts of organic modifier used in CE with proteins, there are still reports of anomalous retention behaviour (i.e., increases in retention) upon addition of organic modifiers. In this article, we describe an investigation of the effects a variety of organic modifiers (acetonitrile, methanol, ethanol, 1- and 2-propanol) have on the analysis of benzoin and propiomazine using human serum albumin (HSA) as a BGE additive. The effects of each modifier on the mobility of analyte are presented. It is shown that the mobility data are rather difficult to interpret. This is principally due to alteration of the BGE's viscosity and dielectric constant by the modifier, which influences the effective mobilities of the analyte and the selector. In the case of 1- and 2-propanol (the most denaturing modifiers [8]), a change in protein conformation may also occur. As in HPLC, the capacity factor can be used in electrokinetic chromatography (EKC) as a measure of the underlying analyte–selector interactions [10]. The effect of the modifiers on k' for benzoin and propiomazine enantiomers is shown, and it is explained in terms of reduction of hydrophobic interactions and possibly specific competition and/or protein denaturation. Some of the pitfalls in the determination of k' are pointed out.

2. Experimental

Unless otherwise stated, all buffer salts were of analytical reagent-grade; the organic modifiers, methanol, ethanol, 1-propanol, 2-propanol and acetonitrile were of HPLC grade (Anachemia, Montreal, Canada). Fatty-acid free fraction V HSA, benzoin and propiomazine were purchased from Sigma (St. Louis, MO, USA).

An Applied Biosystems (Foster City, CA, USA) model 270A-HT capillary electrophoresis system was used and this was equipped with a variable-wavelength UV absorbance detector that was operated at 247 nm with a 0.5-s rise time. Data were analysed using a Spectra-Physics (San Jose, CA, USA) DataJet integrator and stored on a personal computer running Spectra-Physics Winner System software.

Electrophoresis was performed using 72 cm long× 50 μm I.D., 365 μm O.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA); the length of the capillary to the detector window was 50 cm. After installation, the capillary was rinsed for about 30 min with 1 *M* NaOH, followed by water for 12 min and BGE for 10 min. Between runs, the capillary was rinsed with 0.1 *M* NaOH, water and run buffer for 2 min each. Without the NaOH rinse, capillary blockages would sometimes occur when using the protein-containing BGE. All washes and rinses were performed using a vacuum of ≈70 kPa applied to the capillary outlet. Separations were performed at 30 kV, which resulted in a running current of 50–60 μA. The oven temperature was set at 30°C. Samples were introduced into the capillary by vacuum injection for a time of 1.0 s, with a vacuum of 17 kPa. Immediately before the sample, an equivalent volume of water was injected to give a more distinct electroosmotic flow (EOF) marker peak.

All buffers were prepared fresh daily using water from a Milli-Q50 unit (Millipore, Montreal, Canada). Prior to use, all solutions were filtered through a 0.45-μm membrane filter and degassed by sonication. Phosphate buffer for the BGE was prepared by mixing 100 m*M* solutions of analytical grade dibasic sodium hydrogen phosphate and monobasic sodium hydrogen phosphate (BDH, Toronto, Canada) to give

a pH value of 7.0. An appropriate amount of HSA was dissolved in the 100 m*M* sodium phosphate buffer to obtain a 70 μ*M* solution of HSA. Dissolution was aided by sonication. The above solution was diluted with appropriate amounts of water and organic modifier to give a BGE containing 50 m*M* phosphate buffer, 35 μ*M* HSA and the desired volume percentage of organic modifier.

The analytes used in this study were racemic benzoin (100 μ*M*, dissolved in water–methanol, 99:1, v/v) and racemic propiomazine (100 μ*M*, dissolved in water), both from Sigma. The effective mobility of the analyte was calculated using the baseline disturbance from the water marker as an indication of EOF. Mobilities were measured for both benzoin and propiomazine in BGEs containing HSA and various proportions of modifier, as shown in the figures.

In order to calculate the capacity factors, effective mobilities of propiomazine and HSA were also measured in each of the BGEs containing different concentrations of organic modifiers (but without HSA) using similar separation conditions to those described above.

The relative viscosities of each of the BGEs using different concentrations of organic modifiers were measured by timing the elution of a marker sample (10% acetone in water), which was sucked through the capillary using a 17-kPa vacuum (which is precisely controlled on the Applied Biosystems instrument used). The relative viscosity is found by dividing the elution time of the marker in the buffer–organic modifier mixture by that of the marker sucked through the capillary containing a purely aqueous buffer solution. The accuracy of this method has been validated previously [11].

3. Results and discussion

3.1. Effect of the organic modifier on the mobility of the analyte in HSA-containing BGEs

Separations of the analyte enantiomers were performed using increasing concentrations of the organic modifiers mentioned above. Addition of organic modifiers resulted in an alteration in the analyte's mobility and enantioselectivity. Typical electropherograms for analyses of benzoin using 0, 2, 4 and 6% (v/v) ethanol as an organic modifier in the BGE are shown in Fig. 1. Separations with propiomazine were similar to those previously published [5]. There was relatively poor efficiency and peak tailing when no organic modifier was used, but these characteristics improved upon addition of organic modifier. The analyte migration times were measured from the peak maxima. The EOF point was marked by a rather broad deviation from the baseline with first a downward and then an upward-going excursion. The negative peak appears to be an artifact [12] that varies somewhat with the type and concentration of organic modifier. Taking the positive peak as the EOF point gave consistent measurements when using either water or acetone as a marker. The EOF and analyte migration time data were used to calculate the analyte's effective mobilities, μ_{eff}.

The effect of organic modifiers on μ_{eff} for benzoin and propiomazine enantiomers separated using a HSA-containing BGE is illustrated in Figs. 2 and 3. For clarity, each figure is divided into parts A (containing data for methanol and 2-propanol) and B (with data for 1-propanol, ethanol and acetonitrile). Measurements of mobility were made in triplicate, and the relative standard deviation in these measure-

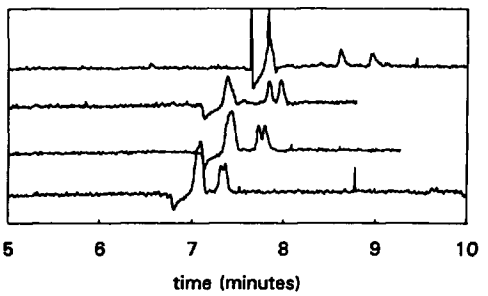

Fig. 1. Effect of the addition of ethanol to the BGE on the separation of benzoin enantiomers using HSA as a chiral selector. Separation conditions are as described in Section 2, with (top to bottom) 0, 2, 4 and 6% (v/v) ethanol in the BGE. The EOF point is marked by the positive peak at the baseline inflection, with the two peaks from the benzoin enantiomers migrating afterwards. With increasing concentrations of ethanol, the benzoin peaks approach the electroosmotic flow point, as the interaction of these neutral enantiomers with HSA is weakened.

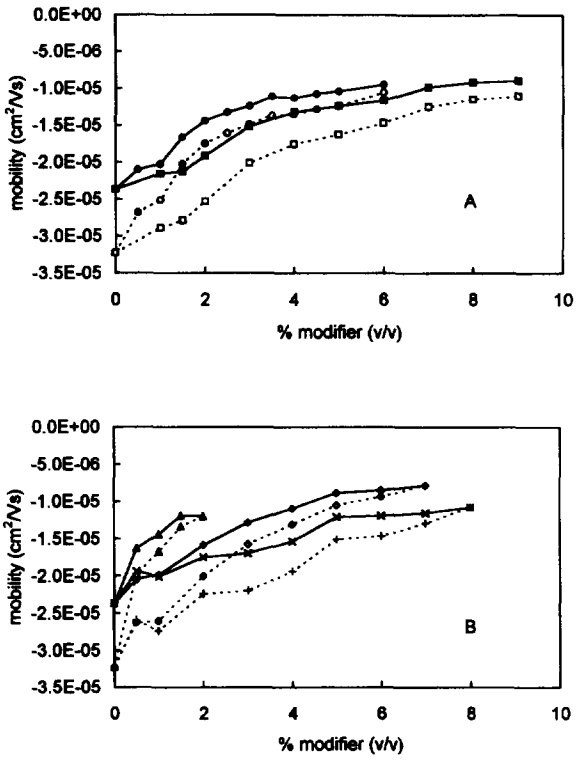

Fig. 2. Effect of the addition of organic modifiers on the effective mobility of the enantiomers of benzoin. Separation conditions are as described in Section 2. A: ■, methanol and ●, 2-propanol. B: ×, acetonitrile, ♦, ethanol and ▲, 1-propanol. Open and closed symbols are used to indicate the mobilities of the two enantiomers.

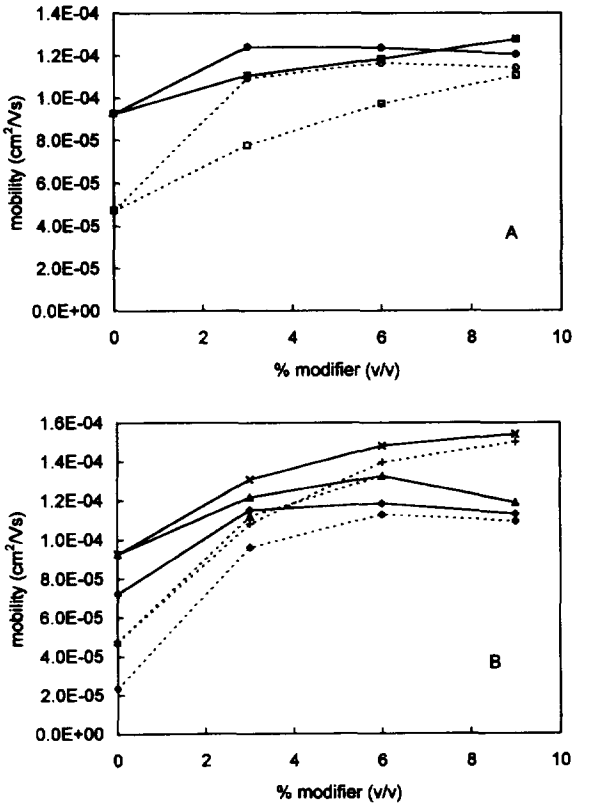

Fig. 3. Effect of the addition of organic modifiers on the effective mobility of the enantiomers of propiomazine. Separation conditions are as described in Section 2. A, ■, methanol and ●, 2-propanol. B, ×, acetonitrile, ♦, ethanol and ▲, 1-propanol. Open and closed symbols are used to indicate the mobilities of the two enantiomers.

ments was typically 5% or less (error bars have been omitted from most of the figures for clarity).

Benzoin is uncharged under the conditions used, thus, with no binding to the HSA, it would migrate by electroosmosis alone; in the presence of the protein, benzoin has a negative mobility. Propiomazine is a cation at the analytical pH, with an effective mobility in BGE with no protein or organic modifier of $\approx 1.8 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. Its binding to HSA reduces its positive mobility. At least at low proportions of modifier, all modifiers appear to cause a reduction in binding, as evidenced by a change in μ_{eff} towards the value obtained in the absence of HSA. With all organic modifiers, μ_{eff} changes rapidly to begin with, however, the effect quickly levels off and, in some cases, there is even an inflection in the mobility curves at higher concentrations of modifier, at least for propiomazine with, e.g., 1- and 2-propanol and ethanol (Fig. 3). This inflection is difficult to explain in terms of the effect of the modifier on protein–analyte binding, since normally one would expect increasing modifier concentrations to cause a weakening of this binding.

In no case does the mobility of benzoin reduce to zero upon addition of organic modifier, indicating a certain residual binding to HSA. In terms of eliminating enantioselectivity (judged by the percentage of modifier needed to eliminate stereoselectivity), the order of effectiveness of the modifiers with benzoin is: 1-propanol, ethanol and 2-propanol, acetonitrile, methanol. With propiomazine, more modifier is needed to eliminate enantioselectivity. This is not surprising, given that the binding constant of benzoin to HSA is approximately an order of

magnitude less than that of propiomazine [3,5]. The order of effectiveness of the modifiers is the same as for benzoin.

3.2. Calculation of capacity factors

Because the effective mobility of the analytes is controlled by several parameters, plots such as those in Figs. 2 and 3 can only give a qualitative understanding of the effect of organic modifiers on the HSA–analyte interaction. Although the inflections in the mobility curves could be interpreted as being due to increases in binding due to alterations in the protein's conformation at higher concentrations of modifier [2,7,8], they could also be the result of changes in the effective mobilities of the analyte and protein because of alterations in the viscosity and dielectric constant of the BGE by the modifier.

The underlying interaction between the protein and analytes can be investigated if the capacity factor, k', is calculated. In terms of effective mobilities,

$$k' = (\mu_{eff} - \mu_0)/(\mu_{comp} - \mu_{eff}) \quad (1)$$

where μ_0 is the effective mobility of the analyte without HSA present in the BGE, and μ_{comp} is the mobility of the HSA–analyte complex [13]. For binding of benzoin, we assume that $\mu_{comp} = \mu_{HSA}$, the effective mobility of HSA, since there is no change in charge and only an insignificant increase in the molecular mass of HSA. The propiomazine–HSA complex will have an overall charge that is +1 greater than that of HSA alone (assuming 1:1 binding) and, thus, one would expect μ_{comp} to be significantly less than that of μ_{HSA}. We have previously measured μ_{comp} for thioridazine (structurally similar to propiomazine and with the same +1 charge) bound to HSA and found this to be 82% of the mobility of the free protein [3]. For calculation of k' for propiomazine in this study, we adjusted the measured HSA mobilities by the same factor (assuming 1:1 binding for both phenothiazines with HSA). Correction of μ_{eff} values for changes in viscosity on addition of the HSA are not necessary, since this is an insignificant effect at the concentrations of HSA used here [5]. However, μ_0 for propiomazine and μ_{HSA} are both affected by the addition of organic modifiers to the BGE. This was determined by taking measurements of the mobilities of HSA and propiomazine in BGEs containing varying percentages of each modifier, but no protein. The necessity of performing such measurements should be stressed, since failure to do so will result in the calculation of a physically meaningless "capacity factor" [14].

It is known that HSA will bind to the walls of a fused-silica capillary [5] and that this HSA will, in effect, be a stationary reservoir of adsorptive sites. However, the amount of surface-adsorbed HSA is small relative to that in solution, and a maximum k' of 0.02 due to wall-adsorbed HSA has been measured for the compounds investigated here [5].

3.3. Effect of the organic modifiers on the free analyte and HSA mobilities

The addition of organic modifiers has a significant effect on mobility, as described by the Hückel equation,

$$\mu = \zeta\epsilon/6\pi\eta \quad (2)$$

Where ζ is the zeta potential, η is the viscosity and ϵ is the dielectric constant. For the purpose of calculating k', one needs to measure μ_0 and μ_{comp}. If one assumes that ζ is invariant with added organic modifier, then μ_0 and μ_{comp} could even be calculated from the values obtained when no modifier is added, if the effect of the various modifiers on the viscosity and dielectric constant of the solution were known. However, it is questionable as to whether ζ is unchanging, particularly for proteins that may bind organic modifiers at their surface [7] or undergo conformational changes [2,7,8]. To investigate this, we compared μ_0 for propiomazine and μ_{HSA} at different concentrations of organic modifier. We also measured changes in the relative viscosity of the BGE due to the addition of organic modifiers, as described in Section 2. Changes in the dielectric constant were taken from the data of Akerlof [15] for aqueous–organic mixtures, except for acetonitrile, for which values were extrapolated from the results of Schwer and Kenndler [16]. From Eq. (2), it is apparent that a plot of $\mu\eta_r$ against ϵ_r (subscript r indicates relative viscosity or dielectric constant) would give a straight line with a slope that is proportional to ζ if ζ was invariant; non-linearity

would reveal a variation in ζ with the composition of the BGE. Such plots are shown for HSA (Fig. 4a) and propiomazine (Fig. 4b). In the case of propiomazine, there is apparently a linear relationship with each of the organic modifiers, and the slopes are similar with all of the modifiers. In contrast, HSA appears to show two different behaviours. The slopes of the lines for acetonitrile, methanol and ethanol are similar. The 1- and 2-propanol data are similar to each other, but quite different from those of the other modifiers. Although there is considerable error (mostly due to errors in the determination of the effective mobilities), it is apparent that for HSA, ζ is lower with 1- and 2-propanol than with the other modifiers. It should be noted that 1- and 2-propanol are more strongly denaturing than the other modifiers used [8] and are known to bind strongly to other acidic globular proteins [7]. 1-propanol is also the most effective at reducing the enantioselectivity of HSA (Figs. 2 and 3).

3.4. Effect of the organic modifiers on capacity factors

Calculation of k' (Eq. (1)) reveals a decrease as a function of the concentration of organic modifier for benzoin and propiomazine. It is instructive to look at plots of log k' vs. the volume fraction of organic modifier, as shown in Fig. 5 Fig. 6. Generally, this relationship is linear in reversed-phase HPLC [17]. The effects of the modifiers acetonitrile, methanol and ethanol on log k' are reasonably linear (Fig. 5), while with 1- and 2-propanol, the effect seems to be distinctly non-linear.

1-Propanol is also by far the most effective at reducing retention and enantioselectivity; all chiral

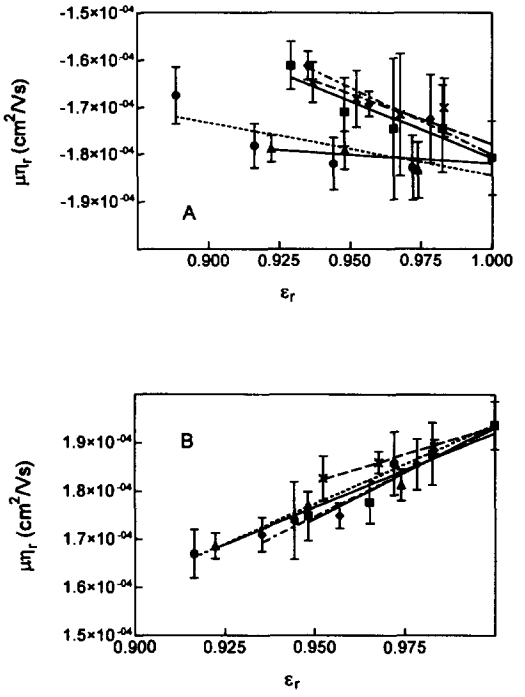

Fig. 4. Plots of $\mu\eta_r$ versus ϵ_r for HSA (A) and propiomazine (B). ■ and solid line, methanol; ● and dotted line, 2-propanol; × and dashed line, acetonitrile; ♦ and dot-dashed line, ethanol; ▲ and solid line, 1-propanol. Lines are linear regression fits to the data for each modifier. While the data for propiomazine seems to be relatively independent of the type of modifier used, that for HSA falls into two groups, indicating modifier-specific effects on the protein's mobility.

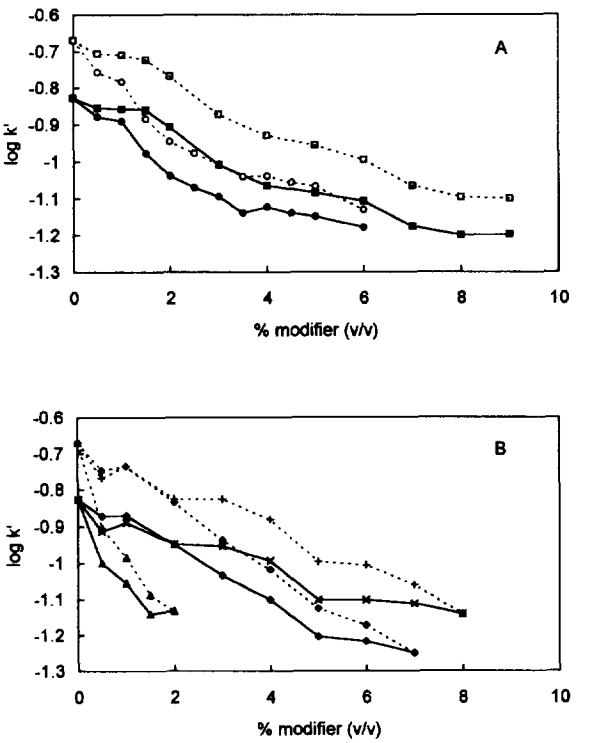

Fig. 5. Variation of log k' for benzoin with addition of organic modifier. Separation conditions are as described in Section 2. A, ■, methanol and ●, 2-propanol. B, ×, acetonitrile, ♦, ethanol and ▲, 1-propanol. Open and closed symbols are used to indicate values of log k' for the two enantiomers.

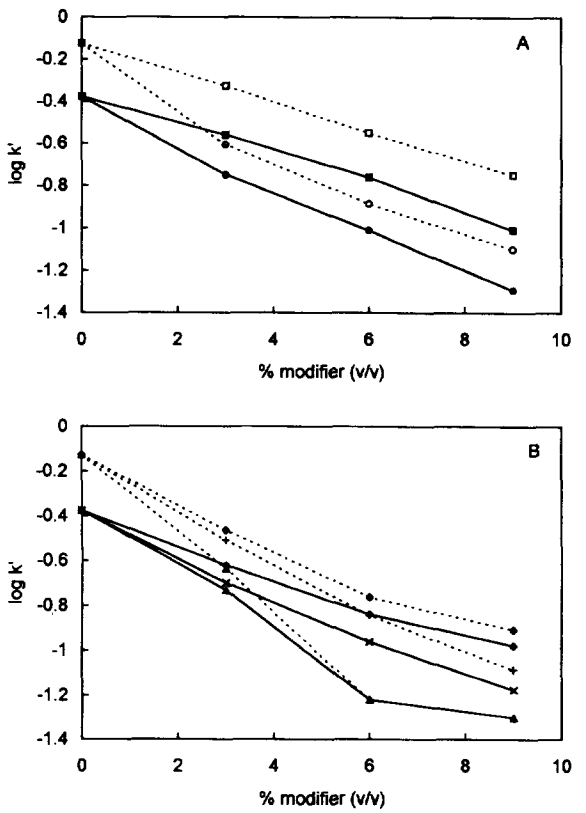

Fig. 6. Variation of log k' for propiomazine on addition of organic modifier. Separation conditions are as described in Section 2. A, ■, methanol and ●, 2-propanol. B, ×, acetonitrile, ♦, ethanol and ▲, 1-propanol. Open and closed symbols are used to indicate values of log k' for the two enantiomers.

separation being lost with only 2% of this modifier. For propiomazine, addition of each of the organic modifiers generally causes a relatively linear decrease in log k' (Fig. 6), although there are some deviations at >6% ethanol and 1-propanol.

With propiomazine, 1- and 2-propanol cause the greatest reduction in k' (k' with 9% of these modifiers is only ≈15% of the value obtained in the absence of modifier). Acetonitrile seems to be a slightly stronger modifier than ethanol, and methanol has the least effect (k' with 9% methanol is about 25% of the value obtained in the absence of modifier). With benzoin, the order is broadly similar except that acetonitrile has a smaller effect than ethanol. As noted in Section 1, k' and the quantity of modifier used here is quite low, compared to the amounts typically used in HPLC with a HSA chiral stationary phase, as a result of the relatively low concentration of protein used in CE [9].

Judging from the capacity factors (Figs. 5 and 6) and the effects of the modifiers on the mobility of the analyte and the protein (Fig. 4), it seems that the inflections in the analyte mobility curves (Figs. 2 and 3) come about because there are significant changes in the viscosity and dielectric constant of the BGE, which cause alterations in the mobilities of the analyte and selector, rather than an increase in binding, which might be attributed to modifier-induced changes in the protein's conformation. It is apparent from the k' data (Figs. 5 and 6) that increases in the concentration of organic modifier always produce a decrease in binding affinity, at least within the range of concentrations used in this study. Since benzoin and propiomazine bind at separate sites on HSA [5], it is not surprising to find some differences in the detail of their behaviour.

CE measurements have previously been reported using a similar system to that described here, i.e., benzoin (as well as some other analytes) with bovine serum albumin and 1-propanol as the modifier [18]. On addition of 1-propanol, enantioselectivity was shown to pass through a maximum, while capacity factors decreased at first and then increased. It is well known that a maxima in enantioselectivity may occur which is dependant on the affinity of the analyte for the selector and on the concentration of selector [19], but an increase in k' is unexpected; for this to be the case, the increase in affinity due to conformational alterations and an improvement of the "fit" between the ligand and the protein must outweigh the general weakening in hydrophobic binding interactions caused by the organic modifier. This observation [18] is in contrast to the data reported here and illustrates that one should be cautious when extrapolating results between bovine and human albumins. There are known differences in their binding properties and different methods of protein preparation may also give rise to dissimilar properties.

4. Conclusions

The effects of a variety of organic modifiers on the

binding of benzoin and propiomazine to HSA have been investigated. Plots of the effective mobility of analyte as a function of the proportion of added modifier are difficult to interpret because of associated changes in the viscosity and dielectric constant of the BGE and thus the effective mobilities of the analytes and selector. Calculation of the capacity factors at different modifier concentrations reveals the underlying ligand–protein interactions. To calculate capacity factors, the effect of changing the composition of BGE on the mobilities of analyte and selector must be accounted for, preferably by direct measurement, since unpredictable changes in mobility may occur with complex selectors, such as proteins, as is shown with the effect of 1- and 2-propanol on HSA. As previously reported [3], the mobility of the complex of a charged analyte and the selector is likely to be significantly different from that of the free protein, and this should also be taken into account when calculating capacity factors. In the systems studied here, the effect of the modifier was always to reduce the strength of analyte–selector binding. In general, there were only moderate differences in the effects of the different modifiers. The exception was 1-propanol, which eliminated enantioselectivity for benzoin at a volume fraction of 2%. It is possible that this modifier has a specific action at the benzoin binding site, or that it causes a change in the protein's conformation. Such effects should be considered when designing CE drug binding studies. To avoid using organic modifiers in such a case, one has some flexibility in simply being able to alter the protein concentration to tune k' to an acceptable value [9].

Acknowledgments

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