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Optimization of Micellar Electrokinetic Chromatography

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The separating power of micellar electrokinetic chromatography (MEC) for neutral solutes is critically examined. A theory for the optimization of resolution (R_s) and resolution per unit time (R_s/t_R) is presented and evaluated. Equations are derived that predict the optimum retention factor (k') and the corresponding surfactant concentration to use. Another equation is derived that relates the analysis time to five parameters: R_s, α (selectivity), k', t_o/t_{mc}, and H/v_{eo} (plate height/electroosmotic velocity). The capacity factor for the best *resolution* is given by $k' = (t_{mc}/t_o)^{1/2}$, where t_o and t_{mc} are the retention times of the aqueous and micellar phases, respectively. The capacity factor for the best *resolution per unit time* ranges from 1.2 to 2. The separations obtained by using the optimum capacity factor for resolution per unit time instead of resolution may be slightly poorer in terms of resolution but are much faster. Provided the optimum conditions are employed, the quality of MEC separations is independent of the solute hydrophobicity. The advantages of a surfactant gradient for the separation of solutes with a wide range of hydrophobicities are discussed. A brief comparison of MEC, micellar liquid chromatography, and conventional high-performance liquid chromatography is also made.

INTRODUCTION

Micellar electrokinetic chromatography (MEC) is a relatively new and important modification of capillary zone electrophoresis (CZE) that allows neutral molecules to be separated via their differential distribution between an aqueous mobile phase and a pseudostationary micellar phase (*1*, *2*), the latter created by increasing the concentration of an ionic surfactant above its critical micelle concentration (cmc). Whereas the bulk aqueous phase migrates at a velocity strictly determined by electroosmotic flow, the migration of the micelles is retarded due to the additional opposing electrophoretic force exerted on the charged micelle. The result of the neutral solutes' differential distribution between the mobile aqueous phase and the slower moving ("stationary") micellar phase is a difference in the migration rates of the solutes and thus a separation.

The fundamental equations to describe the retention time of a neutral solute and the resolutions of a pair of neutral solutes in MEC are (*2*)

$$t_R = \left(\frac{1 + k'}{1 + (t_o/t_{mc})k'}\right)t_o \qquad (1)$$

$$R_s = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{k'_2}{1 + k'_2}\frac{1 - t_o/t_{mc}}{1 + (t_o/t_{mc})k'_1} \qquad (2)$$

where k' is the capacity factor (moles of solute in micelle/moles of solute in bulk water); N is the number of theoretical plates; α is the selectivity; and t_o and t_{mc} are the retention times of the aqueous and micellar phases, respectively. If two peaks in MEC are reasonably close together, i.e., $k_1 \approx k_2 \approx k'$, a convenient approximation to eq 2 is (*2*)

$$R_s = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{k'}{1 + k'}\frac{1 - t_o/t_{mc}}{1 + (t_o/t_{mc})k'} \qquad (2a)$$

Using eq 2 or 2a as a guide, much research has been directed toward the optimization of MEC. A variety of factors have been examined for their effect on N, including applied voltage (*3*, *4*); electroosmotic flow velocity (*4*); column dimensions (*3*); buffer concentrations (*3*); temperature (*4*, *5*); surfactant concentration (*3-5*); retention factor ratio, R (*6*); organic modifier content (*5*, *7*, *8*); and the voltage, time, and/or length associated with sample injection (*4*, *9*).

Likewise, a number of effects on the selectivity, α, in MEC have been examined, such as applied voltage (*10*), pH (*11*, *12*), organic modifier content (*8*, *13*), surfactant identity (*4*, *10*, *14*), and surfactant concentration (*9*, *11*, *12*). Since for a given surfactant the selectivity for *neutral* solutes in MEC is determined almost exclusively by the water/micelle partition coefficients (vide infra), it is not surprising that all the variables except surfactant identity had very little, if any, effect. Moreover, with only one exception (*14*), the use of *different* surfactants provided only moderate, not dramatic, changes in the separation selectivity for neutral solutes. Thus, for neutral analytes at least, it appears that there is less control of the separation selectivity in MEC than in modern liquid chromatography (high-performance LC).

Finally, the significance of the last two terms of eq 2, which may collectively be referred to as the retention term in MEC, was discussed by Terabe et al. in an earlier paper (*2*). From their qualitative discussion, it is evident that for a given value of t_o/t_{mc}, there is an optimum, although unspecified, value of k' that maximizes the retention term and hence, for a given value of N and α, maximizes the resolution itself.

Other variables important to MEC that have also been identified previously include (i) the ratio t_o/t_{mc} (*2*, *7*, *10*, *14*), which appears in the retention term of eqs 2 and 2a; and (ii) the electroosmotic velocity, v_{eo} (*2*, *15*), which affects the time required for analysis. Both variables were found to be largely independent of the surfactant concentration, at least for the surfactants examined.

Unfortunately, despite the abundance of research noted above, there has been very little work on (i) the fundamental optimization of what is arguably the most important variable in MEC, the concentration of surfactant; and (ii) the optimization of resolution per unit time. The purpose of the present paper is to consider the optimization of the surfactant concentration in considerable detail from the viewpoint of both resolution and resolution per unit time. Equations are derived that predict the optimum capacity factor and the corresponding surfactant concentration to use for each approach. Provided the optimum conditions are employed, the results are shown to be independent of the solute water/micelle partition coefficients.

Note that in the present paper we purposely restrict ourselves to discussions involving neutral solutes and positive values of t_o/t_{mc}. This has been done for three reasons: First and foremost, the fundamental equations of MEC (eqs 1 and 2) upon which our theory is based were derived with these restrictions (*1*, *2*). Second, the selectivity of charged species does not appear to be sufficiently independent of the surfactant concentration (*11*, *12*) to permit the application of our theory in its present form. Third, although negative values of t_o/t_{mc} are certainly possible (ref 15, when the micelles migrate in a direction opposite to the electroosmotic flow),

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they are not desirable in MEC as it is currently practiced, since those solutes that partition significantly into the micelle ($k' > |t_{mc}/t_o|$) would then travel away from the detector after injection, thus making detection with conventional fixed-position detectors impossible. Discussions of MEC separations involving charged solutes and negative values of t_o/t_{mc} are planned for future papers.

THEORY

The theory for the optimization of the surfactant concentration for maximum (i) resolution and (ii) resolution per unit time is now presented. Each derivation will be performed in three stages. First, a relationship will be developed between the surfactant concentration and the capacity factor, phase ratio, and solute partition coefficient. Second, equations will be derived that predict the *optimum capacity factors* for the best resolution and resolution per unit time. Finally, by using the results of the second stage, expressions for the corresponding resolution, analysis time, and resolution per unit time will be obtained.

Relationship of k' to Surfactant Concentration. As in liquid chromatography, the capacity factor, k' (moles of solute in micelle/moles of solute in bulk water), in MEC is given by

$$k' = P_{wm}\beta \tag{3}$$

where β is the phase ratio and P_{wm} is the partition coefficient of the solute between water and the micellar phase (a constant for a given solute/surfactant). The phase ratio in MEC can be expressed explicitly in terms of the surfactant concentration as (2)

$$\beta = \frac{V([\text{SURF}] - \text{cmc})}{1 - V([\text{SURF}] - \text{cmc})} \tag{4}$$

where V is the partial molar volume of the surfactant; [SURF] is the molar concentration of surfactant; and cmc is the critical micelle concentration, the surfactant concentration above which surfactant molecules will self-aggregate to form micelles.

Substitution of eq 4 into eq 3 and solving of the surfactant concentration yields

$$[\text{SURF}] = \frac{k' + V\text{cmc}(k' + P_{wm})}{V(k' + P_{wm})} \tag{5}$$

Equation 5 is very important. It shows the exact concentration of surfactant required for a given capacity factor. Assuming that an optimum k' exists (which we shall verify presently), it enables us to predict a priori the optimum concentration of surfactant to use in any MEC separation. This is possible, since all of the other parameters in eq 5 are either known or experimentally measurable. Since $P_{wm} \gg k'$ (except for very hydrophilic compounds), a useful approximation to eq 5 is

$$[\text{SURF}] = \frac{k'}{P_{wm}V} + \text{cmc} \tag{6}$$

Optimization of the Capacity Factor. We now derive equations that predict the optimum capacity factor for the best resolution and best resolution per unit time. Both derivations utilize eq 2a and the following simplifying assumptions: For a given surfactant, both the efficiency (N) and the selectivity (α) of neutral solutes (the solutes of interest in the present study) are independent of k' (i.e., the surfactant concentration).

The above assumptions involving N and α are important and merit further discussion. With regard to selectivity (α), the results of Row et al. (*9*) and Fujiwara et al. (*11*, *12*) clearly illustrate that for a given surfactant and neutral solutes (the solutes of interest in the present study), the selectivity *is* virtually independent of k', i.e., the surfactant concentration.

With regard to efficiency (N), however, the assumption that N is independent of k' in MEC is clearly a first-order approximation at best, since most studies have reported or predicted at least a slight dependence of N on the surfactant concentration or k' (*3*–*6*). Nevertheless, we believe that the assumed lack of dependence of N on k' is a legitimate approximation in view of the following:

First, neither an experimental nor a theoretical consensus has yet been reached on the effect of the surfactant concentration or k' on N. Some studies have noted an almost direct dependence, whereas others have shown that a more complicated and sometimes inverse relationship exists. Sepaniak et al. (*3*) and Balchunas et al. (*5*) reported a moderate dependence of N for a wide range of surfactant concentrations, with higher concentrations resulting in higher N values due to a reduction in the distance between adjacent micelles and thus less band broadening due to intermicellar mass transfer. On the other hand, the findings of Terabe et al. (*4*) show that intermicellar mass transfer (and hence surfactant concentration) does not contribute significantly to the total plate height in MEC. The more recent random-walk theory of Davis (*6*) corroborates the results of Terabe et al. with regard to *micellar* mass transfer but concludes that another process, *transchannel* mass transfer, is likely to be the dominant one under most circumstances. Still other researchers have reported *extracolumn* effects in CZE that are also applicable to MEC that are much more significant than any variations in N caused by a dependence on k' (*16*–*18*).

Second, one of the more definitive papers on band broadening in MEC seems to indicate (ref 4, Figure 6 and related text) that the dependence of N on k' is only slight over a fairly wide range of useful linear velocities (1–3 mm/s) and k' values (0–5). Another theoretical treatment also supports this general view (*6*), although the effect of linear velocity is not explicitly discussed.

Finally and perhaps most importantly, the resolution depends only on the square root of N (eqs 2 and 2a), and thus any dependence of N on k' that *may* exist will be reduced accordingly.

In summary, although the assumption that α is independent of k' for a given surfactant system is entirely appropriate for neutral solutes, the parallel assumption involving N is clearly an approximation, but one we believe is appropriate and valid under most experimental conditions. In future papers we will, however, assume some explicit dependence of N on k' and compare those results (*19*) with those presented here.

(I) Capacity Factor for the Best Resolution. Assuming that N and α are independent of k' (see above), the dependence of the resolution on k' is limited to the last two terms of eq 2a, which may be referred to collectively as the retention term shown below:

$$f(k') = \frac{k'}{1 + k'} \frac{1 - t_o/t_{mc}}{1 + (t_o/t_{mc})k'} \tag{7}$$

The optimum k' value can be determined explicitly by differentiating eq 7 with respect to k' and setting the resulting expression (eq 7) equal to zero. The only physically significant root is

$$k'_{opt}(\text{maximum } R_s) = (t_{mc}/t_o)^{1/2} \tag{8}$$

The word "maximum" is omitted hereafter to condense these terms. Equation 8 tells us the best resolution will be obtained if the surfactant concentration (or other parameter) is adjusted to give an average capacity factor of $(t_{mc}/t_o)^{1/2}$. To calculate the optimum concentration of surfactant, we merely substitute eq 8 into eq 5 or 6 to obtain

$$[\text{SURF}]_{opt}(R_s) = \frac{(t_{mc}/t_o)^{1/2} + V\text{cmc}((t_{mc}/t_o)^{1/2} + P_{wm})}{V((t_{mc}/t_o)^{1/2} + P_{wm})} \tag{9}$$

or

$$[\mathrm{SURF}]_{\mathrm{opt}}(R_s) = \frac{(t_{mc}/t_o)^{1/2}}{P_{wm}V} + \mathrm{cmc} \qquad (10)$$

Equations 9 and 10 show that the concentration of surfactant to use for the best resolution of neutral solutes is essentially predetermined in MEC and can be calculated from P_{wm}, the average of their partition coefficients, and four parameters related to the MEC/surfactant: t_o, t_{mc}, V, and cmc.

(II) Capacity Factor for the Best Resolution per Unit Time. Surprisingly, the optimization of resolution per unit time, R_s/t_R, has largely been unexplored in MEC until now. We utilize the approach that has been almost universally employed in previous derivations of optimum resolution/time for the conventional column chromatographic methods (gas chromatography (GC), high-performance LC, supercritical fluid chromatography (SFC), etc.) and yields an optimum k' of 2 for these methods (*20*). That result, of course, does not apply to MEC, since it is based on a different expression for the resolution.

The linear velocity of a neutral solute in MEC is given by (*14*)

$$v_{\mathrm{solute}} = \frac{v_{eo}(1 + (t_o/t_{mc})k')}{1 + k'} \qquad (11)$$

where v_{eo} is the electroosmotic velocity of the aqueous phase. Substitution of eq 11 into the well-known chromatographic expression, $t_R = L/v_{\mathrm{solute}} = NH/v_{\mathrm{solute}}$, yields the following equation for the retention time in MEC:

$$t_R = \frac{NH(1 + k')}{v_{eo}(1 + (t_o/t_{mc})k')} \qquad (12)$$

If eq 2a is solved for N, the resulting equation is

$$N = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{1 + k'}{k'}\right)^2\left(\frac{1 + (t_o/t_{mc})k'}{1 - t_o/t_{mc}}\right)^2 \qquad (13)$$

Substitution of eq 13 into eq 12 yields (upon simplification)

$$t_R = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{1}{1 - t_o/t_{mc}}\right)^2\frac{H}{v_{eo}} \times \frac{(1 + k')^3(1 + (t_o/t_{mc})k')}{(k')^2} \qquad (14)$$

Equation 14 relates the analysis time to seven variables in MEC: R_s, α, t_o, t_{mc}, H, v_{eo}, and k'. For a given surfactant, all parameters except for R_s and k' will be essentially constant as the surfactant concentration (and hence k') is varied. (Note that the assumption of a constant plate height (H) is consistent with the assumption of the independence of N on k'.) Furthermore, if we constrain the resolution to a fixed value (e.g., $R_s = 1.5$), the retention time will go through a minimum as k' is varied through its optimum value. This is equivalent to maximizing the resolution per unit time.

Differentiating eq 14 with respect to k' while holding R_s constant and equating the resulting expression with zero gives an expression which in turn yields only one physically significant root:

$$k'_{\mathrm{opt}}(R_s/t_R) = \frac{-(1 - t_o/t_{mc}) + ((1 - t_o/t_{mc})^2 + 16(t_o/t_{mc}))^{1/2}}{4(t_o/t_{mc})} \qquad (15)$$

For $1 < t_{mc}/t_o < \infty$, k'_{opt} ranges from 1.2 to 2. Substitution of the value of k' obtained via eq 15 into eq 5 or 6 permits the surfactant concentration for maximum R_s/t_R to be calculated. Using eq 6, one obtains

$$[\mathrm{SURF}]_{\mathrm{opt}}(R_s/t_R) = \frac{k'_{\mathrm{opt(eq\ 15)}}}{P_{wm}V} + \mathrm{cmc} \qquad (16)$$

Equation 16 shows that the concentration of surfactant to use for the best resolution per unit time is essentially predetermined in MEC and can be calculated from a solute property, the average partition coefficient of two solutes (P_{wm}), and four parameters related to the MEC/surfactant system: t_o, t_{mc}, V, and cmc.

Equations for Resolution, Analysis Time, and Resolution per Unit Time. The results of the previous section can be used to derive equations for the resolution, analysis time, and resolution per unit time. These equations, which are given below, will be applicable whenever the capacity factor is optimized as discussed earlier. Note that although we believe that the optimization of the capacity factor is best accomplished by adjusting the surfactant concentration, *our theory (eqs 8, 15, and 17–22) can also be applied to the optimization of other variables, such as the amount of organic modifier, that also affect the capacity factor, provided that any concurrent changes in t_{mc}/t_o are accounted for.*

For the resolution (R_s) approach, the equations for the analysis time and resolution are obtained by substituting k'_{opt} from eq 8 into eqs 1 and 2, respectively. These equations can in turn be manipulated to give an expression for the resolution per unit time. The results are

$$t_R = \left(\frac{1 + k'_{\mathrm{opt(eq\ 8)}}}{1 + (t_o/t_{mc})k'_{\mathrm{opt(eq\ 8)}}}\right)t_o = k'_{\mathrm{opt(eq\ 8)}}t_o = (t_{mc}/t_o)^{1/2}t_o = (t_{mc}t_o)^{1/2} \qquad (17)$$

$$R_s = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{(t_{mc}/t_o)^{1/2} - (t_o/t_{mc})^{1/2}}{2 + (t_{mc}/t_o)^{1/2} + (t_o/t_{mc})^{1/2}} \qquad (18)$$

$$R_s/t_R = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{1 - t_o/t_{mc}}{t_o(2 + (t_{mc}/t_o)^{1/2} + (t_o/t_{mc})^{1/2})} \qquad (19)$$

For the resolution per unit time (R_s/t_R) approach, the corresponding equations are obtained by substituting k'_{opt} from eq 15 into eqs 1 and 2, followed by the appropriate manipulations:

$$t_R = \left(\frac{1 + k'_{\mathrm{opt(eq\ 15)}}}{1 + (t_o/t_{mc})k'_{\mathrm{opt(eq\ 15)}}}\right)t_o \qquad (20)$$

$$R_s = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{k'_{\mathrm{opt(eq\ 15)}}}{1 + k'_{\mathrm{opt(eq\ 15)}}}\frac{1 - t_o/t_{mc}}{1 + (t_o/t_{mc})k'_{\mathrm{opt(eq\ 15)}}} \qquad (21)$$

$$R_s/t_R = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{k'_{\mathrm{opt(eq\ 15)}}(1 - t_o/t_{mc})}{t_o(1 + k'_{\mathrm{opt(eq\ 15)}})^2} \qquad (22)$$

RESULTS AND DISCUSSION

Although most of the comments below will be equally appropriate for multicomponent samples, the discussion that follows is based on the objective of optimizing the separation of a *pair* of solutes.

Optimum Capacity Factors. Shown in Figure 1 is a plot of the optimum capacity factor needed for (i) the best resolution and (ii) the best resolution per unit time as a function of t_{mc}/t_o. It is apparent in Figure 1 that $k'_{\mathrm{opt}}(R_s) > k'_{\mathrm{opt}}(R_s/t_R)$ for all values of t_{mc}/t_o, with the differences in the capacity factors (and corresponding surfactant concentrations) becoming greater as t_{mc}/t_o increases. In addition, whereas $k'_{\mathrm{opt}}(R_s)$ approaches infinity as t_{mc}/t_o increases, $k'_{\mathrm{opt}}(R_s/t_R)$

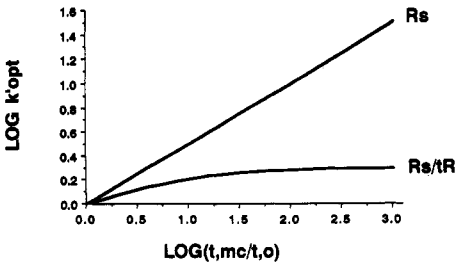

Figure 1. Optimum capacity factor for the best resolution (eq 8) and the best resolution per unit time (eq 15).

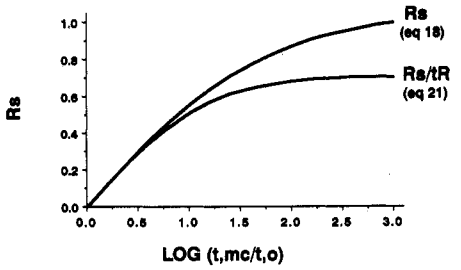

Figure 2. Resolution as a function of t_{mc}/t_o for two approaches (R_s and R_s/t_R) to the optimization of the surfactant concentration. The calculations were performed by using eqs 17–22 and assuming N = 75 000, log P_{wm} = 2.5, $\alpha = \Delta(\log P_{wm}) = 0.05$, and t_o = 1 min. The results were normalized to unity for purposes of comparison.

never exceeds a value of 2. This upper limit of 2 for $k'_{opt}(R_s/t_R)$ was fully expected, since the resolution and retention time equations in MEC reduce to the more familiar relationships observed in conventional column chromatography (for which $k'_{opt}(R_s/t_R) = 2$, ref 20) as t_{mc}/t_o approaches infinity (2).

Although necessary for plotting purposes, the log scale of the ordinate exaggerates the differences between $k'_{opt}(R_s)$ and $k'_{opt}(R_s/t_R)$ for small values of t_{mc}/t_o. For example, at t_{mc}/t_o = 4 (log (t_{mc}/t_o) = 0.6), the optimum capacity factors are 2 (R_s) and 1.4 (R_s/t_R), respectively. For t_{mc}/t_o = 10 (log (t_{mc}/t_o) = 1), the capacity factors are 3.2 and 1.63. These relatively small differences in k'_{opt} at low values of t_{mc}/t_o do not translate into a significant difference in the resolution (see below). However, for $t_{mc}/t_o \geq 20$ (log $(t_{mc}/t_o) \geq 1.3$), the differences in k'_{opt} *do* become significant.

Dependence of Resolution on t_{mc}/t_o. Shown in Figure 2 is the (normalized) resolution obtained by using the optimum values of capacity factors from Figure 1. The resolution curves illustrate two important points.

First, the resolution obtained in MEC is highly dependent on t_{mc}/t_o, and there is a significant advantage in increasing t_{mc}/t_o from values of 4 (log $(t_{mc}/t_o) \approx 0.6$) or so typically observed in the literature (e.g., refs 2, 5, 7, 10, and 13–15) to at least a value of 30 but ideally to 100 or larger. This point has been made *qualitatively* by others (*2, 7, 10, 14*). As shown in Figure 2, if t_{mc}/t_o were increased from 4 to 100 or greater, the resolution or resolution per unit time would be increased by a factor of 2–3.

Second, the difference in resolution obtained by using $k'_{opt}(R_s/t_R)$ instead of $k'_{opt}(R_s)$ is small, at most 33% for $t_{mc}/t_o = \infty$. This is important, because as eqs 17 and 20 show, the time required for the best resolution approaches infinity as t_{mc}/t_o increases, whereas the time needed for the best resolution per unit time approach is always short and finite, never exceeding 3 times the retention time of an unretained solute ($t_R \leq 3t_o$).

Preferred Values for the Capacity Factor. The curves of Figure 1 serve as boundaries for three distinct regions of values for the capacity factor in MEC. These regions are shown in Table I along with a qualitative evaluation (+ = good; − = bad) using the criteria of resolution, analysis time, and resolution per unit time (eqs 17–22).

Table I. Regions for the Capacity Factor in Micellar Electrokinetic Chromatography

region	value of capacity factor[a]	qualitative evaluation[b] R_s	t_R	R_s/t_R
I	$0 < k' < k'_{opt}(R_s/t_R)$	−	+ +	−
II	$k'_{opt}(R_s/t_R) \leq k' \leq k'_{opt}(R_s)$	+	+	+
III	$k'_{opt}(R_s) < k' < \infty$	−	−	−

[a] The values of $k'_{opt}(R_s/t_R)$ and $k'_{opt}(R_s)$ depend on t_{mc}/t_o (see eqs 8 and 15). For t_{mc}/t_o = 10, $k'_{opt}(R_s/t_R)$ = 1.63 and $k'_{opt}(R_s)$ = 3.16. [b] Based on eqs 8, 15, and 17–22 and related text and Figures 1 and 2.

In general, intermediate values of k' (region II) are preferable in MEC, since they provide satisfactory results according to all three criteria. Low values of k' (region I) are the next most desirable, since they may occasionally be useful for easy-to-separate samples because of the very fast analysis times that result. Finally, large capacity factors (region III) are the least desirable and generally unacceptable, since they yield the poorest separations according to all three criteria.

In contrast to MEC, which has three regions for the capacity factor, conventional column chromatographic techniques (high-performance LC, GC, and SFC) have only two such regions, analogous to regions I and II of Table I. Thus Table I illustrates an important fundamental difference between MEC and conventional column chromatography in the relationship of resolution and retention: Whereas with conventional column chromatography it is impossible to experience a loss in resolution due to excessive retention (although detectability and analysis time may suffer), with MEC there will be a loss in resolution if k' is increased to a value above $k'_{opt}(R_s)$. In other words, from the viewpoint of resolution, it is possible to have excessive retention in MEC but not in conventional column chromatography.

Note that the ranges of k' values of the regions in Table I depend on the magnitude of t_{mc}/t_o as shown by eqs 8 and 15. The larger the value of t_{mc}/t_o, the larger are regions I and II, especially region II. This is yet another advantage of large values of t_{mc}/t_o in addition to those pointed out earlier: a larger range of k' values in which to fine-tune a given separation.

Trade-Off between Resolution and Analysis Time (Solute Retention). A common feature of capacity factor regions I and II in Table I is that a shift in k' does not result in a simultaneous improvement for all three optimization criteria. In region I, an increase in k' results in improved resolution and resolution per unit time at the expense of a longer analysis time. In region II, an increase in k' results in improved resolution at the expense of analysis time and resolution per unit time. Thus, within regions I and II it is possible, on the basis of the requirements of a particular separation, to select a suitable compromise between resolution, analysis time, and resolution per unit time. For example, if the resolution is more than adequate, k' can be decreased to reduce the analysis time. On the other hand, if more resolution is needed, k' may be increased to improve the resolution. As noted earlier, in general, region II is expected to provide the best overall results. Within region II, given that an increase in k' results in only moderate improvements in the resolution but very large increases in the analysis time (Figures 1 and 2 and eqs 17 and 20), the best initial value to select for k' should be much closer to $k'_{opt}(R_s/t_R)$ than to $k'_{opt}(R_s)$.

Ease with Which Retention Can Be Optimized in MEC. For a given system (value of t_{mc}/t_o), eqs 8 and 15 can

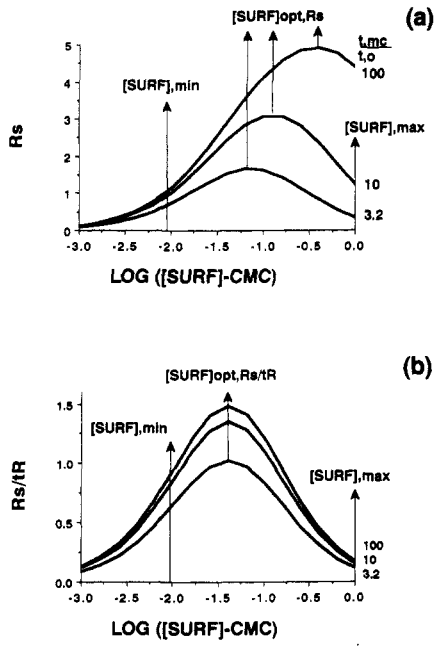

Figure 3. Practical range of the surfactant concentration as determined by surfactant properties ($[SURF]_{min}$ and $[SURF]_{max}$) and chromatographic goals ($[SURF]_{opt}(R_s/t_R)$ and $[SURF]_{opt}(R_s)$). The R_s and R_s/t_R curves were drawn separately for purposes of clarity. The calculations were performed by assuming an aggregation number of 62 for the surfactant, log P_{wm} = 2, and $\alpha = \Delta(\log P_{wm}) = 0.04$. The other conditions are as in Figure 2, except that the results were not normalized to unity.

be used to calculate the limits of the optimum range of capacity factors (region II, Table I). And if the retention is being controlled via the surfactant concentration, eqs 10 and 16 can be used to predict a priori the concentration for optimum resolution or resolution per unit time. This is possible since all the parameters in eqs 10 and 16 are either known or experimentally measurable. Partial molar volumes and critical micelle concentrations have been tabulated for numerous surfactants (e.g., ref 21). Solute/micelle partition coefficients (association constants) have been measured for selected compounds by numerous groups (e.g., refs 22–25). In addition, we have recently published a compilation of over 150 solute/micelle association constants (*26*). By using these data, it may be possible to *estimate* partition coefficients for still additional compounds.

Practical Range of Surfactant Concentration. Assuming the desirability of controlling solute retention in MEC primarily by the concentration of surfactant, what are the practical minimum and maximum values? Obviously the concentration of surfactant cannot be varied over an infinitely wide range, if only because of the properties of the surfactant itself. The lower usable concentration limit will be determined by the need to generate reproducible concentrations of micelles so that retention times, etc., will be reproducible. This limit will therefore depend on the cmc and the precision with which it is known, but for our purposes we will assume it to be 10% above the cmc, i.e., $[SURF]_{min}$ = 1.1cmc. The upper concentration limit, in all likelihood, will be determined by adverse solution properties (high viscosity, etc.) caused by high concentrations of surfactant. From experimental results in the literature, a concentration of 1 M would appear to be a reasonable upper limit, i.e., $[SURF]_{max}$ = 1 M.

Having established a practical range of surfactant concentration strictly on the basis of surfactant properties (1.1cmc < [SURF] < 1 M), it is then appropriate to consider any additional restrictions on the surfactant concentration range that might be imposed by chromatographic goals. Given the

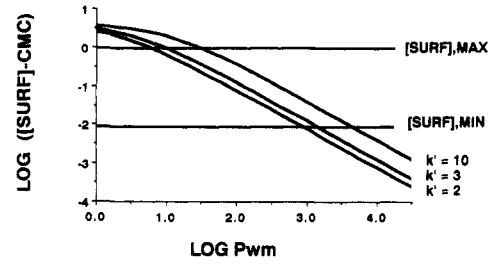

Figure 4. Optimum surfactant concentration as a function of solute partition coefficient and capacity factor. The values selected for the capacity factor correspond to those that would typically be needed for the optimization of the resolution per unit time ($k' = 2$) or the resolution ($k' = 3$, 10). The calculations were performed by assuming an aggregation number of 62 for the surfactant. The other conditions are as in Figure 2.

above discussion of the trade-off between resolution and analysis time, it is clear that additional constraints are very likely. By using eq 5 to convert from capacity factor values to surfactant concentrations, a graph (Figure 3) of resolution and resolution per unit time was constructed as a function of surfactant concentration assuming cmc = 0.0081 M (sodium dodecyl sulfate at 25 °C), log P_{wm} = 2 (average solute partition coefficient), and t_{mc}/t_o = 3.16, 10, and 100. The lower and upper limits of the surfactant concentration as determined by surfactant properties, $[SURF]_{min}$ and $[SURF]_{max}$, are indicated by the vertical lines at log ([SURF] − cmc) = −2.05 and 0, respectively.

As expected from eqs 5, 9, and 16–22, all of the R_s and R_s/t_R curves in Figure 3 go through a maximum value. Vertical lines drawn from the maxima of the R_s and R_s/t_R curves to the concentration axis define the lower and upper surfactant concentrations, $[SURF]_{opt}(R_s/t_R)$ and $[SURF]_{opt}(R_s)$, for a given value of t_{mc}/t_o and log P_{wm}. These concentrations correspond to the capacity factor limits of region II (Table I), the most desirable range. In the present example (log P_{wm} = 2), it is evident that the chromatographic limits for all values of t_{mc}/t_o shown in Figure 3 fall within the concentration boundaries established by the surfactant properties. For t_{mc}/t_o = 3.2, the lower and upper chromatographic limits of the surfactant concentration are 50 and 80 mM, respectively. For t_{mc}/t_o = 10 and 100, the upper limit increases to 135 and 385 mM, respectively, while the lower limit remains at 50 mM. Note that even for t_{mc}/t_o = 100, the practical chromatographic range of the surfactant concentration is much narrower (50–385 mM) than the range dictated by surfactant properties (10–1000 mM). This difference will be even more dramatic for surfactants with lower cmc values than that used in the present example (lower $[SURF]_{min}$).

In summary, the desire for an optimum separation in MEC imposes an additional set of restrictions on the surfactant concentration beyond those imposed by the surfactant itself. Although in the above example the inherent surfactant and chromatographic restrictions do not conflict with each other for the hypothetical solutes in Figure 3, for certain solutes they will (vide infra). In these instances, it will probably be necessary to switch to a different surfactant.

Solutes That *Cannot* Be Separated by MEC with Present Surfactants. As shown in eqs 9 and 16, the optimum concentration of surfactant to use for a given separation depends on the solute partition coefficients and on t_{mc}/t_o. Given that the surfactant concentration cannot be varied over an infinitely wide range (vide supra), an important consequence of eqs 9 and 16 is that some solutes may not be separated very well with a given surfactant.

Assuming an aggregation number of 62 for the surfactant, the concentration required for the separation of a pair of solutes is shown in Figure 4 for three values of k': 2, 3, and

10. These values correspond to those that might typically be needed for the optimization of R_s/t_R ($k' = 2$) or R_s ($k' = 3$, 10). The results show that the optimum surfactant concentration, although different for each k' value, varies in a similar fashion (≈parallel slopes) according to the solute partition coefficient. Although differences in the concentrations may appear to be slight ($\Delta = 0.5$ between $k' = 2$ and $k' = 10$), the reader is reminded that the concentration scale is logarithmic.

The upper and lower limits of the surfactant concentration in Figure 4 are indicated by horizontal lines and were calculated as in Figure 3. As shown in Figure 4, there are thus two groups of solutes for which MEC separations cannot be optimized under these conditions: very hydrophilic solutes (log $P_{wm} < 1$) and moderately to highly hydrophobic solutes (log $P_{wm} > 3$).

For both groups of solutes, a different surfactant system will probably be required for satisfactory resolution. To accommodate the very hydrophilic solutes, the new surfactant system should be more hydrophilic in order to promote larger solute partition coefficients, which would then permit an experimentally accessible surfactant concentration ([SURF] < $[SURF]_{max}$). For the hydrophobic solutes, the new surfactant should have a very low and well-defined cmc, which as discussed earlier would allow the use of much lower surfactant concentrations while still remaining above $[SURF]_{min}$. The accommodation of both groups of solutes represents a future research challenge in MEC.

Optimization When t_{mc}/t_o Depends on Surfactant Concentration. In general, little dependence of t_{mc}/t_o on the surfactant concentration has been observed in MEC thus far (*2*, *13–15*). However, if such a dependence is established, the optimization of the surfactant concentration would proceed in an iterative fashion via eq 9 or 16 until convergence is achieved (measure or estimate t_{mc}/t_o, adjust [SURF] to its optimum value via eq 9 or 16, remeasure t_{mc}/t_o, readjust [SURF], etc.). Given that the dependence of t_{mc}/t_o on the surfactant concentration will be only moderate at most, it will probably take only one iteration (two calculations) to obtain convergence.

Limitations of Isocratic MEC. It has already been reported (*5*) that some type of gradient elution is needed in MEC when solutes of a very wide polarity are to be separated. Our theory corroborates those results. Thus MEC suffers from the same general elution problem as all other elution-based chromatographic methods. The theory we have developed should be of great benefit for isocratic MEC separations and probably extends the useful range of solutes separable by isocratic MEC by an additional log P_{wm} unit, where P_{wm} is the solute partition coefficient. Nonetheless, for samples in which the range of solute log P_{wm} values exceeds 2, some type of gradient elution is likely to be necessary. If a gradient of the original surfactant is to be employed, our theory shows that the surfactant concentration should be decreased with time. As shown in Figure 4, in order to achieve roughly the same resolution for a range of solutes with an evenly spaced distribution of log P_{wm} values, a *negative* surfactant gradient {linear in log ([SURF] − cmc)} will be necessary. We are currently investigating ways in which this type of gradient can be experimentally achieved.

CONCLUSIONS

MEC represents an alternative to conventional high-performance LC (hydroorganic mobile phases), micellar LC (micellar mobile phases), and size-exclusion chromatography (SEC) for the separation of neutral compounds. Although an exhaustive comparison is beyond the scope of this paper, a few similarities and differences are noteworthy.

(1) MEC is an interesting hybrid of CZE, micellar LC, conventional high-performance LC, and SEC. In terms of its well-defined time limits for a separation (t_o and t_{mc}), MEC most closely resembles SEC. In fact, if a micellar solution is used as a mobile phase in SEC, the retention mechanisms of MEC and SEC are remarkably similar (*27*). Despite these similarities as well as other similarities with micellar and conventional high-performance LC, however, MEC is a distinct method of separation that requires its own theory in order to be utilized efficiently. The theory presented here was developed for this purpose.

(2) From the viewpoint of column efficiency, MEC appears to be superior to SEC, conventional high-performance LC, and micellar LC. Plate counts in MEC substantially exceed those of SEC and also conventional or micellar LC with packed columns or open capillaries due to fewer significant sources of band broadening (*3*, *4*, *6*). The theory we have developed for the optimization of the surfactant concentration should be especially beneficial for isocratic MEC separations. On the other hand, MEC suffers from the same "general elution problem" as conventional high-performance and micellar LC and, like these methods, would benefit greatly from some type of gradient elution. The fact that gradient elution is experimentally more difficult to accomplish in MEC at present is a significant, though not insurmountable, disadvantage of MEC relative to conventional and micellar LC.

(3) Whereas retention (k') in micellar and conventional high-performance LC is controlled primarily by adjusting the partition coefficient, retention in MEC is controlled by adjustment of the phase ratio (eq 3). The adjustment of the partition coefficient in high-performance LC is typically accomplished via a change in the mobile phase, whereas the change in the phase ratio is performed by changing the volume of the pseudostationary phase, with little change in the mobile phase volume at reasonable surfactant concentrations.

(4) Whereas changes in the retention via solvent strength adjustments in conventional high-performance LC frequently result in dramatic changes in the selectivity, similar changes in the retention of neutral solutes in MEC for a given surfactant system do *not*, provided that the retention is adjusted via the surfactant concentration as we have suggested.

(5) A final difference between micellar LC and MEC is that the use of micellar solutions in the former results in three partitioning processes, only two of which are independent (*22*, *23*), whereas their employment in MEC results in only one partitioning process. In instances where the two independent partitioning processes in micellar LC are correlated, the separation is usually significantly poorer than what might be otherwise expected (*28*). The fact that no such correlation is possible in MEC (because there is only one partitioning process) represents a distinct advantage of MEC over micellar LC.

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Indirect Stereoselective Determination of the Enantiomers of a Thieno[2,3-*b*]thiopyran-2-sulfonamide in Biological Fluids

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A sensitive (12.5 ng/mL) and stereoselective high-performance liquid chromatographic (HPLC) assay for the *S* and *R* enantiomers of 5,6-dihydro-4-((2-methylpropyl)amino)-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide ((*S*)-1 and (*R*)-1), in whole blood and urine has been developed. The assays are based on derivatization with homochiral (*S'*)-(+)-1-(1-napthyl)ethyl isocyanate ((*S'*)-(+)-NEIC) formation of the diastereomeric derivatives and their separation and quantification using HPLC with ultraviolet (UV) detection. Several other homochiral derivatizing reagents were explored allowing separation of the (*S*)-1 and (*R*)-1 enantiomers in the form of their respective diastereomeric derivatives. Because of inefficient derivatization at the requisite concentrations and limited sensitivity for the UV detection, these derivatization reactions were abandoned in favor of the (*S'*)-(+)-NEIC derivatization. The (*S'*)-(+)-NEIC derivatives of (*S*)-1 and (*R*)-1 were independently synthesized, their structures confirmed, and their spectral properties evaluated. Details of this stereoselective assay procedure, which has been applied routinely for the multisample analyses from human clincal studies, are described. Formation of the major hydroxy metabolite, (S_m)-2 and (R_m)-2, has been confirmed and found to be stereoselective. A minor modification of the original assay procedure allowed full separation of the (*S'*)-(+)-NEIC diastereomeric derivatives of the (S_m)-2 isomer of the hydroxy metabolite from the (R_m)-2 isomer and from the endogenous sample and reagent-related impurities. The preliminary data obtained indicated that the disposition of the isomers of (1) is highly stereoselective, with the (*R*)-1 and (R_m)-2 isomers being cleared much faster than (*S*)-1 and (S_m)-2. No interconversion of the isomers was observed in vivo.

INTRODUCTION

The stereoselective disposition of the isomers of chiral drugs is a well-established phenomenon in the field of pharmacokinetics and biopharmaceutics (*1-5*). Optical isomers usually exhibit differences in biological activity and in pharmacokinetic behavior. The desired pharmacological activity often resides predominantly in one enantiomer (*6*). Nevertheless, of the 486 synthetic chiral pharmaceuticals surveyed, 82% were used as racemates (*7*). In addition, isomers of the parent drug can be formed metabolically. In most of these cases the pharmacokinetic properties have been predominantly evaluated from concentration measurements on the racemates using nonstereoselective assays. Such data do not reflect the behavior of either enantiomer and are, therefore, inappropriate for the pharmacokinetic evaluation of a drug. For these reasons the availability of the stereoselective assay methodologies in biological fluids is of prime importance in the current bioanalytical chemistry field.

Separation of enantiomers can be accomplished either directly or indirectly after derivatization to diastereomers with homochiral reagents. If possible, the direct mode is usually preferred (*8*). For direct separation, either chemically bonded chiral stationary phases (CSP's) or conventional stationary phases with chiral mobile phase additives are usually used. Reviews on the direct chromatographic separation of enantiomers using these two approaches are available (*9-14*). Most of the work on direct chiral separation has been focused on analytes in a simple sample matrix; little has been published on the direct separation of drug enantiomers in biological fluids (*15-18*). Various direct separation approaches have also been extensively evaluated for the pair of enantiomers studied in this paper. The results of these studies will be reported separately.

Although direct separation of enantiomers by HPLC is a very useful and elegant technique, its application to biological samples seems to be rather limited. Constraints on the chromatographic conditions increase the difficulty in resolving endogenous interferences from the compound of interest (*19*). Chiral separations in the direct mode usually require chromatographic conditions in which normal-phase mobile phases are utilized precluding the use of some selective detectors, e.g., the electrochemical detector. There is also a necessity for the analyte to be transferred from an aqueous to an organic phase before injection can be made. In addition, the efficiency of chiral columns (plate number) is usually low and the low flow rates that are required result in long retention times. Also, loading the chiral compound on the column can be limited (e.g., protein columns (*20*)), and finding an appropriate internal standard is usually more complicated.

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