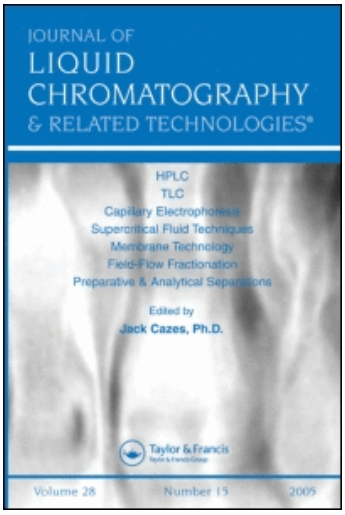

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DEVELOPMENT OF A CYCLODEXTRIN MODIFIED MICELLAR ELECTROKINETIC CHROMATOGRAPHIC METHOD FOR THE SEPARATION OF β-METHYL ADC-13-ENOLPHOSPHATE DIPHENYL ESTER AND ITS α-METHYL DIASTEREOMER

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ABSTRACT

A cyclodextrin modified micellar electrokinetic chromatographic method for the separation of β-methyl ADC-13 enolphosphate diphenyl ester from its α-methyl diastereomer was developed. The influence of the organic modifier, sodium dodecyl sulfate (SDS), and γ-cyclodextrin concentration in the background electrolyte on the relative contributions of the efficiency, selectivity, and capacity function to the resolution between the diastereomers were studied. The results of these studies showed that, although, the resolution could be increased by varying the organic modifier and SDS concentrations, sufficient resolution for accurate determination of low levels of α-enol phosphate in the presence of higher levels of β-enol phosphate was not obtained. However, addition of γ-cyclodextrin to the background electrolyte (BGE)

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resulted in an almost two-fold increase in the resolution obtained. Optimized conditions allowed the detection of α-enol phosphate at levels as low as 0.1 area-% vs. β-enol phosphate. The method was validated in terms of sensitivity, linearity, and precision.

INTRODUCTION

β-methyl ADC-13 enolphosphate diphenyl ester (β-enol phosphate), a neutral, hydrophobic compound, is a key raw material used in the synthesis of the active pharmaceutical ingredient contained in the novel carbapenem antibiotic ertapenem sodium. (Note that Ertapenem sodium is known under the name INVANZ™, which is a trademark of Merck and Co., Inc., Whitehouse Station, New Jersey, USA). As shown in Fig. 1a, this compound contains four chiral carbons. The final chiral center that is introduced during synthesis (carbon atom labelled with an asterisk) has an R configuration. During the synthesis, partial epimerization may occur at this center, resulting in the formation of the α-methyl diastereomer (α-enol phosphate), which has an S configuration (Fig. 1b). It is very important to be able to ensure that the level of the α-methyl diastereomer contained in the β-enol phosphate key raw material is less than 0.1 area-%. The presence of higher levels of α-enol phosphate could affect the quality and potency of the ertapenem sodium that is manufactured. Due to the high hydrophobicity of the diastereomers, adequate separation could not be obtained using reversed phase HPLC with a non-chiral column without impractical extensions in analysis time. Adequate separation for detection of 0.1 area-% α-enol phosphate was achieved under normal phase conditions, using a non-chiral column.[1]

Micellar electrokinetic chromatography (MEKC), first introduced by Terabe in 1984,[2] can be used to determine a large number of mildly hydrophobic to hydrophobic compounds. Additionally, unlike free solution capillary electrophoresis, where uncharged solutes co-migrate with the electroosmotic flow (EOF), MEKC is an electrokinetic technique that can be employed for the separation of electronically neutral as well as ionic compounds. The separation mechanism for neutral solutes in MEKC is based solely on their differing affinities for the micelles that are formed upon addition of a suitable amount of surfactant to the background electrolyte (BGE). Micelles are formed after the critical micelle concentration (CMC) is exceeded and act as a dynamic "pseudostationary phase" that, when the electrodes are configured in standard mode, migrate towards the positively charged anode and against the EOF. Micellar electrokinetic chromatography can provide a high degree of separation efficiency and numerous surfactant systems have been developed to control selectivity.[3,4] Additionally, like other related techniques, MEKC offers the

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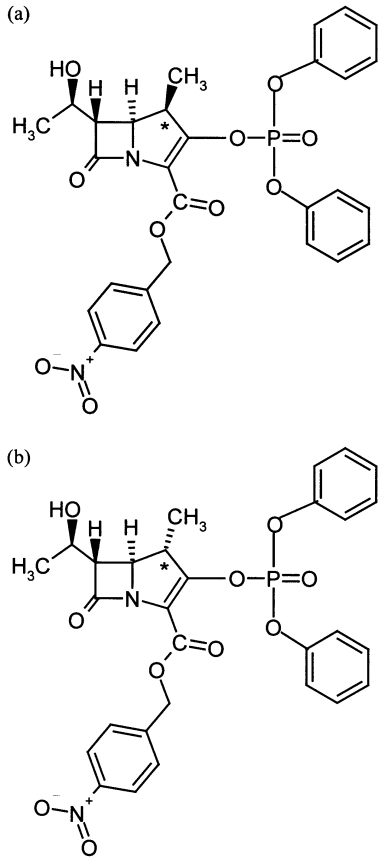

Figure 1. Structures of (a) β-methyl ADC-13 Enolphosphate Diphenyl Ester (β-enol phosphate) and (b) its α-methyl diastereomer (α-enol phosphate).

practical advantage of lower operating costs, since there is minimal solvent usage and bare fused silica capillaries are much less expensive than HPLC columns.

In MEKC, optimal resolution is obtained for solutes having capacity factors (k') of approximately 2.[5] Being very hydrophobic compounds, both α and β enol phosphate have very high capacity factors ($k' > 20$) when a BGE containing only sodium dodecyl sulfate (SDS) in borate buffer is used. This is due to strong interactions with the SDS micelles in the BGE. Both compounds stay solubilized in the micelle, resulting in insufficient resolution for accurate quantitation of residual amounts of the later eluting α-diastereomer.

One way to decrease solute/micelle interactions is to increase the polar nature of the micelle. This can be accomplished, for example, by using more polar surfactants such as bile acids,[6,7] by adding a second surfactant such as Tween 20 or Brij-35, to form more polar mixed micelles,[8,9] or by addition of alkyl polyalcohols such as 1,2-hexanediol, which act as "class I" organic modifiers.[10,11] Another approach, is to decrease the polar nature of the aqueous BGE, thereby, increasing the solubility of hydrophobic solutes. This can be done by adding organic modifiers such as acetonitrile, methanol, or urea to the BGE.[5] The amount of such modifiers that can be added to the BGE is limited however, by their effect on micelle integrity and the increase in analysis time that results from a decrease in EOF.

Cyclodextrin modified MEKC (CD-MEKC) was first introduced by Terabe.[12] By adding a suitable cyclodextrin to the BGE, partitioning of hydrophobic solutes between the aqueous and micellar phases can be shifted by inclusion in a third phase, the cyclodextrin cavity. If the cyclodextrin is neutral, it comigrates with the EOF and, depending on its degree of interaction with the cyclodextrin, the capacity factor of a hydrophobic solute is decreased due to solubilization in the cyclodextrin and decreased interaction with the micelles. It is important to choose a cyclodextrin that allows a goodness of fit between the analyte molecule and the cyclodextrin cavity. Additionally, cyclodextrins can improve the selectivity between stereoisomers due to the formation of inclusion complexes of differing stabilities. Cyclodextrin modified MEKC has been used to separate a wide range of enantiomers, diastereomers, and geometric isomers in drug substances and intermediates used during their synthesis.[13–16]

The development of a CD-MEKC method for the separation of β-methyl ADC-13 enolphosphate diphenyl ester and its α-methyl diastereomer is described. Initially, the effect of varying the organic modifier and SDS concentration in the BGE on the efficiency, selectivity, capacity function, and resultant resolution between the diastereomers was investigated and the results are presented and discussed. Likewise, results showing the effect of adding either underivatized β-cyclodextrin or γ-cyclodextrin to the BGE on the factors contributing to resolution is discussed. Finally, optimization of the method for detection of 0.1 area-% α-methyl diastereomer is described. The optimized method was validated in terms of sensitivity, linearity, and precision.

EXPERIMENTAL

Chemicals and Reagents

Background electrolyte solutions were prepared using stock solutions of 100 mM sodium dodecyl sulfate (SDS)/50 mM sodium borate, pH 9.3 and

50 mM sodium borate, pH 9.3 purchased from Agilent Technologies (Waldbronn, Germany). Solutions of sodium hydroxide, 0.1 N and 1.0 N, for conditioning of capillaries were also purchased from Agilent Technologies. Acetonitrile, ethanol, and methanol were HPLC grade and were purchased from EM Science (Merck KgaA, Darmstadt, Germany). Sudan III was purchased from Sigma (St. Louis, MO, USA). β-cyclodextrin hydrate, and γ-cyclodextrin hydrate were purchased from Aldrich Chemical (Milwaukee, WI, USA). Deionized water was obtained from a Picotech Hydro Ultrapure Water System (Garfield, NJ, USA). β-Methyl ADC-13 enolphosphate diphenyl ester was obtained from Kaneka Corporation (Osaka, Japan) and α-methyl diastereomer was provided by the Process Research and Development Department, Merck Research Laboratories (Rahway, NJ, USA).

Instrumentation and Separation Conditions

An Agilent G1602A capillary electrophoresis system (Waldbronn, Germany) equipped with a diode array detector was used for all studies. Fused silica capillaries (48.5 cm/40 cm total/effective length, 50 μm i.d. with extended light path) were also purchased from Agilent. All studies were performed using 200 nm as the detection wavelength, 30°C as the capillary cassette temperature, and 25 kV as the applied voltage. Typical currents, depending on the BGE composition, ranged from 35 to 49 μA. Hydrodynamic injection (50 mbar, 2 s) was used in all studies. All experiments were carried out in either duplicate or triplicate. The average values are reported.

Data aquisition, integration, and calculation of resolution and efficiency (Foley–Dorsey) were performed using a PE Nelson Turbochrom system (Cupertino, CA).

Methods

Background Electrolyte Solutions

Background electrolyte solutions were prepared by pipetting aliquots of stock solutions of 100 mM SDS/50 mM sodium borate, pH 9.3, 50 mM sodium borate, pH 9.3, along with organic modifier and diluting to volume with deionized water. Background electrolyte solutions containing CD were prepared by weighing out the appropriate amount of β-cyclodextrin or γ-cyclodextrin, adding aliquots of SDS and sodium borate stock solutions and organic modifier, sonicating to effect dissolution of the CD, and then diluting to volume with deionized water. All BGE solutions were filtered through a 0.2 μm nylon filter before use.

Sample Solutions

Separate stock solutions of each diastereomer were prepared in acetonitrile, methanol, or ethanol. Aliquots of these solutions, along with an aliquot of a 8.5 mM solution of Sudan III in methanol, were diluted with BGE not containing organic modifier to prepare sample solutions for development of separation conditions. These solutions contained approximately 0.03 mg/mL α-methyl diastereomer and 0.1 mg/mL β-enol phosphate.

Capillary Conditioning

New capillaries were conditioned with 1 N sodium hydroxide for 30 min, deionized water for 10 min, 0.1 N sodium hydroxide for 10 min, deionized water for 10 min, and BGE for 30 min. Each day before use, capillaries were rinsed with 0.1 N sodium hydroxide for 10 min, deionized water for 10 min, and BGE for 15 min. Between injections, the capillary was rinsed with BGE for 5 min.

Calculations

In MEKC, the capacity factor of an analyte, k', is defined by the following equation:[5]

$$k' = \frac{t_r - t_0}{t_0(1 - (t_r/t_{mc}))} \tag{1}$$

where t_r is the migration time of the analyte, t_0 is the migration time of an unretained marker (methanol was used in these studies), and t_{mc} is the migration time of a compound that is completely solubilized in the micelles and, therefore, migrates at the same velocity as the micelles (Sudan III was used in these studies). This definition is similar to that used in HPLC, except that it takes into account that the micelles act as a "pseudostationary phase" since they are actually migrating through the BGE during the analysis. If t_{mc} becomes infinite (i.e., the micelles act as a true stationary phase) then Eq. (1) reduces to the definition of k'used in HPLC:

$$k' = \frac{t_r - t_0}{t_0} \tag{2}$$

Resolution (R_s) in MEKC is defined by the following equation:[5]

$$R_s = \frac{\sqrt{N}}{4} \frac{\alpha - 1}{\alpha} \frac{k'_2}{1 + k'_2} \frac{1 - (t_0/t_{mc})}{1 + (t_0/t_{mc})k'_1} \tag{3}$$

where N is the average number of theoretical plates, $k'_1$and k'_2 are the capacity factors of the earlier and later eluting components, respectively, and α is the selectivity factor (k'_2/k'_1). This equation is similar to that used in HPLC, except for the addition of a fourth term, which accounts for the migration of the micelles through the BGE. The last two terms in Eq. (3) can be combined to give $f(k)$, the capacity function, which is unique to MEKC:[5]

$$f(k) = \frac{k'_2}{1 + k'_2} \frac{1 - (t_0/t_{mc})}{1 + (t_0/t_{mc})k'_1} \tag{4}$$

The electrophoretic mobility of the EOF, μ_{eof}, was calculated using Eq. (5):[18]

$$\mu_{eof} = \frac{L_e L_t}{V t_0} \tag{5}$$

where L_e is the effective capillary length (i.e., the length of the capillary between the injection end and detector), L_t is the total capillary length, and V is the applied voltage.

The electrophoretic mobility of the micelles μ_{mc}, was calculated using Eq. (6):[18]

$$\mu_{mc} = \left(\frac{1}{t_{mc}} - \frac{1}{t_0}\right) \frac{L_e L_t}{V} \tag{6}$$

RESULTS AND DISCUSSION

Initial Experiments

Sodium dodecyl sulfate was chosen as the surfactant due to its UV transparency, and excellent solubility characteristics. Due to the hydrophobicity of the α and β diastereomers and their high affinity for SDS micelles, it was desirable to keep the phase ratio as low as possible in order to minimize capacity factors. The CMC of SDS in pure water is approximately 8 mM, though it has been found that in solutions containing electrolytes (e.g., buffered BGE solutions) this value can be as low as 2.6 to 5.0 mM.[17] In order to maintain a low phase ratio, while ensuring micelle integrity, an initial SDS concentration of 20 mM was chosen. In order to obtain reproducible electroosmotic flow, a high pH was used. At pH values greater than 9, the silanol groups of the fused silica capillary are predominately deprotonated and the EOF is less sensitive to small variations in pH. Sodium borate was chosen as the buffer since it has a $pK_a = 9.24$ and, being a relatively large molecule, could be used at higher concentrations with less generation of current and, hence, lower Joule heating at high operating

voltages.[18] To ensure maximum buffer capacity, a sodium borate concentration of 25 mM and a pH of 9.3 were chosen.

Using the above BGE, it was possible to obtain separation of the diastereomers (Fig. 2a). However, the resolution obtained was not sufficient for the determination of residual amounts of α-enol phosphate in the presence of much higher amounts of β-enol phosphate. In order to obtain adequate detectability of low amounts of α-enol phosphate, it would eventually become necessary to use higher sample concentrations and/or longer injection times. Due to volume or mass overloading, significant band broadening could occur with a corresponding loss in resolution.[6]

As can be seen in the electropherogram given in Fig. 2a, both analytes had migration times close to that of Sudan III, indicating a high affinity for the SDS micelles. Capacity factors (k') of 21 and 27 were obtained for β- and α-enol phosphate, respectively. The selectivity that was obtained can be attributed mostly to the differing degrees of hydrophobic interactions of the two diastereomers with the SDS micelles.

Influence of Organic Modifier Concentration on Resolution

In order to decrease solute/micelle interactions, an organic modifier was added to decrease the polar nature of the BGE, thereby, increasing the solubility of the diastereomers. The effect of adding methanol to the BGE (20 mM SDS, 25 mM sodium borate, pH 9.3) on resolution was investigated. The volume % of methanol in the BGE was varied as follows: 0, 2, 5, 10, and 20%. Upon increasing the methanol concentration in the BGE, the average theoretical plate number remained constant ($N \approx 120{,}000$). The relative contributions of selectivity, (α) and $f(k)$, to the resolution (Eqs. 3 and 4) as a function of methanol concentration were examined.

There was a steady decrease in selectivity between the analytes as the methanol concentration was increased (Fig. 3). This can be attributed to a decrease in the selective interactions of the diastereomers with the SDS micelles, due to increased solubilization of the diastereomers in the surrounding aqueous phase. This decrease in selectivity may have also been partially due to a change in micelle properties.

The effect of methanol concentration on the $f(k)$ term of the resolution equation (Eq. 4) is shown in Fig. 4. Upon increasing the methanol concentration from 0 to 20% v/v, there was almost a five-fold increase in $f(k)$. The data in Table 1 shows that this can be attributed to a simultaneous increase in the migration time window (t_{mc}/t_0) and decrease in the capacity factors of the analytes. The increase in $t_{mc}/t_0$was due to the decrease in the electroosmotic mobility (u_{eof}) that resulted from an increase in the viscosity and a decrease in both the dielectric constant of the

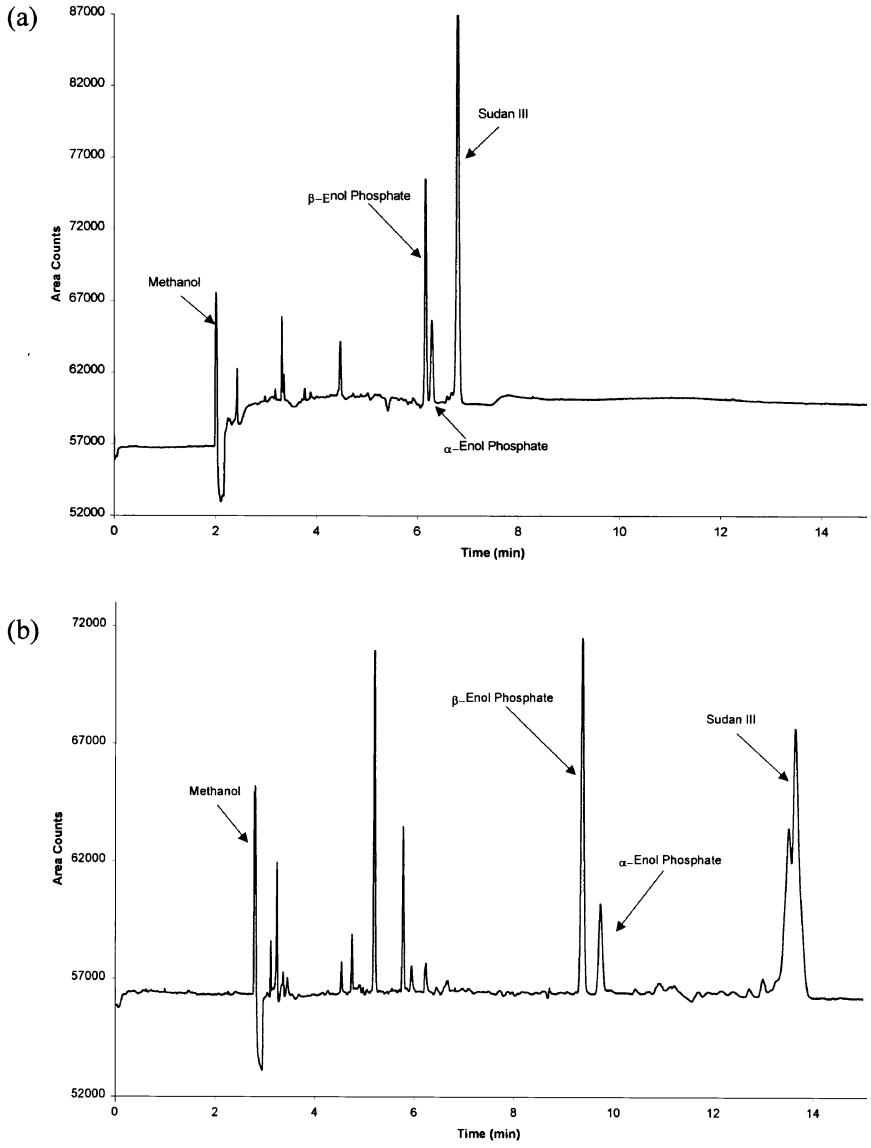

Figure 2. Electropherogram of α and β enol phosphate mixture (0.03 and 0.1 mg/mL, respectively, dissolved in BGE). (a) BGE: 20 mM SDS, 25 mM sodium borate, pH 9.3; capillary: 48.5 cm/40 cm, total/effective length, 50 μm i.d., extended light path; applied voltage: 25 kV (current 49 μA); UV detection wavelength: 200 nm; capillary cassette temperature: 30°C; hydrodynamic injection, 50 mbar, 2 s. (b) Same conditions as (a) except with 10% methanol added to BGE (current 41 μA).

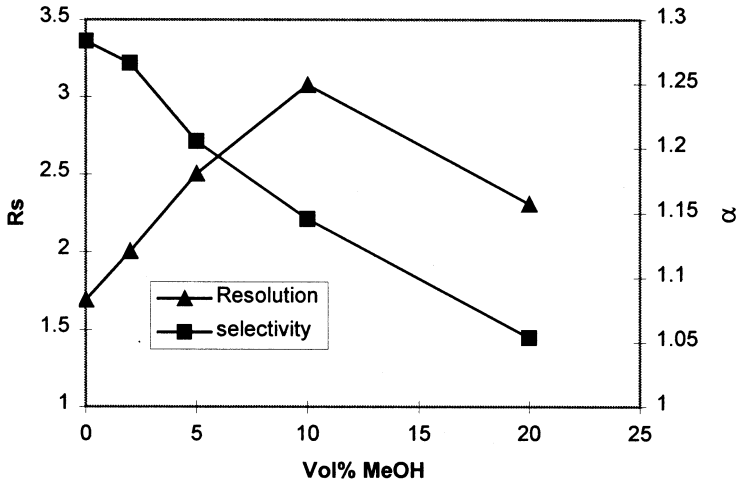

Figure 3. Effect of methanol concentration on selectivity (α) and resolution (R_s) between diastereomers. BGE: 20 mM SDS, 25 mM sodium borate with 0 to 20 volume % methanol added, pH_{app} 9.3; applied voltage: 25 kV (current 35 to 49 μA). Other conditions as in Fig. 2a.

BGE and the zeta potential at the capillary wall. The decrease in u_{eof} produced an increase in analysis time from less than 6.5 min (0 volume % methanol) to more than 12 min (20 volume % methanol). The change in the effective micelle mobility (u_{mc}) was also due to the increased viscosity of the BGE, but also to changes in micelle structure upon addition of increasing amounts of methanol.[19] In Table 1, $|u_{mc}|$ is given, since in the normal mode of MEKC (i.e., $t_{mc}/t_0 > 0$), the SDS micelles migrate in the direction opposite to that of the EOF.

The initial increase in resolution that was obtained upon increasing the volume % of methanol from 0 to 10% v/v could be attributed to an increase in $f(k)$. As the methanol concentration was increased further to 20%, the positive contribution of $f(k)$ was offset by a loss in selectivity and the resolution decreased. The best resolution ($R_s = 3.0$) was obtained using 10 volume % methanol in the BGE. An electropherogram showing the separation that was obtained when this amount of methanol was added to the BGE is given in Fig. 2b.

The use of ethanol and acetonitrile as organic modifiers was also investigated. Although R_s could be increased when an optimal amount of either modifier was added to the BGE, the resolution obtained in both cases was less than that obtained using methanol (maximum $R_s = 2.5$ and 2.7 for ethanol and acetonitrile, respectively). Methanol was, therefore, retained as the organic modifier for the remainder of the studies.

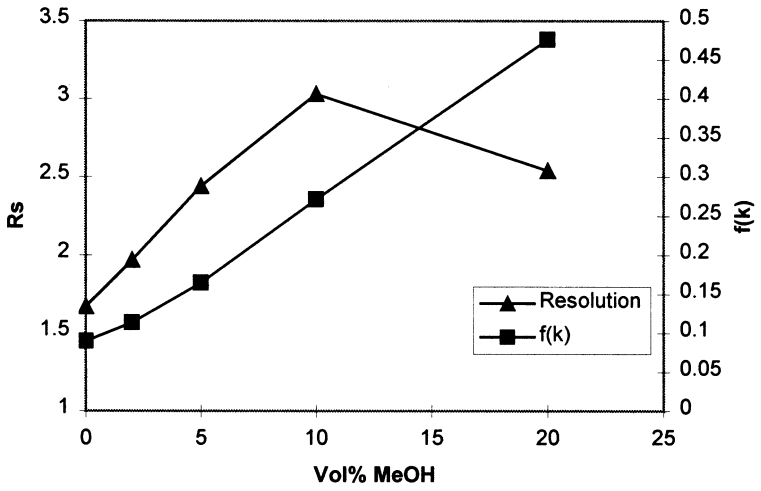

Figure 4. Effect of methanol concentration on $f(k)$ and resolution (R_s) between diastereomers. BGE: 20 mM SDS, 25 mM sodium borate with 0 to 20 volume % methanol added, pH_{app} 9.3; applied voltage: 25 kV (current 35 to 49 μA). Other conditions as in Fig. 2a.

Influence of Sodium Dodecyl Sulfate Concentration on Resolution

In order to study the effect of the SDS concentration on R_s, the concentration of SDS in the BGE (25 mM sodium borate, 10% methanol) was varied as follows: 10, 20, 30, 40 mM. The influence that the SDS concentration had on R_s is shown in Fig. 5.

Table 1. Electroosmotic Flow Mobility, $u_{eof} \times 10^{-5}(cm^2V^{-1}s^{-1})$, Effective Micelle Mobility, $|u_{mc}| \times 10^{-5}(cm^2V^{-1}s^{-1})$, Migration Time Window (t_{mc}/t_0), k', and $f(k)$ Values Obtained Upon Varying Methanol Concentration

% v/v Methanol	u_{eof}	$\lvert u_{mc}\rvert$	t_{mc}/t_0	k'_1(Beta)	k'_2(Alpha)	$f(k)$
0	64.03	45.03	3.37	22.2	28.5	0.090
2	61.01	43.55	3.50	17.5	22.2	0.114
5	54.80	40.81	3.92	12.7	15.3	0.165
10	46.52	36.60	4.69	7.5	8.6	0.271
20	33.42	29.11	7.76	3.2	3.3	0.476

BGE: 20 mM SDS, 25 mM sodium borate, with 0 to 20 volume percent methanol added, pH_{app} 9.3; applied voltage: 25 kV (current 35 to 49 μA); other conditions as in Fig. 2a.

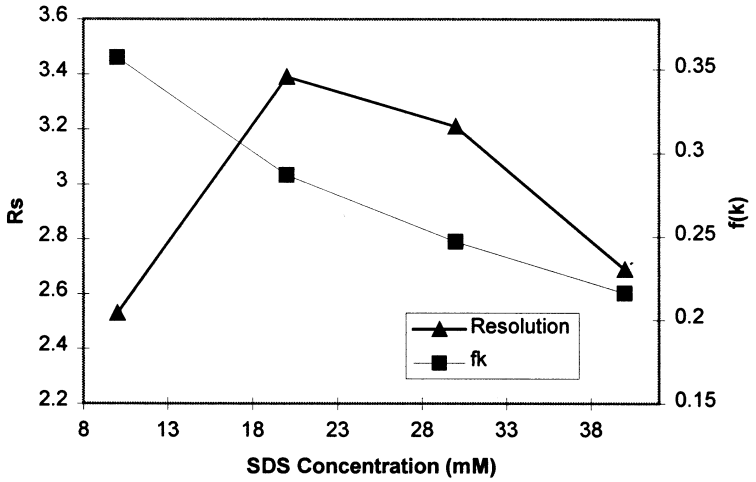

Figure 5. Effect of SDS concentration on f_k term and resolution between diastereomers. BGE: 10 to 40 mM SDS, 25 mM sodium borate, 10% v/v methanol, pH_{app} 9.3; applied voltage 25 kV (current 41–48 μA). Other conditions as in Fig. 2a.

Upon increasing the SDS concentration from 10 to 20 mM, there was a large increase in the efficiency obtained. With 10 mM SDS added to the BGE, the average number of theoretical plates obtained was 68,000. The number of theoretical plates increased as more SDS was added and, at concentrations greater than 20 mM, the number exceeded 140,000. The low efficiency obtained at an SDS concentration of 10 mM could be attributed to the fact, that since this concentration was closer to the CMC of SDS, the concentration of micelles in the BGE was much lower than that obtained using more normal SDS concentrations (>25 mM).[5]

Upon increasing the SDS concentration and, hence, the phase ratio, there was a linear increase in the average capacity factors of both analytes with correlation coefficients greater than 0.999 for both analytes. This is because the capacity factor k', under normal conditions, can be linearly related to the surfactant concentration as follows:[19]

$$k' = K_c v(C_{SF} - CMC) \tag{7}$$

where K_c is the distribution coefficient of the analytes v is the partial molar volume of the micelles, C_{SF} is the surfactant concentration, and CMC is the critical micelle concentration. The average capacity factor ranged from 3.6 (10 mM SDS) to 15.6 (40 mM SDS) and the analysis time increased from less than 6 min to greater than 12 min upon increasing the SDS concentration. Over the concentration range studied, the selectivity, α, remained constant at 1.15.

Figure 5 shows the influence of the SDS concentration on $f(k)$. Upon increasing the surfactant concentration, $f(k)$ decreased since the phase ratio was increased and, hence, so were the capacity factors of the analytes (Eq. 4). This is consistent with what other workers have observed for strongly hydrophobic compounds as the SDS concentration is increased.[19]

Overall, as the surfactant concentration was increased, there was an initial increase in R_s upon going from 10 mM to 20 mM SDS. Although lower capacity factors and, hence, higher $f(k)$ values were obtained using a BGE containing 10 mM SDS, lower efficiency was obtained. Upon increasing the SDS concentration to 20 mM, the efficiency improved and so did R_s. As the SDS concentration was increased further beyond 20 mM, the phase ratio increased and R_s decreased, due to the reduction in $f(k)$ that resulted from increased capacity factors. A BGE containing 20 mM SDS provided the highest R_s and was retained for the remainder of the studies.

Influence of Cyclodextrin Concentration on Resolution

Studies were performed using underivatized β-cyclodextrin as a BGE additive. Addition of increasing amounts of β-cyclodextrin decreased the capacity factors of both analytes at almost the same rate. This indicates that the diastereomers may have been partially included in the β-cyclodextrin cavity, but not to a degree that would allow for selective interactions to occur. Overall, the resolution was only slightly improved, and was not adequate for determination of residual amounts of the α-methyl diastereomer.

γ-Cyclodextrin was the second choice as a BGE additive, due to its larger inner diameter (8.3 A) compared to β-cyclodextrin (6.4 A), and it was thought that it would provide a better goodness of fit for the enol phosphate diastereomers.[3] Also, γ-cyclodextrin has much better solubility characteristics than β-cyclodextrin. A concentration study was done in which γ-cyclodextrin was added to the BGE that, up to this point, provided the best resolution (20 mM SDS, 25 mM sodium borate, 10% methanol, pH_{app} 9.3). The γ-cyclodextrin concentration was varied as follows: 0, 1.4, 2.8, 5.6, and 11.2 mM.

The graph in Fig. 6 shows that the addition of γ-cyclodextrin to the BGE had a pronounced effect on the resolution obtained. At γ-cyclodextrin concentrations greater that 5.6 mM, the resolution is increased nearly two-fold compared to that obtained using BGE with no added γ-cyclodextrin. This increase in resolution could be attributed to simultaneous increases in the capacity function $f(k)$ and selectivity factor, α.

The influence that the γ-cyclodextrin concentration had on $f(k)$ (Eq. 4) is shown in Fig. 7. There was an increase in $f(k)$ upon addition of γ-cyclodextrin that reached a maximum value at a concentration of approximately 6 mM. This increase in $f(k)$ accounted for approximately a 1.4 increase in the resolution

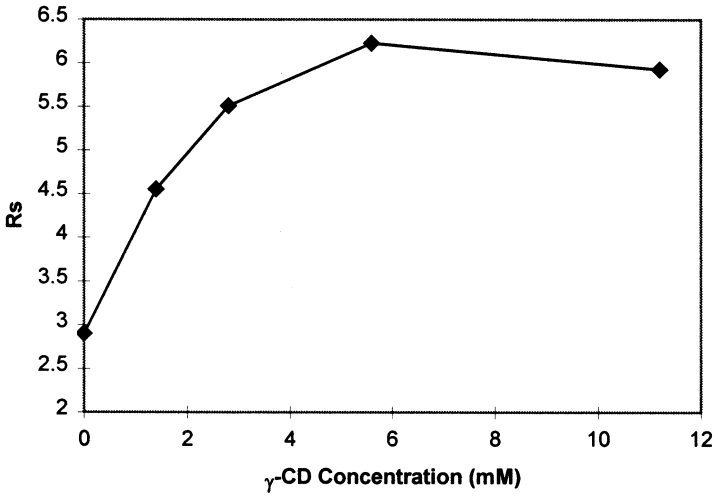

Figure 6. Effect of γ-cyclodextrin concentration on resolution (R_s) between diastereomers. BGE: 20 mM SDS, 25 mM sodium borate, 10% v/v methanol, 0 to 11.2 mM γ-cyclodextrin, pH_{app} 9.3; applied voltage 25 kV (current 41 μA). Other conditions as in Fig. 2a.

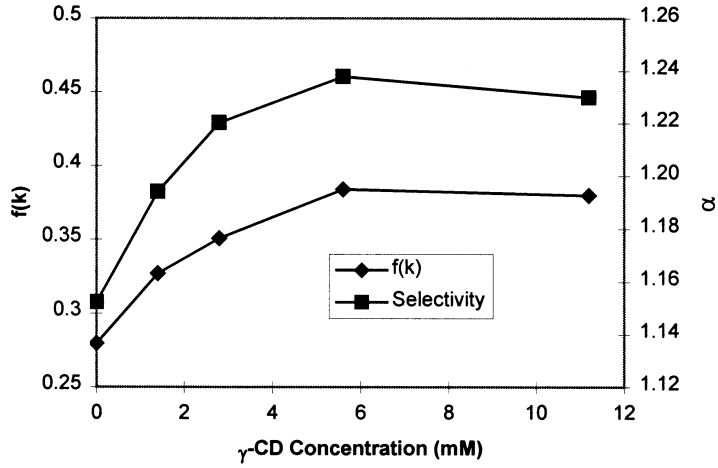

Figure 7. Effect of γ-cyclodextrin concentration on $f(k)$ and selectivity (α). BGE: 20 mM SDS, 25 mM sodium borate, 10% v/v methanol, 0 to 11.2 mM γ-cyclodextrin, pH_{app} 9.3; applied voltage 25 kV (current 41 μA). Other conditions as in Fig. 2a.

obtained. The increase in $f(k)$ was due to a reduction of the capacity factors that resulted from inclusion of the diastereomers into the cyclodextrin cavity. Since γ-cyclodextrin had the same mobility as the EOF, the migration times and, hence, the capacity factors of the diastereomers were reduced. The migration time window, t_{mc}/t_0, decreased slightly over the concentration range studied, mostly due to increased BGE viscosity and, hence, a reduction in the electroosmotic flow mobility, u_{eof}. The optimal capacity factor, k'_{opt}, for a given migration time window can be calculated to be $(t_{mc}/t_0)^{1/2}$.[19] From the migration time windows obtained in this study, k'_{opt} was calculated to be 2.2. From the plot in Fig. 8, the γ-cyclodextrin concentration that provided capacity factors closest to k'_{opt} could be estimated to be 9 mM. However, at concentrations greater than 5.6 mM, other sample components began to co-migrate with the diastereomers.

As the γ-cyclodextrin concentration was increased, there was an increase in the selectivity, α. When γ-cyclodextrin was not present in the BGE, an α value of 1.15 was obtained. However, upon increasing the γ-cyclodextrin concentration to 5.6 mM, the α increased to a value of 1.24 and began to level off at higher concentrations. When the logarithms of the capacity factors were plotted against the γ-cyclodextrin concentration from 0 to 5.6 mM, linear relationships (correlation coefficients >0.991) were obtained for both diastereomers. The slopes obtained from the linear regression analysis revealed that the capacity factor of β-enol phosphate decreased at a rate 7.5% faster than that of the α-diastereomer as the γ-cyclodextrin concentration was increased. This indicates, that β-enol phosphate forms a more stable inclusion complex with γ-cyclodextrin than the α-methyl diastereomer. The increase in selectivity that was obtained upon adding γ-cyclodextrin to the BGE could alone account for a 1.5 fold increase in R_s.

In summary, the nearly two-fold increase in R_s that was observed upon adding γ-cyclodextrin to the BGE was due to combined increases in $f(k)$ and selectivity. The increase in R_s began to level off at a γ-cyclodextrin concentration of approximately 6 mM. When the γ-cyclodextrin concentration was increased beyond 6 mM, other components began to interfere with the separation of the diastereomers. A BGE containing 6 mM γ-cyclodextrin was thus chosen for the final method. This permitted an analysis time of less than eight minutes. An electropherogram showing the separation obtained using the final BGE composition (20 mM SDS, 25 mM sodium borate, 10% methanol, 6 mM γ-cyclodextrin, pH_{app} 9.3) is shown in Fig. 9a.

Final Method Conditions

Although diode array analysis of the diastereomer peaks showed that both had absorption maxima at approximately 270 nm, much better response was obtained at a wavelength of 200 nm. At this wavelength, the UV transparency of the BGE was

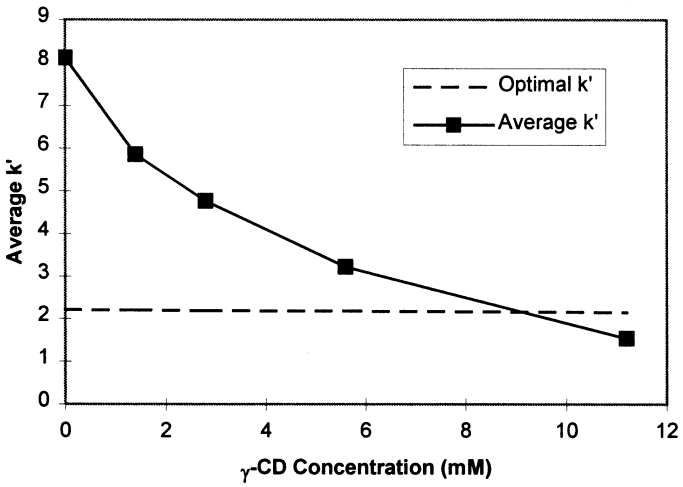

Figure 8. Effect of γ-cyclodextrin concentration on average capacity factor. BGE: 20 mM SDS, 25 mM sodium borate, 10% v/v methanol, 0 to 11.2 mM γ-cyclodextrin, pH_{app} 9.3; applied voltage 25 kV (current 41 μA). Other conditions as in Fig. 2a.

still sufficient enough for accurate detection and quantitation of residual amounts of the α-diastereomer. Therefore, 200 nm was used as the detection wavelength in the final method. The capillary cassette temperature used throughout these studies, 30°C, was retained in the final method. Use of higher capillary temperatures resulted in co-migration of the diastereomers with other sample components.

In order to obtain a lower detection limit for α-enol phosphate, a sample concentration of 1 mg/mL was used. The final BGE, which contained 10% v/v methanol, did not provide sufficient solubility for this sample concentration and could not be used as sample diluent. Background electrolyte containing 45% v/v methanol was required to solubilize the sample. However, peak broadening of the α-enol phosphate occurred using this diluent composition, resulting in lower detectability. Therefore, other diluent compositions were investigated. The best peak shape and sensitivity was obtained using a sample diluent containing 35% v/v acetonitrile and 33 mM sodium borate pH 9.3. Hydrodynamic injection at 50 mbar for 2 seconds was used in the final method.

Validation Studies

The limit of detection (LOD) of the method was evaluated by spiking aliquots of a 0.17 mg/mL solution of α-enol phosphate into a 1.02 mg/mL

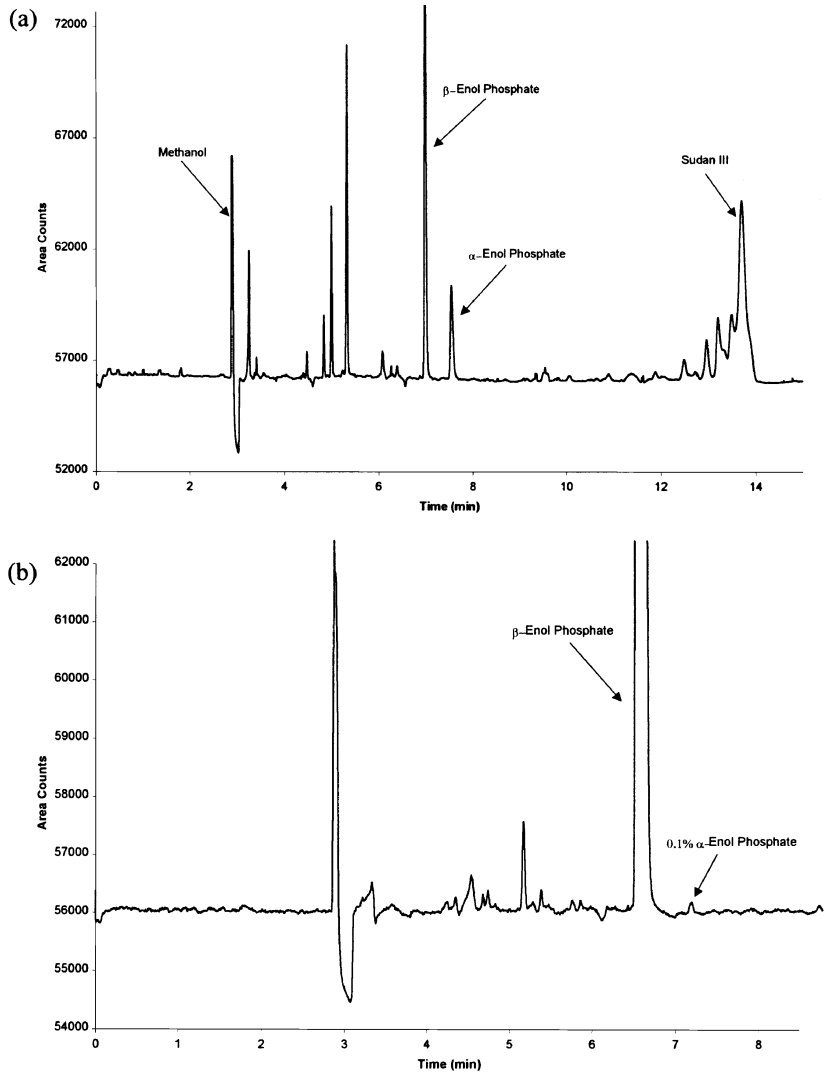

Figure 9. (a) Electropherogram of mixture α and β enol phosphate (0.03 and 0.1 mg/mL, respectively, dissolved in BGE). BGE: 20 mM SDS, 25 mM sodium borate, 6 mM γ-cyclodextrin, 10% methanol, pH_{app} 9.3; capillary: 48.5 cm/40 cm, total/effective length, 50 µm i.d., extended light path; applied voltage: 25 kV (current 41 µA); UV detection wavelength: 200 nm; capillary cassette temperature: 30°C; hydrodynamic injection, 50 mbar, 2 s. (b) β-enol phosphate key raw material spiked with 0.1% α-enol phosphate. Conditions same as (a) except sample concentration: 1 mg/mL in 35% acetonitrile/33 mM sodium borate pH 9.3.

solution of β-enol phosphate. The limit of detection for α-enol phosphate was determined to be 0.1 area-% vs. β-enol phosphate. At this level, a signal to noise ratio (S/N) of four was obtained. An electropherogram of sample spiked with 0.1% α-enol phosphate is given in Fig. 9b. The limit of quantitation (LOQ) was taken to be three times the LOD and was, therefore, determined to be 0.3 area-%.

The linearity of response of α-enol phosphate was evaluated by spiking a 1.02 mg/mL solution of β-enol phosphate with aliqouts of α-enol phosphate to obtain concentrations ranging from 2.57 μg/mL to 17.1 μg/mL. This corresponds to an area-% range of 0.3% to 1.7% α-enol phosphate. Duplicate injections were done of five solutions and the corrected area response (area counts/migration time) was found to be linear (correlation coefficient = 0.9949) throughout the entire range of concentrations studied. The linearity of response of β-enol phosphate was evaluated by injecting solutions of β-enol phosphate having concentrations ranging from 0.0102 mg/mL to 1.28 mg/mL. Duplicate injections were done of five solutions and the corrected area response was found to be linear (correlation coefficient = 0.9984) over the concentration range studied.

The injection precision at the LOQ was evaluated by making five injections of a 1.02 mg/mL solution of β-enol phosphate spiked with 0.3 area-% α-enol phosphate. The % RSD for the corrected area counts was 4.7% for α-enol phosphate.

The reproducibilty of migration times was evaluated using the data from the linearity study for α-enol phosphate. This represented a total of ten injections. The % RSD of the migration times of α-enol phosphate and β-enol phosphate were 0.90% and 1.01%, respectively.

CONCLUSIONS

A CD-MEKC method for the determination of α-methyl diastereomer impurity in β-methyl ADC-13 enolphosphate diphenyl ester key raw material was developed. This method can be used to detect α-methyl diastereomer at levels of 0.1 area-% in less than eight minutes. Studies showed that, although, the resolution between the diastereomers could be increased by adding an organic modifier to the BGE and optimizing the SDS concentration, sufficient resolution for accurate determination of low levels of the α-methyl diastereomer was not obtained. However, addition of a sufficient amount of γ-cyclodextrin to the BGE resulted in an almost two-fold increase in resolution, thus, allowing for more accurate detection and quantitation of the α-methyl diastereomer impurity. This increase in resolution was found to be due to combined increases in selectivity and $f(k)$. Results of the validation studies show that the method is sensitive, linear, and precise.

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