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Separation of piperidine-2,6-dione drug enantiomers by micellar electrokinetic capillary chromatography using synthetic chiral surfactants

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Abstract

The enantioselective separation of five racemic piperidine-2,6-dione compounds was accomplished using chiral micelle electrokinetic capillary chromatography. This class of drugs includes glutethimide, aminoglutethimide, cyclohexylaminoglutethimide, pyridoglutethimide, and phenglutarimide. The resolution of all five compounds with simultaneous enantioselective separation of four of the five was obtained in a single run, using the synthetic chiral surfactant (*S*)-N-dodecoxycarbonylvaline. An enantioselective separation of the fifth compound was obtained by a second synthetic chiral surfactant, (*R*)-dodecoxycarbonylproline. It was observed that surfactant type and concentration, pH, and sample matrix all effect enantiomeric resolution. The migration order of cyclohexylaminoglutethimide enantiomers was confirmed by injecting a sample comprised mostly of the (−)-enantiomer. In addition, the separation of a sixth related sample (a thalidomide mixture) is shown to be achiral and not enantiomeric, as verified by the lack of enantiomer migration order reversal.

Keywords: Enantiomer separation; Chiral surfactants; Micellar electrokinetic chromatography; Piperidine-2,6-dione compounds

1. Introduction

The piperidine-2,6-dione compounds have been used therapeutically since the early 1950's for the treatment of Parkinson disease [1], breast cancer [2,3], and hormone dependent tumors [4,5]. This biologically important class of compounds includes glutethimide, aminoglutethimide, cyclohexylaminoglutethimide, pyridoglutethimide, phenglutarimide, and thalidomide. Although piperidine-2,6-dione drugs are routinely administered as the racemic mixtures, it has been shown in at least one instance that the enantiomers of these compounds possess different biological activity [6]. It is for this reason that the separation of the various piperidine-2,6-dione

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enantiomers has been studied [7–15]. These enantioselective separations have been accomplished using high-performance liquid chromatography (HPLC) employing either a cellulose tris(3,5-dimethylphenyl carbamate) stationary phase operated in a "normal phase" mode (hexane–ethanol mobile phase [7–12], or a silica-based cyclodextrin bonded stationary phase operated in both a normal- and reversed-phase mode [13–15]. Capillary electrophoretic (CE) separation of the enantiomers of glutethimide, aminoglutethimide, and related analogs using cyclodextrin buffer modifiers has also been reported [16]. The methods employed to date, however, do not have the selectivity or efficiency to separate complex mixtures of several components. In addition, the HPLC methods require the use of large volumes of non-aqueous solvents, and neither the HPLC or the cyclodextrin CE methods are capable of exact enantiomer migration order reversal.

To overcome some of the potential disadvantages of various enantioselective HPLC methods, and to provide confirmatory orthogonal information, researchers have investigated the use of capillary electrophoresis for the separation of enantiomeric mixtures. CE is an attractive technique because of the high efficiencies (in excess of 100 000 theoretical plates) possible for the separation of small molecules. Many different approaches to the analysis of chiral compounds by CE have been reported in the literature, and the subject has been recently reviewed [17,18]. Several chiral natural products have been used as additives for CE including cyclodextrins [19–23], various biological compounds such as proteins [24–26], and crown ethers [27]. Natural product additives are somewhat limited in their applicability, however, and many exhibit high detector background levels which result in diminished sensitivity and dynamic range. In addition, natural product additives are only available as the single enantiomer, making peak reversal difficult.

For added selectivity and to separate both changed and neutral components of a mixture, a mode of CE known as micellar electrokinetic capillary chromatography (MECC) can be employed [28,29]. The separation of chiral compounds by MECC has been achieved through the use of bile salts [30–32], and synthetic chiral surfactants such as (*S*)-dodecanoylvaline [33–37] or dodecyl-β-D-glucopyranoside [38]. Mixed achiral and chiral micelles [39] and combinations of natural products such as cyclodextrins with achiral micelles [40,41] have also been used. Bile salts, like other natural products, are not capable of providing peak reversal, however, and selectivity is limited. Furthermore, synthetic chiral surfactants such as (*S*)-dodecanoylvaline have limited solubility and a high UV detector background, that, like the natural product additives, limit sensitivity and linearity.

We have recently reported the use of MECC employing new synthetic chiral surfactants as buffer additives for enantioselective separations [42,43]. Using the novel synthetic chiral surfactants (*S*)- and (*R*)-dodecoxycarbonylvaline [(*S*)- and (*R*)-DDCV], the enantioselective separation of several racemic drugs [42] and N-protected amino acid mixtures [43] has been accomplished. The advantages of using synthetic chiral surfactants in the MECC mode over the more traditional HPLC methods include: high efficiencies, simultaneous chiral and achiral compound separation, a simple and straightforward method development process, the ability to reverse enantiomer migration order, tolerance of complex sample matrices, and the use of small quantities of predominately aqueous solvents. Because of the advantages of this technique, our work using novel synthetic chiral surfactants was extended to a new class of compounds, the piperidine-2,6-diones. Method development leading to the enantiomeric separation of four of the six compounds studied (aminoglutethimide, cyclohexylaminoglutethimide, pyridoglutethimide, and phenglutarimide) using (*R*)-DDCV is reported and the enantiomeric separation of a fifth compound (glutethimide) utilizing a new synthetic chiral surfactant, (*R*)-dodecoxycarbonylproline [(*R*)-DDCP] is also described. Finally, the separation obtained for a sixth related sample (a thalidomide mixture) is shown to be achiral and

not enantiomeric. This determination was made possible by using the migration time reversal properties of (*S*)- and (*R*)-DDCV.

2. Experimental

2.1. Apparatus

A Waters Quanta™ 4000E capillary electrophoresis system was used throughout (Waters Corporation, Milford, MA, USA). The electrophoretic system was controlled by the Millennium Chromatography Manager (Waters) which was also used for data collection (five points per second) and processing. All separations were performed on standard untreated AccuSep™ capillaries, 50 μm internal diameter by 60 cm (52.5 cm effective length) (Waters). Prior to use, the capillaries were prepared by rinsing (purging by vacuum) with 0.5 *M* sodium hydroxide (10 min), followed by water (10 min), and run buffer (10 min). A 3-min purge of fresh buffer was routinely employed between individual runs. Hydrostatic (10 cm height) injections for 5–30 s with an applied voltage of +16 000 V were employed. All analyses were performed at 30°C with UV detection at 214 nm.

2.2. Chemicals and supplies

Electrophoresis buffers were purchased commercially from either Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) in the highest purity available and were used without further purification. Racemic aminoglutethimide (Batch No. 9614586) and racemic glutethimide (Batch No. 25726) were obtained from Ciba-Geigy (Basle, Switzerland). Racemic phenglutarimide-HCl was kindly supplied by Professor P.J. Nicholls (University of Wales, School of Pharmacy, Cardiff, UK). Racemic pyridoglutethimide was obtained from Dr. R. McCague (Chiros Limited, Cambridge, UK). Racemic and (−)-cyclodexylaminoglutethimide was a gift from Professor R.W. Hartmann (University of Saarland, Saarbrucken, Germany). Thalidomide (FDA-Lot A, presumed to be a racemic mixture) was supplied by the Division of Drug Analysis, Food and Drug administration (St. louis, MO, USA).

2.3. Buffer solutions

(*S*)- and (*R*)-Dodecoxycarbonylvaline and (*S*)-dodecoxycarbonylproline were synthesized in our laboratories as described previously [42]. Buffers were prepared in Milli-Q water (Millipore, Bedford, MA, USA) with surfactant and disodium phosphate and/or disodium tetraborate. The pH was adjusted with either sodium hydroxide or phosphoric acid. Buffers were filtered (0.45-μm Millex filters, Millipore) and degassed under vacuum daily.

2.4. Capacity factor, selectivity, and resolution calculations

Capacity factors (k) and selectivity (α) were calculated as described by Terabe et al. [44] using the following formulas:

$$k' = (t_r - t_{aq})/t_{aq}(1 - t_r/t_{mc}) \quad (1)$$

$$\alpha = k_2/k_1 \quad (2)$$

where t_r is the observed migration time of the solute, t_{mc} is the migration time of a solute completely partitioned into the micelle, and t_{aq} is the migration time of the solute if it does not interact with the micelle. The t_{aq} value is calculated as follows [42]:

$$t_{aq} = 1/(1/t_{CZE} + 1/t_{os,MECC} - 1/t_{os,CZE}) \quad (3)$$

where t_{CZE} is the migration time of the compound in the free-zone mode without surfactant (all other conditions identical), $t_{os,MECC}$ is the electroosmotic flow migration time in the MECC experiment, and $t_{os,CZE}$ is the electroosmotic flow time in the free-zone experiment. Optimum partitioning was determined by the ratio of t_{mc}/t_{aq} according to the following equation [45]:

$$k'_{optimum} = (t_{mc}/y_{aq})^{1/2} \quad (4)$$

Methanol was used as the electroosmotic flow

marker in both the free-zone and MECC experiments, and the hydrophobic compound sulconazole was used as the micelle marker.

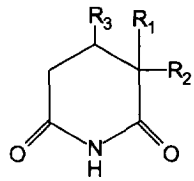
Resolution was calculated automatically by the data station according to Eq. 3.5:

$$R_s = 2(T_{m_2} - T_{m_1})/(W_1 + W_2) \quad (5)$$

Where T_m is the peak migration time and W is the peak width. Peak width was measured by drawing tangent lines from 50% of the peak height through the baseline intercept.

3. Results and discussion

As shown previously [42], the key variables used to optimize selectivity using (*S*)- and (*R*)-DDCV include surfactant concentration, pH, and the organic modifier concentration. To obtain and optimize an enantiomeric separation, it is necessary to optimize partitioning into the micelle using both hydrophobicity (surfactant and organic modifier concentration) and/or solute charge (pH). Partitioning is measured by k' (Eq. 1) and reaches an optimum value when calculated according to Eq. 4. When partitioning is not optimized, resolution is compromised. The structures of the six piperidine-2,6-diones used in this study are shown in Fig. 1. Racemic mixtures of aminoglutethimide, cyclohexylaminoglutethimide, pyridoglutethimide, glutethimide, and phenglutarimide were used to study the effects of both surfactant concentration and pH on enantiomeric resolution. Since the pK_as of most of these compounds are unknown, free solution capillary electrophoresis at pH 9.0 was used to determine their charge. The results of these experiments indicate that all of these compounds are either neutral (pyridoglutethimide, glutethimide, aminoglutethimide and cyclodexylglutethimide), or positively charged (phenglutarimide) over the useful pH range of the surfactants employed (pH > 6.5). Fig. 2 shows the effect on enantiomeric resolution as the concentration of (*R*)-DDCV is increased from 0 to 100 m*M* at pH 9.0. For three of the five compounds studied (glutethimide, pyridoglutethimide and aminoglutethimide), resolution steadily increased with an increase in surfactant concentration. However, for two of the compounds (cyclohexylglutethimide and phen-



Compound	R_1	R_2	R_3
Aminoglutethimide	Ethyl	p-Aminophenyl	H
Cyclohexyl-aminoglutethimide	Cyclohexyl	p-Aminophenyl	H
Pyridoglutethimide	Ethyl	4-Pyridyl	H
Phenglutarimide	2-Diethylaminoethyl	Phenyl	H
Glutethimide	Ethyl	Phenyl	H
Thalidomide	H	H	

Fig. 1. Chemical structure of the piperidine-2,6-diones used in this study.

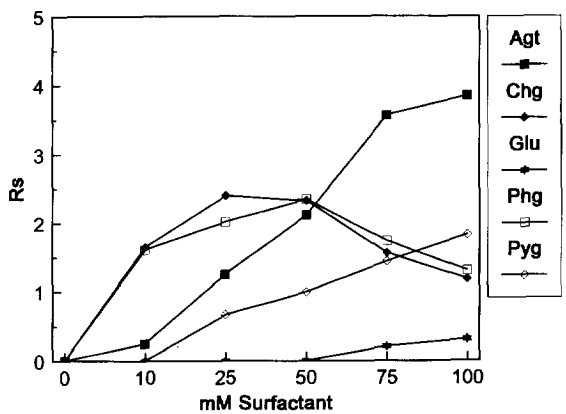


Fig. 2. Effect of surfactant concentration on enantiomeric resolution. Buffer consisted of 25 m*M* Na_2HPO_4/NaB_4O_7, pH 9.0, with varying amounts of (*S*)-DDCV. Sample concentration was 100 μg/ml each in water–methanol (90:10), and a 5-s hydrostatic (10 cm) injection was used. All other conditions as described previously (Experimental). Abbreviations are: aminoglutethimide (Agt), cyclodexylaminoglutethimide (Chg), glutethimide (Glu), phenglutarimide (Phg) and pyridoglutethimide (Pyg).

glutarimide), resolution plateaued at 25–30 m*M*, and then decreased. These results suggest that hydrophobic interactions may play an important role since cyclohexylglutethimide possesses a more hydrophobic side chain and ring structure than any of the other four compounds studied to this point (Fig. 1). Additionally, the optimum k' has been surpassed and additional partitioning leads to decreased resolution (Table 1). For subsequent pH investigations, a compromise concentration of 80 m*M* surfactant was chosen in an attempt to baseline resolve (resolution of 1.5) as many of the compounds as possible in a single run, as well as minimize run time (higher surfactant concentrations lead to longer run times). In Fig. 3, the effect of increasing pH on enantiomeric resolution is shown. As would be expected, resolution of the neutral enantiomers is mostly unaffected. The resolution of these enantiomers is governed predominately by the extent of partitioning based upon hydrophobicity and hydrogen bonding. However, for phenglutarimide and aminoglutethimide, resolution increased above pH 9.0. For phenglutarimide, this is likely due to the fact that as pH increases, the compound becomes less positive, and less partitioning with the negatively charged micelle is obtained. In the case of aminoglutethimide, optimum partitioning has not yet been achieved. The increased resolution may be due in part to observed higher efficiencies at elevated pH, as well as a wider elution window due to the addition of sodium hydroxide for pH adjustment. At pH values greater than 9.5, however, decreased signal-to-noise ratios were obtained due to outgassing caused by increased Joule heating. Therefore all subsequent work was carried out at a surfactant concentration of 80 m*M* and a pH of 9.25 as the best compromise between individual enantiomeric resolution of each of the five compounds, shortest run time, and largest signal-to-noise ratio. A separation of the racemic mixtures of phenglutarimide, glutethimide, pyridoglutethimide, aminoglutethimide and cyclohexylglutethimide under these conditions is shown in Fig. 4. For all five of these compounds, enantioselective recognition is obtained through a combination of optimum partitioning into the micelle, and the differential stabilities associated with the transient diastereometric complexes formed between the micelle and each of the enantiomers. In the case of glutethimide, pyridoglutethimide, aminoglutethimide and cyclohexylaminoglutethimide, formation of the complex is based most likely on hydrophobicity, hydrogen bonding, and dipole–dipole interactions. However, in the case of phenglutarimide, there is the additional mechanism of ionic interaction between the positive charge on the amino

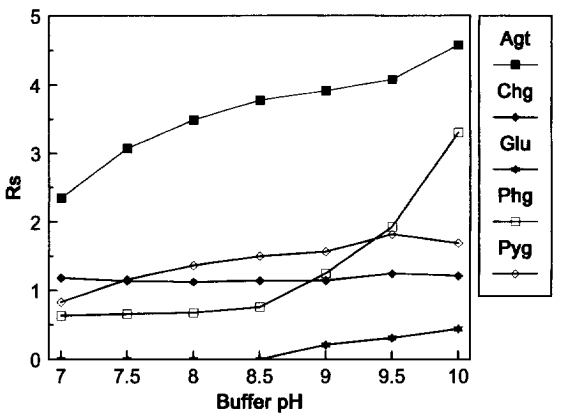


Fig. 3. Effect of pH on enantiomeric resolution. Buffer consisted of 25 m*M* Na_2HPO_4/NaB_4O_7 and 80 m*M* (*S*)-DDCV at varying pH. Sample concentration was 100 μg/ml each in water–methanol (90:10), and a 5-s hydrostatic (10 cm) injection was used. All other conditions as described previously (Experimental). Abbreviations as in Fig. 1.

Table 1
Summary of k', α and R_s for Piperidine-2,6-diones

Compound	k'_1	k'_2	α	k'_{opt}	R_s
Pyridoglutethimide	0.81	0.86	1.06	2.86	2.81
Aminoglutethimide	1.63	1.80	1.10	2.86	3.37
Glutethimide	6.48	6.48	1.00	2.86	0.00
Phenglutarimide	16.45	17.43	1.06	3.67	1.13
Cyclohexylaminoglu	38.16	42.52	1.11	2.86	0.76

Free-zone conditions: 25 m*M* Na_2HPO_4/NaB_4O_7 pH 9.25. CMECC conditions: 25 m*M* Na_2HPO_4/NaB_4O_7 pH 9.25, and 80 m*M* (*S*)-DDCV. Sample concentration was 100 μg/ml each in water–methanol (90:10), and a 15-s hydrostatic (10 cm) injection was used. All other conditions and calculations as described previously (Experimental).

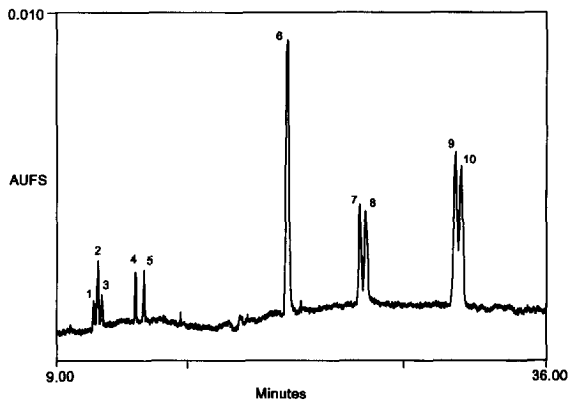


Fig. 4. Chiral micelle electrokinetic capillary chromatographic enantioselective separation of piperidine-2,6-dione enantiomers. Buffer consisted of 25 m*M* Na_2HPO_4/NaB_4O_7 pH 9.25, and 80 m*M* (*S*)-DDCV. Sample concentration was 100 μg/ml each in water–methanol (90:10), and a 15-s hydrostatic (10 cm) injection was used. All other conditions as described previously (Experimental). Peaks: 1 = Pyr enantiomer #1; 2 = unknown impurity from Agt, Glu, and Phg samples; 3 = Pyr enantiomer #2; 4,5 = Agt enantiomer pair; 6 = Glu enantiomer comigration; 7,8 = Phg enantiomer pair; 9 = (−)-Chg enantiomer; 10 = (+)-Chg enantiomer.

group with the negatively charged micelles. These observations coincide with the migration order of the five compounds (Fig. 4).

The above conclusions drawn from Figs. 2 and 3 are further supported by the data presented in Table 1, where k', selectivity (α), and $k'_{optimum}$ values for each of the enantiomers separated in Fig. 4 are summarized. It is evident from Table 1 that buffer conditions exist that would increase the resolution of each of the enantiomeric pairs individually. For pyridoglutethimide and aminoglutethimide, increasing the partitioning by increasing the surfactant concentration and/or increasing the pH lead to increased resolution. For cyclohexlaminoglutethimide, lowering the surfactant concentration results in less partitioning, thereby lowering k' and increasing resolution. The same holds true for phenglutarimide; partitioning also would be optimized by a decrease in surfactant concentration as well as an increase in pH (within limits). The interesting exception is glutethimide, discussed in more detail below. Due to the high α-values, however, adequate resolution is obtained under compromise buffer conditions resulting in the enantioselective separation of all five compounds in a single run (Fig. 4).

During the course of this study, the effect of the sample matrix on enantiomeric resolution was found to be significant. Due to the hydrophobic nature of these compounds, it was necessary to prepare stock solutions in methanol, and then dilute with buffer to provide working standards. When prepared in this manner, the methanol concentration in the individual samples could be kept to under 10%, thereby holding sample matrix effects to a minimum. These conditions were necessary to achieve the resolution shown in Figs. 2 and 3. However when a mixture of all five compounds was first prepared, the sample matrix consisted of 50% methanol, and separations of the type shown in Fig. 5 were obtained. As can be seen, the high level of methanol in the sample matrix completely deteriorated enantiomeric resolution and peak shape in general. In order to achieve the level of resolution shown in Fig. 4, it was necessary to prepare more concentrated stock solutions and then dilute the solutions appropriately with buffer to keep the methanol concentration in the sample to a minimum. Keeping the injection volume or time to a

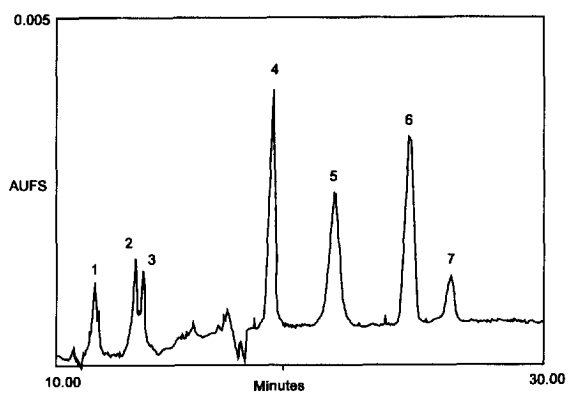


Fig. 5. Effect of sample matrix on the chiral micelle electrokinetic capillary chromatographic enantioselective separation of piperidine-2,6-dione enantiomers. Conditions as in Fig. 4, except sample concentration was 100 μg/ml each in water–methanol (50:50). Peaks: 1 = co-migration of Pyr enantiomers plus unknown impurity from Agt, Glu, and Phg samples; 2,3 = Agt enantiomer pair; 4 = Glu enantiomers; 5 = Phg enantiomers, 6 = Chg enantiomers; 7 = unknown.

minimum also improved resolution. This injection matrix effect has been observed previously [20] as efficiency is known to decrease dramatically as injection volume increases. To investigate enantiomeric elution order an enantiomeric mixture comprised mostly of the (−)-cyclohexylaminoglutethimide enantiomer was injected under the conditions used in Fig. 4. It was found that the (−)-enantiomer migrated first.

3.1. Separation of glutethimide enantiomers

Under the conditions used in Fig. 4, the enantiomers of glutethimide were not baseline resolved. The lack of an enantiomeric separation can be due to one or a combination of two situations: either partitioning is too high, or there is no selectivity. For a neutral compound such as glutethimide, partitioning is manipulated by varying surfactant and organic modifier concentration. Using (*R*)-DDCV, the best resolution obtained was approximately 0.6, achieved using the highest pH (10.0) and surfactant concentration (100 m*M*) reasonable, as supported by the data in Figs. 2 and 3. The data in Table 1 suggests, however, that a lowering of k' to optimize partitioning (a change in the phase ratio by a decrease in surfactant concentration) is called for. Therefore, what may be contributing to the partial resolution (Figs. 1 and 2) is actually an increase in the elution window (t_{mc}/t_{aq}). This hypothesis (a change in the phase ratio) was further tested by adding methanol to the running buffer. The addition of up to 30% methanol, however, did not significantly improve the resolution. At higher methanol concentrations, resolution was actually worse. Therefore selectivity was not sufficient. To change the selectivity of the buffer, it was necessary to change the surfactant type. When the surfactant (*S*)-N-dodecocycarbonylproline [(*S*)-DDCP] was used, resolution of the glutethimide enantiomers was obtained as shown in Fig. 6. As seen in Fig. 1, the structure of glutethimide differs from the other compounds by the lack of a nitrogen functionality adjacent to the chiral center in either a side chain or a ring structure. This difference in solute structure together with the change in surfactant

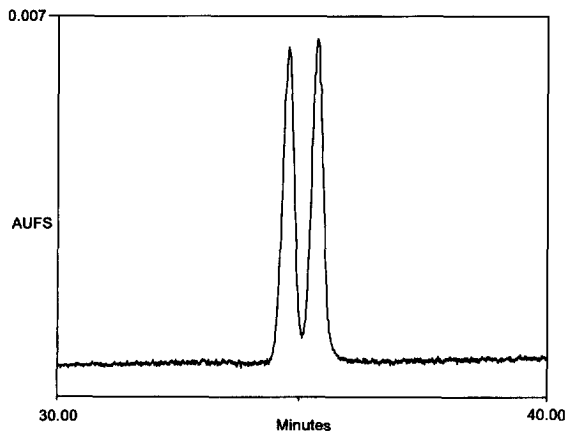


Fig. 6. Chiral micelle electrokinetic capillary chromatographic enantioselective separation of glutethimide enantiomers using (*S*)-DDCP. Buffer consisted of 25 m*M* Na_2HPO_4/NaB_4O_7 pH 8.80 and 75 m*M* (*S*)-DDCP. Sample concentration was 100 μg/ml in water–methanol (90:10) and a 5-s hydrostatic (10 cm) injection was used. All other conditions as described previously (Experimental).

structure leads to the enantioselective separation. Additional work is underway in our laboratories utilizing both molecular modeling and NMR studies to more fully characterize the types of interactions necessary for enantiomeric separations and will be reported at a later date.

3.2. Migration order reversal and the CMECC separation of a thalidomide sample mixture

The ability to reverse the migration order of enantiomers has been shown to be important in quantitation [46] and to assist in enantiomer identification [42,43,47]. For maximum sensitivity and reproducibility, it is desirable to have the minor component elute before the major component since most large peaks have significant tailing. Peak reversal has also been proposed as an aid in the identification of enantiomers when a complex separation of both chiral and achiral compounds is obtained [47]. We have previously reported that for the separation of enantiomers, peak reversal can be accomplished by changing between (*R*)- and (*S*)-DDCV as the buffer additive [42]. That is, if the resulting separation is the resolution of enantiomers, switching between the

two surfactants [(*R*)- and (*S*)-DDCV] will cause the enantiomers to change elution order. Performed in this manner, peak reversal can be exploited to provide better quantitation, and facilitate the identification of enantiomers in a complex mixture.

One of the compounds originally included in the enantiomeric mixture separated in Fig. 4 was a related compound, thalidomide, whose structure is also shown in Fig. 1. Initial attempts at the enantioselective separation of this sample using (*R*)-DDCV lead to the results presented in Fig. 7A. Since three peaks were obtained, peak assignments could not be readily made. Therefore, exact migration order reversal was used in this instance to ascertain whether or not an enantiomeric separation was obtained, or whether the peaks in Fig. 7A correspond to the resolution of impurities and/or degradation products in the thalidomide sample. By employing (*S*)-DDCV, the results illustrated in Fig. 7B were obtained. as can be seen, the same peak height profile relative to migration time is obtained. Since the same migration order is obtained, the compounds comprising the peaks in Figs. 7A and B are not representative of the separation of enantiomers. Had this been the separation of enantiomers, the peak height profile between the electropherograms presented in Figs. 7A and B would be different. Free solution CE experiments showed that the thalidomide sample mixture was negatively charged at the pH employed for these separations. The inability to separate the enantiomers of thalidomide using (*S*)-DDCV may be due to an inability to fully optimize partitioning due to charge repulsion with the negatively charged micelles and/or a lack of enantioselectivity. In addition, multiple

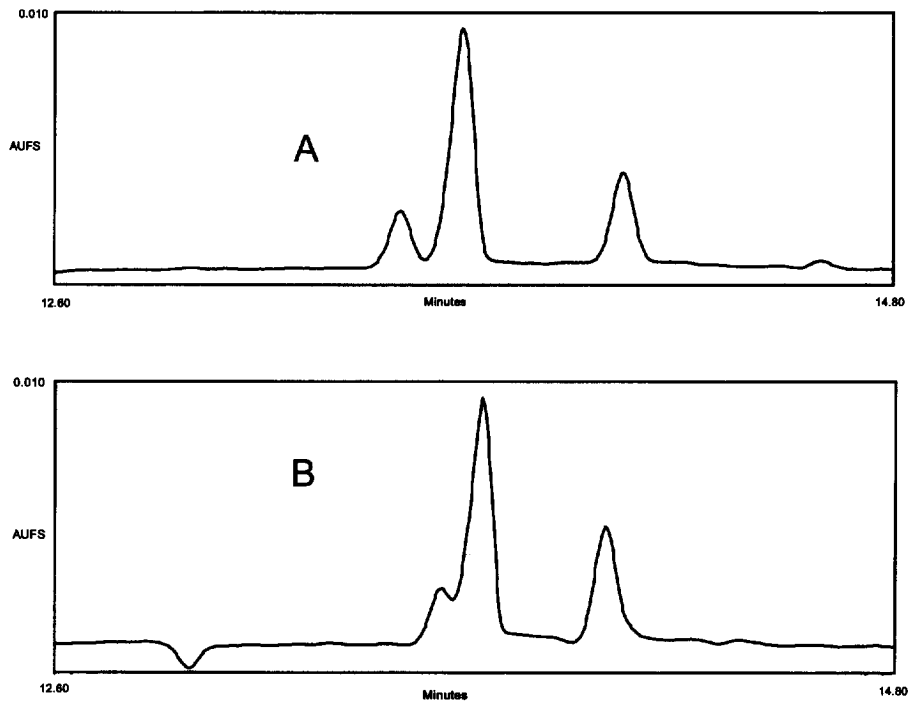


Fig. 7. Chiral micelle electrokinetic capillary chromatographic separation of thalidomide sample mixture. Buffer consisted of 25 m*M* Na_2HPO_4/NaB_4O_7 pH 9.25, and 80 m*M* (*R*)-DDCV (A) and (*S*)-DDCV (B). Sample concentration was 100 μm/ml in water–methanol (90:10), and a 5-s hydrostatic (10 cm) injection was used. All other conditions as described previously (Experimental). Peak identities could not be confirmed.

peak electropherograms for this sample mixture raise questions related to thalidomide stability. Efforts are underway to better understand these results.

4. Conclusion

Using minimal method development, enantiomeric separations of five out of six piperidine-2,6-dione compounds were obtained. This work further extends the applicability of the novel synthetic surfactants, (*R*)- and (*S*)-DDCV and (*S*)-DDCP, to a new class of compounds, and our knowledge of the structural relationships necessary for achieving enantiomeric separations. Using a compound for which a single enantiomer standard was available (cyclohexylaminoglutethimide), migration order was confirmed. In addition, the utility of exact migration order reversal was used to determine that the separation of a thalidomide sample mixture was not enantioselective.

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