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Separation of stereoisomers of aminoglutethimide using three capillary electrophoretic techniques

Vincent C. Anigbogu[a], Christine L. Copper[b], Michael J. Sepaniak[b,*]

[a]*Department of Chemistry, Agnes Scott College, Decatur, GA 30030, USA*

[b]*Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA*

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Abstract

A novel approach to capillary electrokinetic chromatography involving differential distribution between neutral and charged cyclodextrins is described for the separation of the enantiomeric pair of the drug aminoglutethimide. For comparison purposes, micellar electrokinetic capillary chromatography and capillary electrophoresis with neutral cyclodextrin additives are also evaluated for this separation. The three techniques are compared in terms of their ability to resolve the two enantiomers. At pH 9, where aminoglutethimide is neutral, the enantiomers were resolved using a running buffer containing 5 m*M* carboxymethyl-β-cyclodextrin, 1 m*M* β-cyclodextrin, and 50% (v/v) methanol. At the same pH, micellar electrokinetic capillary chromatography, using a running buffer containing 50 m*M* sodium dodecyl sulfate and 17.5 m*M* β-cyclodextrin, only partially resolved the enantiomers. However, at pH 3, where aminoglutethimide is ionized, the enantiomeric pair was separated using capillary electrophoresis with either 10 m*M* α-cyclodextrin or 5 m*M* γ-cyclodextrin added to the running buffer. Enantiomeric separations, by way of the three electrophoretic capillary chromatographic techniques mentioned above, are presented and mechanisms of chiral recognition of aminoglutethimide by cyclodextrins under various experimental conditions are also discussed.

1. Introduction

The difference in the pharmacodynamic activities of enantiomers has created a need to study the pharmacological and toxicological properties of optically active compounds including drugs, agrochemicals, pesticides, herbicides, halogenated hydrocarbons and/or their stereoisomeric metabolic products [1–3]. Unfortunately, such studies are hampered by difficulties in obtaining both enantiomers of the target compound in optically pure forms either through stereospecific synthesis or chromatographic separation. Nevertheless, it is widely believed that in the near future, the pharmaceutical and agrochemical industries will be required by regulatory agencies, such as The Food and Drug Administration, to provide detailed information regarding the enantiomeric purity of drugs and therapeutic or toxic effects of individual enantiomers [4,5]. Hence, the development of rapid and accurate methods for stereochemical resolution of drugs is expected to remain an important issue for some time.

A racemic mixture of aminoglutethimide (AGT) (see Fig. 1 for structures), also known as

* Corresponding author.

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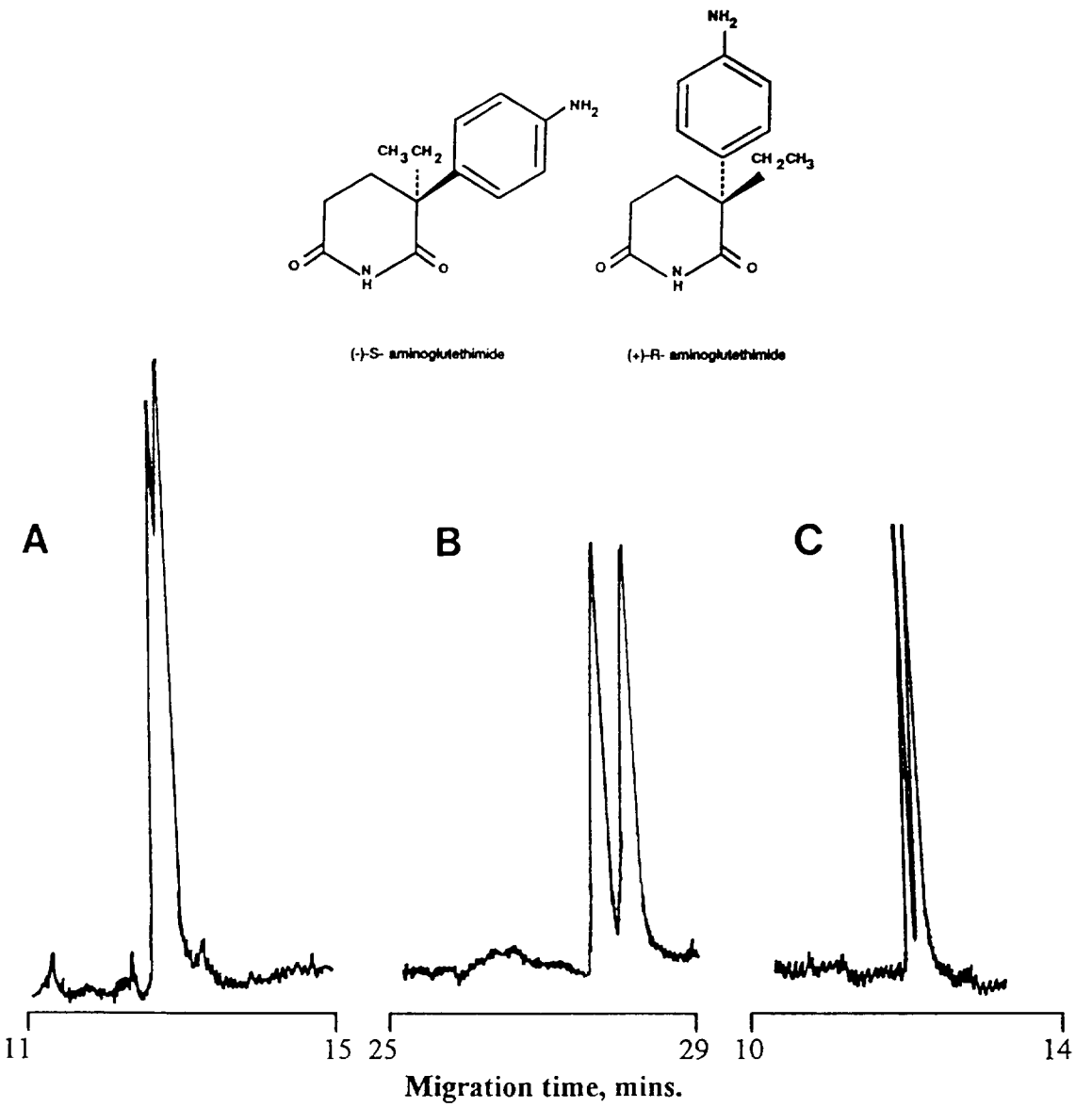

Fig. 1. Dual CD-phase CE (dual-CD-CE) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 9) with 5 m*M* CM-β-CD and 1 m*M* β-CD; (A) 0% methanol and 10 kV applied voltage; (B) 50% (v/v) methanol at 10 kV; and (C) 50% (v/v) methanol at 20 kV.

(±)-3-(aminophenyl)-3-ethyl-2,6-piperidinedione, is clinically used for the treatment of adrenocortical tumors, metastatic breast cancer, and Cushing's syndrome [6,7]. HPLC has been used to resolve the enantiomers of AGT and its acetylated metabolite using a 10-cm long α_1-acid glycoprotein column [8]. However, the method was found to be time-consuming and very sensitive to mobile-phase conditions. Enantiomers of AGT have also been resolved using Chiralcel OD (cellulose tris-3,5-dimethylphenyl carbamate) and Chiralcel OJ (cellulose tris-(4-methylphenyl benzoate) ester) columns with some success [9]. More recently, the use of

Chiralcel OD and OJ columns in series was reported for the resolution of AGT stereoisomers and its acetylated metabolite in urine [10]. Besides suffering from the typical disadvantages of HPLC, the use of these specialty columns is very expensive. To circumvent these problems, various capillary electrophoretic techniques can be employed.

Capillary electrophoresis (CE) has been used extensively to obtain highly efficient separations (up to 10^6 plates/m) of charged solutes. However, neutral compounds cannot be separated using CE unless a charged "secondary phase" is employed to produce a capillary electrokinetic chromatographic mode of separation. Surfactants, above their critical micelle concentration (cmc), were first utilized in electrophoretic separations of neutral compounds by Terabe et al. in 1984 [11]. This technique, often dubbed micellar electrokinetic capillary chromatography (MECC), is instrumentally and operationally similar to CE [12]. In MECC, solutes are separated based on a differential distribution between the running buffer and the electrophoretically retarded micellar phase. MECC exhibits a finite "elution window", bordered by the column void time (t_0) and the effective elution time of the micelle (t_m). The existence of this elution window can limit peak capacity and hinder the separation of hydrophobic compounds, since they tend to completely associate with the micellar phase and co-elute near t_m [12].

Enantiomeric resolution by MECC can be achieved by using chiral surfactants, such as bile salts, or by adding chiral selectors to the running buffer. In fact, the combination of cyclodextrins (CDs) and micelles, referred to herein as CD-MECC, is widely reported for successful separation of enantiomers [13–15]. Native CDs are neutral, cylindrically shaped molecules consisting of a hydrophillic exterior and a hydrophobic cavity. Cavity diameters vary depending on the number of glucose units present (6, 7, or 8 units for α-, β-, and γ-CDs, respectively) [16]. Various CD derivatives (neutral and ionizable), such as hydroxypropyl (HP) and carboxymethyl (CM) variations, are also available and have been utilized in electrophoretic separations.

Terabe et al. first used carboxylated CDs as a charged secondary phase, which functioned in a manner similar to negatively charged micelles in MECC. They separated non-optically active, water-soluble compounds using this capillary electrokinetic chromatography format [17]. More recently, reports of charged CDs as running buffer additives in CE to separate enantiomers have appeared in the literature [18–20]. For example, Schmitt and Engelhardt [20] employed carboxylated CDs in different modes (charged or uncharged according to the pH of the buffer system) for the separation of neutral, cationic or anionic enantiomeric drugs.

It is likely that a comparable, but more predictable, alternative to the above-mentioned techniques would prove advantageous for some enantiomeric separations. In this report, we describe a dual (neutral and charged) CD-phase mode of capillary electrokinetic chromatography, referred to herein as dual-CD-CE, for the separation of the enantiomeric pair of AGT. Enantiomeric separations of AGT via CD-MECC and neutral CD-modified CE (CD-CE) are also presented for comparative purposes.

2. Experimental

2.1. Apparatus

An in-house constructed system, which consisted of a Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply and a Linear (Reno, NV, USA) Model 204 UV–Vis absorbance detector operated at 205 nm and 0.001 AUFS, was used in this work. Separations were performed using unmodified, fused-silica capillaries that were 50 cm × 50 μm I.D. (40 cm to the detector) and purchased from Polymicro Technologies (Phoenix, AZ, USA). Samples were hydrostatically injected for 10 s by raising the anodic end of the capillary 10 cm above the cathodic reservoir. The applied voltage ranged from 10 to 20 kV and the observed current ranged from 5 to 40 μA. All separations were carried out in a 10 m*M* NaH_2PO_4–6 m*M* $Na_2B_4O_7$ running buffer at various pHs (which

were reached by addition of phosphoric acid). Electropherograms were recorded with a Kipp and Zonen (Delft, Netherlands) strip-chart recorder.

2.2. Reagents

The *d,l*-aminoglutethimide (98% purity) sample was purchased from Sigma (St. Louis, MO, USA) and was used as received. The bile salt, sodium cholate, was purchased from Aldrich (Milwaukee, WI, USA). Buffer salts and other conventional chemicals were purchased from Baxter Scientific (McGaw, IL, USA). All cyclodextrin samples used were gifts from American Maize Products (Hammond, IN, USA) or CTD (Gainesville, FL, USA).

2.3. Procedure

A 1.0 m*M* stock solution of AGT was prepared in the appropriate buffer solution (1.4 mg AGT into 5.0 ml of buffer). The mixture was sonicated for about 30 min to insure complete dissolution. Working solutions containing 0.1 m*M* AGT were found to be stable for about two days.

At the beginning of each day, the column was rinsed with 100 m*M* NaOH and water. The column was then filled with the appropriate running buffer and allowed to equilibrate by applying 10 kV for several minutes before making the first injection. The column was also rinsed with 10 m*M* NaOH, followed by water, at regular intervals to insure consistent electroosmotic flow.

3. Results and discussion

Charged CDs alone, including carboxymethyl β-CD (CM-β-CD), have been employed to separate enantiomers of some optically active species. However, when charged CDs by themselves do not provide enantioselectivity, adding another chiral selector to the running buffer is a logical decision. The addition of a second optically active phase, such as a neutral CD, can result in enantiomeric resolution. This creates a capillary electrokinetic chromatographic mode of separation involving a charged CD secondary phase and a primary phase composed of running buffer with neutral CD. In separations of neutral, water-soluble compounds, interactions with the running buffer are significant. Conversely, water-insoluble compounds interact with the primary phase based solely on association with the neutral CD [21].

In a dual phase system containing negatively charged and neutral CDs (such as CM-β-CD and β-CD), the mobility of each enantiomer is altered depending upon its interaction with each of the two chiral phases. The magnitude of the difference between the mobilities of the enantiomers strongly influences the resolution observed. This difference can be amplified, thus improving resolution, by altering the types and concentrations of the enantioselective charged and/or neutral CD phases, or by extending the elution window. By analogy with MECC, the elution window is bordered by t_o and the effective migration time of the charged CD phase, t_{ch}. The elution window can be extended by adding certain organic solvents to the running buffer [12]. It should be noted that the polydispersity of the charged CD phase renders the value of t_{ch} poorly defined. It also can be a source of band broadening if the solute–charged CD association–dissociation kinetics are not rapid [21].

Since the enantiomers of AGT have no intrinsic mobility under neutral or basic pH conditions, negatively charged CM-β-CD was employed to impart an effective mobility to the enantiomers upon complexation. Despite an obvious complexation, indicated by an increase in migration time, the presence of CM-β-CD alone provided no enantiomeric resolution. Therefore, combinations of CM-β-CD and several neutral CDs (α, β, γ, HPβ, and HPγ) were tested. In these cases, complexation with the neutral CD inhibited complexation with the charged CD and reduced migration time. It was discovered that only the CM-β-CD/β-CD system imparted some enantioselectivity on AGT. The best resolution was observed at concentrations of 5 m*M* CM-β-CD and 1 m*M* β-CD (Fig. 1A).

Increasing the β-CD concentration above 1 m*M* further reduced migration time (i.e., further reduced the effective capacity factor of AGT), but degraded resolution. The dual-CD-CE technique is expected to mimic MECC in that the dependency of resolution on capacity factor exhibits an optimum that is influenced by the size of the elution window [17]. Because of a strong inclusion with β-CD, concentrations greater than 1 m*M* result in capacity factors less than the optimum. Further improvement in resolution was achieved through extension of the elution window by adding 50% (v/v) methanol (Fig. 1B). The ability to add such a large amount of methanol is unique to dual-CD-CE as compared to MECC, which can only tolerate up to 25% (v/v) organic modifier before micellization is disrupted [22]. The addition of methanol also resulted in a sharp drop in current (11 μA to 5 μA) which allowed a higher voltage (up to 20 kV) to be applied across the capillary. This led to a two-fold decrease in analysis time with no degradation of efficiency (Fig. 1C).

As stated above, the presence of an elution window in the dual-CD-CE system makes it comparable to the familiar MECC technique. For this reason, optimization of enantiomeric separation of AGT using a sodium dodecyl sulfate (SDS) micellar system was attempted in order to directly compare these similar techniques. Since SDS micelles alone cannot impart chiral recognition, neutral CDs were investigated as additives to the SDS mobile phase. It was found that β-CD, at a concentration of 17.5 m*M*, exhibited maximum, yet poor, resolution of the AGT enantiomers (Fig. 2A). In an attempt to improve resolution via an extension of the elution window, methanol was added to the running buffer. Unlike the dual-CD-CE case described above, the addition of methanol decreased selectivity (see Fig. 2B), even at concentrations as low as 5% (v/v). Other means of extending the elution window include using coated capillaries or employing surfactants other than SDS. However, these options were not explored in this work.

Bile salt surfactants, unlike SDS, are chiral in nature and have been used alone in MECC to provide enantiomeric resolution [15]. However, an attempt to resolve the neutral AGT enantiomers using 10, 20, and 50 m*M* concentrations of sodium cholate, with and without methanol, proved unsuccessful.

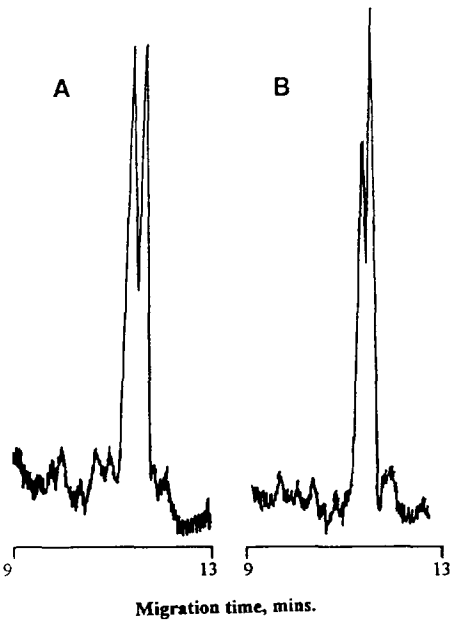

Fig. 2. Neutral CD-modified MECC (CD-MECC) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 9) with 50 m*M* SDS at 15 kV applied voltage; (A) 17.5 m*M* β-CD; (B) conditions same as (A) with 5% (v/v) methanol.

A comparison of the dual-CD-CE system to CD-MECC for the enantiomeric separation of AGT enantiomers gives rise to several general advantages of the former technique. Firstly, the dual-CD-phase system is more resilient to the addition of organic solvents. This is a result of CDs being stable molecules while micelles are dynamic aggregates which are continually exchanging with free surfactant. This equilibrium has been shown to be affected by the addition of organic solvents. Therefore, larger quantities of organic solvents can be used in dual-CD-CE to extend the elution range or to increase solute solubility in the primary phase. Secondly, solute–CD interactions are generally more selective than solute–micelle interactions. Solutes form inclusion complexes with CDs based on their

size, geometry, and physiochemical properties, while interactions with micelles are largely based on solute hydrophobicity [12,16]. Therefore, with the specificity and wide variety of CDs available, the possibilities of unique selectivities in dual-CD-CE are expected to outnumber those of CD-MECC.

While dual-CD-CE and CD-MECC are both potentially applicable to the separation of neutral and charged stereoisomers, CD-CE can result in enantiomeric resolution of charged solutes only. In a pH 3 running buffer, AGT is positively charged and its enantiomers can be separated by addition of various neutral CD additives, as illustrated in Fig. 3. As expected, no enantiomeric resolution was observed in the absence of CD (Fig. 3A). The long migration time of the AGT cation is a consequence of the very slow electroosmotic flow at pH 3. Further increases in migration time result from a reduction in the positive mobility of the AGT cation upon complexation with the neutral CD. Near baseline enantiomeric resolution resulted from the addition of 10 m*M* α-CD while 5 m*M* γ-CD provided complete resolution (see Figs. 3B and 3C, respectively). However, any concentration of β-CD resulted in an increased migration time for AGT with no enantiomeric resolution observed.

While the CD-CE separation performance of AGT enantiomers is comparable to that of dual-CD-CE, this may not be the case in separations of other enantiomeric compounds. Resolution in CD-CE requires solutes to be ionized giving rise to several problems. First, at very low pHs, electroosmotic flow is reduced leading to increased analysis time. Second, the compound of interest may be unstable at extreme pH.

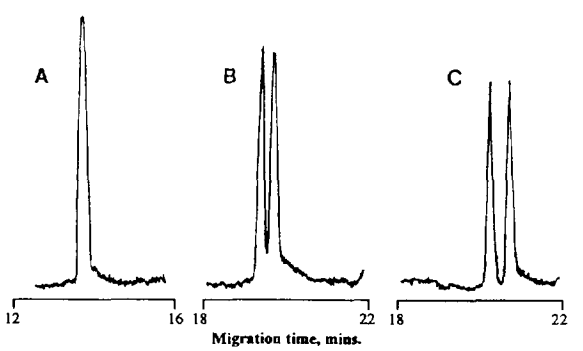

Fig. 3. Neutral CD-modified CE (CD-CE) separations of the enantiomers of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 3) at 15 kV applied voltage (A) no CDs added; (B) conditions same as (A) with 10 m*M* α-CD; and (C) conditions same as (A) with 5 m*M* γ-CD.

It seems that pH plays an important role in each of the separation methods described above. Specifically, AGT enantiomers interact differently with native cyclodextrins depending upon the pH of the running buffer. In dual-CD-CE and CD-MECC at pH 9, only β-CD exhibited enantioselectivity. However, at pH 3 in the CD-CE mode, both α- and γ-CDs exhibited stereoselectivity towards AGT while β-CD did not. It is likely that these behavioral differences of AGT, in the presence of the various native CDs, are reflections of its conformational changes at different pHs. However, they may also stem from more complex occurrences. For, example, in dual-CD-CE, the AGT enantiomers may form complexes of equal energies with the neutral β-CD and these diastereomers are resolved by differentially interacting with the charged CM-β-CD. However, detailed confirmation of such behavior has yet to be determined experimentally.

It is quite obvious that for ionizable stereoisomers, the more straight forward technique is CD-CE, as evidenced by the ease of separation of the AGT enantiomers illustrated above. However, for neutral molecules, CD-MECC or dual-CD-CE is the method of choice. A comparison of these two techniques for the separation of AGT enantiomers revealed that the latter is easier to optimize than the former. But this is not without a price. The prerequisite to enantiomeric separation of neutral compounds using dual-CD-CE is that the enantiomers must form inclusion complexes with the charged CD phase. The degree to which a solute molecule is complexed by a charged CD depends on the size of the CD cavity, steric hindrances at the entrance of the cavity, and the types and charges of substituents on the solute and CD molecules [23]. Since a wide variety of charged CDs, with varying degrees of substitution, are available, it

is important to be able to predict the encapsulating ability of specific CD derivatives with molecules to be separated. Previously, we have used computer-aided molecular modeling to study CD–solute interactions [14,24]. We expect that similar studies involving derivatized CDs will provide insight into their usefulness in capillary electrophoretic separations.

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