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Chiral separation of highly negatively charged enantiomers by capillary electrophoresis

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Abstract

The separation of two highly negatively charged enantiomeric organic disulfates containing two chiral centers was investigated by capillary electrophoresis using cyclodextrin based chiral selectors added to the run buffer. The optimum separation for the enantiomers was achieved in less than 3 min at 25 °C with a run buffer of 10 mM glycine pH 2.4 and 5 mM QA-β-CD, which is a positively charged quaternary ammonium β-cyclodextrin derivative. The method resulted in baseline resolution, excellent linearity, and highly reproducible migration times allowing facile evaluation of the enantiomeric purity of the individual isomers. Detection limits for the enantiomeric pair were determined to be 0.3 ng/μl (S/N = 3). The nature of the selector–enantiomer interaction and a quantitative measurement of the apparent stability constants that governed chiral discrimination of the enantiomers with QA-β-CD were also investigated by UV-Vis spectroscopy and electrospray ionization mass spectrometry.

Keywords: Enantiomer separation; Stability constants; Organosulfur compounds; Cyclodextrins

1. Introduction

Chiral separations are relied upon as key analytical components for drug development in the pharmaceutical industry. Drug–receptor interactions are highly stereoselective with one enantiomer of a racemic pair providing the desired pharmacological activity, while the other enantiomer may exhibit in the worst case negative side effects [1–3]. Chiral discrimination and knowledge of enantiomeric purity are therefore essential [4].

Analytical strategies for chiral separations have been widely examined using high-performance liquid chromatography (HPLC) and have been extensively reviewed [5–8]. Numerous highly selective chiral recognition molecules have been developed as either mobile phase additives or as recognition elements bonded directly to stationary phases making HPLC the mainstay for chiral separations. More recently, capillary electrophoresis (CE) [9–15] has gained popularity for chiral separations since the first report by Gassmann et al. [16] in 1985. In CE, a chiral stationary phase is not required, thus reducing peak broadening due to slow mass transfer between the stationary and mobile phases. In combination with the high separation efficiency inherent in CE, this strategy achieves greater resolution relative to HPLC. Although CE is viewed in some cases as less robust than HPLC, CE is more flexible for readily modifying separation conditions for methods development and more economical and environmentally friendly in the consumption of materials [17].

Addition of an appropriate chiral selector to the run buffer is a prerequisite for effective chiral separation in CE. Macrocyclic antibiotics [15,18–21], cyclodextrin (CD) derivatives [15,22–25], chiral ligand-exchangers [15,26], crown ethers [15,23], polysaccharides [15,27], and peptides/proteins [28,29] have been employed as chiral selectors depending on the properties of the enantiomers to be separated. The basis of such separations requires differences in the

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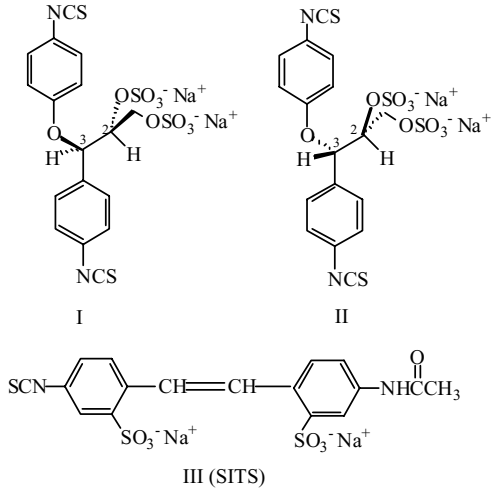

Fig. 1. Structures of **I**, **II**, and **III**. SITS (**III**) is 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate.

effective mobilities of the free and selector-complexes of each enantiomer and substantial differences in the stability constants for the enantiomer–selector interaction [15,23]. The greater the differences in either or both, the better the chiral discrimination by CE.

Enantioselective and regioselective syntheses of compounds **I** and **II** (Fig. 1) were carried out in order to evaluate the biochemical and pharmacological effects of these unique stereoisomers. These compounds are structural analogues of the disulfonate stilbenes (e.g., SITS, **III**) that possess a number of interesting membrane-active properties [30]. While the purified compounds **I** and **II** have large, equal and opposite optical rotations indicating that synthetic products are each largely composed of a single enantiomer, the presence of small and potentially unacceptable amounts of a contaminating enantiomer is not easily determined. Neither the measured optical properties nor any of the other spectroscopic properties of these compounds can be used conveniently to detect traces of contaminating enantiomers.

Although many kinds of chiral separations have been carried out using CE, most have featured separation of simple enantiomers that possess uncharged or weakly charged functional groups attached to or in close proximity to the stereogenic center [31]. In this work, enantiomers **I** and **II** feature two chiral centers and two strongly hydrophilic anionic sulfates. The chiral center opposite to the disulfate end of the molecule possesses a more hydrophobic character. One of these chiral centers could dominate the interaction with a chiral selector, but more likely both would make a contribution to the overall separation provided that the two strongly anionic sulfate functional groups, only one of which is attached to a chiral center, do not inhibit the required differentiating interactions with the chiral selector. To balance the negative charge of the sulfate groups, a positively charged quaternary ammonium derivative of β-CD, QA-β-CD was chosen as a chiral selector to effect separation of **I** and **II**. Despite the potential difficulties described above we have been able to achieve excellent resolution of the enantiomers by CE enabling a highly sensitive determination of enantiomeric purity. The results of the separation are reported herein with an investigation of the enantiomer–selector interaction.

2. Materials and methods

2.1. Materials

β-CD was purchased from Sigma (St. Louis, MO, USA). Quaternary ammonium-β-cyclodextrin (QA-β-CD) with 3.2 quaternary ammonium substitutents (QA: 2-hydroxyl-3-trimethylammoniumpropyl) per cyclodextrin unit and an average molecular weight of 1620 g/mol was a gift from Cerestar USA (Hammond, IN, USA). D_2O (99.9%) was obtained from Cambridge Isotope Labs. (Andover, MA, USA). All other reagents were analytical reagent grade and were used without further purification. Aqueous solutions were prepared with distilled deionized water purified to a resistivity of at least 17 MΩ cm by a Barnstead B pure water purification system.

Compounds **I** and **II** [disodium 3-(*p*-isothiocyanatophenoxy)-3-(*p*-isothiocyanatophenyl)propane-1,2-disulfate] were synthesized by stereoselective and regioselective strategies in multiple steps beginning with *trans*-*p*-nitrocinnamaldehyde prepared from the condensation of acetaldehyde with *p*-nitrobenzaldehyde [32]. Briefly, *p*-nitrocinnamaldehyde was reduced to the corresponding allylic alcohol and stereoselectively epoxidized using the Sharpless procedure [33]. Each of the epoxide enantiomers (−35° and +34° rotations, respectively) was prepared. The epoxide with −35° rotation was converted to **I**, while its optical antipode was converted to **II** using the following procedures. Each epoxide was regioselectively opened through nucleophilic attack at the benzyl carbon of the epoxide with *N*-*tert*-butoxycarbonyl (*N*-*t*-BOC) protected *p*-aminophenoxide. The *t*-BOC group was removed during acid-work-up subsequent to disulfonation of the bis-alcohol intermediate with sulfur trioxide in pyridine. Selective reduction of the nitro group with sodium formate and Pd/C gave the diamino-disulfonate precursors of **I** and **II**. The latter compounds were then obtained by isothiocyanylation of each of the diamines with thiophosgene in water. Compound **I**: mp 195–196 °C (decomp.); ^{1}H NMR (^2H_2O) 4.18 (dd, 1 H, J = 11.08, 3.52 Hz), 4.32 (dd, 1H, J = 11.08, 4.14 Hz), 4.66 (m, 1H), 5.45 (d, 1H, 5.94 Hz), 6.75 (d, 2H, J = 8.96 Hz), 6.92 (d, 2H, J = 8.96 Hz), 7.08 (d, 2H, J = 8.39 Hz), 7.31 (d, 2H, J = 8.39 Hz); ^{13}C NMR (^2H_2O) 66.68 (CH_2), 78.13 (CH), 79.49 (CH), 118.20 (ArCH), 124.87 (ArC), 126.78, 127.91, 129.82 (ArCH), 131.68 (ArC), 133.73, 135.33 (NCS), 136.34 (ArC), 156.65 (ArC); IR (KBr) 2116 cm^{-1}; $[\alpha]_D$ −96° (H_2O, C = 1). Anal. calcd. for $C_{17}H_{12}N_2O_9S_4Na_2$ (+1 H_2O) C, 35.17; H, 2.43; N, 4.83; S, 22.09; Na, 7.92. Found: C, 35.30; H, 2.60; N, 4.82; S, 22.12, Na, 7.85. Compound

II: mp 195–196 °C (decomp.); ^{1}H NMR, ^{13}C NMR, and IR data are the same as for compound **I**. $[\alpha]_D + 90°$ (H_2O, C = 1). Anal. calcd. for $C_{17}H_{12}N_2O_9S_4Na_2$ (+1 H_2O) C, 35.17; H, 2.43; N, 4.83; S, 22.09. Found: C, 35.2; H, 2.48; N, 4.83; S, 22.31. Full synthetic details for the preparation of **I** and **II** will be given elsewhere.

2.2. Instrumentation

Electrophoretic separations were performed on a P/ACE MDQ capillary electrophoresis system (Beckman Scientific Instruments, Fullerton, CA, USA) and were analyzed with P/ACE MDQ software on an IBM Pentium computer. A 31 cm fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 50 μm and an outer diameter of 365 μm was employed. The distance from the inlet to the detector was 21 cm. UV-Vis spectra were obtained with a Varian Cary-5 UV-Vis spectrophotometer in a 1 cm cuvette. ^{1}H or ^{13}C NMR experiments were conducted on either a 400 MHz Varian VXR-400 or a Bruker AC-F 300 MHz spectrometer using tetramethylsilane (TMS) as an internal standard. NMR data from the VXR-400 was processed with ACD/Labs Spec Manager Version 4.09 software. Infrared spectra were collected on a Nicolet 5S FT-IR spectrophotometer as a KBr pellet. Optical rotations were measured in a 1 ml cell with an Autopol III polarimeter (Rudolph Research, Flanders, NJ, USA). Mass spectra were obtained on a Hewlett-Packard (HP) Esquire-LC Ion Trap LC–MS system equipped with advanced electrospray ionization (ESI), a Hewlett-Packard Series 1100 HPLC system, and HP 3-D ChemStation software. Melting points were obtained with a Fisher–Jones melting point apparatus without correction, while elemental analyses were determined by Atlantic Microlab (Norcross, GA, USA).

2.3. Methods

2.3.1. Capillary electrophoresis

Stock solutions of the enantiomers were prepared in the appropriate buffer solution, stored at 4 °C, and diluted for separation and limit of detection studies just before use. All solutions were filtered with 0.2 μm Nalgene surfactant free cellulose acetate syringe filters and pressure injected for 3 s at 3.4 kPa (0.5 psi), which corresponds to an injection volume of 5 nl. All separations were conducted at 25 °C unless otherwise noted. New capillaries were conditioned by pressure rinses at 0.14 MPa (20 psi) unless otherwise stated.

Investigation of QA-β-CD as the chiral selector used glycine buffers (10 or 50 mM, pH 2.4) with QA-β-CD concentrations from 0.5 to 5.0 mM. The final buffer pH was achieved for all solutions by the addition of HCl. Prior to use, new capillaries were first sequentially treated with 1 and 0.1 M HCl, and water for 5 min each, and then a 10 min rinse with run buffer. This was followed by a voltage rinse at +20 kV with run buffer until a stable baseline was achieved. The detection wavelength was 200 nm. The separation voltage was +20 kV.

2.3.2. Spectroscopic methods

Stock solutions (15 μM) of **I**, **II**, and QA-β-CD in 300 μM glycine buffer at pH 2.4 were prepared for absorption analysis. Aliquots of the stock solutions were used to prepare solutions with volume ratios (isomer/QA-β-CD) of 9/1, 8/2, 7/3, 6/4, 5/5, 4/6, 3/7, 2/8, 1/9. The absorbance at 271 nm versus a 300 μM glycine buffer pH 2.4 blank was then measured for each mixture and the stock solutions. Job's plots were obtained from plots of ΔA_{271} versus [QA-β-CD]/([QA-β-CD] + [**I**] or [**II**]). Apparent stability constants (K) for binding of **I** and **II** with QA-β-CD and β-CD were determined by the method of Irwin et al. [34] by monitoring the bathochromatic shift in the absorption spectrum as a function of selector concentration. The concentration of **I** and **II** was kept constant at 15 μM while the concentration of QA-β-CD (0–2000 μM) or β-CD (0–1200 μM) was varied.

For mass spectral analysis, solutions of 5 μM **I** or **II** were prepared in pH 2.4 glycine buffer (0–100 μM) with varying amounts of QA-β-CD (0–50 μM) or β-CD (0–50 μM). Each solution was injected at a rate of 450 μl/h. ESI-MS were collected in the positive ion mode with a nebulizer pressure of 0.21 MPa (30 psi), a dry gas flow rate of 7 l/min, and a dry temperature of 270 °C. The final buffer pH was achieved for all solutions for spectroscopic analysis by the addition of HCl.

3. Results and discussion

3.1. HPLC separation

Separation of enantiomers **I** and **II** was initially attempted by HPLC under a wide variety of experimental conditions. Reversed phase and specialized commercial columns for chiral separations were used with a range of mobile phases under both isocratic and gradient conditions. Commercial chiral columns such as ChiralPak AS, Cyclobond I 2000, and Chirobiotic T were investigated for this separation. These columns contain stationary phases modified with a sugar moiety, cyclodextrin, and a chiral macrocyclic glycopeptide, respectively, as the chiral recognition elements. Normal-phase columns with quinine as a mobile phase additive were also employed. However, in all cases separation was not satisfactorily achieved by HPLC.

3.2. CE separation

Cyclodextrin derivatives have frequently been used as chiral selectors in CE [15,22–25] and were investigated for the separation of **I** and **II**. Using the native cyclodextrins, α-, β-, or γ-CD, separation of **I** and **II** was not observed. Chiral recognition with CD analogues results from the inclusion of the hydrophobic portion of the analyte into the

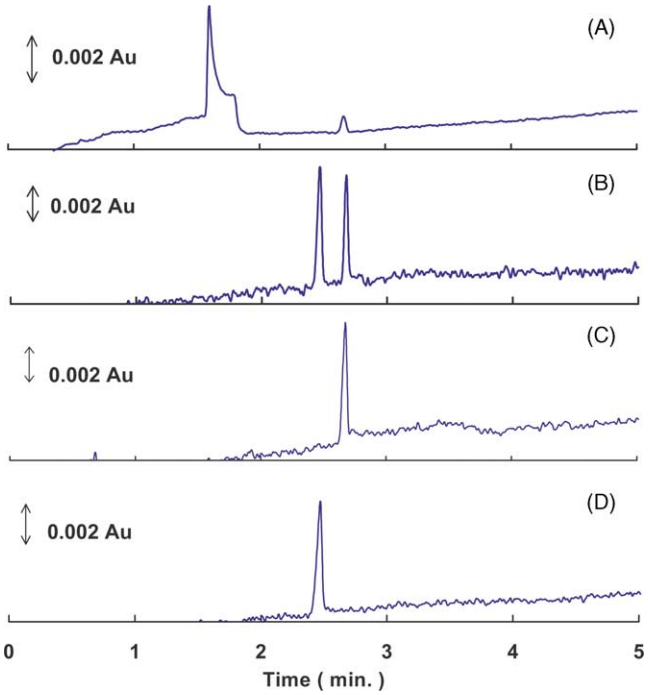

Fig. 2. Electrophereograms of: (A) a mixture of 5 μM **I** and 5 μM **II**, (B) a mixture of 5 μM **I** and 5 μM **II** with 0.5 mM QA-β-CD added to the run buffer, (C) 5 μM **I** with 0.5 mM QA-β-CD added to the run buffer, (D) 5 μM **II** with 0.5 mM QA-β-CD added to the run buffer. Run buffer: 10 mM glycine, pH 2.4; separation temperature, 25 °C; separation voltage, (A) −20 kV, (B–D) +20 kV; UV detection, 200 nm; capillary, 50 μm (i.d.) × 31 cm.

CD cavity as well as interactions with the hydroxyl groups at the rim of the cavity [5]. It could be argued that in the case of **I** and **II**, size considerations should allow both enantiomers to be included into the cavity of the neutral CDs and permit separation. However, the strong anionic character of **I** and **II** most likely creates much different requirements to achieve separation. To counter the effect of these substituents, a quaternary ammonium derivative of β-cyclodextrin, QA-β-CD, was employed to take advantage of ion-pair interactions in the formation of the inclusion complex. Comparative electropherograms at the optimized separation conditions (10 mM glycine, pH 2.4 with 0.5 mM QA-β-CD and a separation voltage of +20 kV) are summarized in Fig. 2 and demonstrate that QA-β-CD effectively discriminates the enantiomers of **I** and **II**.

The run buffer was also quite important for this separation. For example, when a phosphate based run buffer was used detection of either enantiomer was not observed within a 40 min run time. In contrast, a 10 mM glycine run buffer at pH 2.4 resulted in baseline separation of **I** and **II** at QA-β-CD concentrations ranging from 0.5 to 5.0 mM in less than 7 min (Table 1). QA-β-CD is quite soluble and stable over a wide range of pH, which allowed freshly prepared solutions of **I** and **II** to be readily analyzed under these conditions. Acid-catalyzed decomposition of **I** and **II** occurs rapidly at lower pH but is sufficiently slow at pH 2.4 to permit analysis. At the optimized conditions, detection limits (S/N = 3) of 0.3 ng/μl for **I** and **II** were determined. Figures of merit for separation of **I** and **II** under these conditions are summarized in Table 2. It is important to note that at pH 2.4 the electroosmotic flow in the capillary is suppressed such that detection of **I** or **II** in the absence of QA-β-CD required reversing the separation voltage polarity. Although resolution improved with an increase in the concentration of

Table 1
Resolution as a function of QA-β-CD concentration[a]

[QA-β-CD] (mM)	$m_{\mathbf{I}}$ (min)	$m_{\mathbf{II}}$ (min)	Resolution
5.0	6.91	5.77	5.2
2.5	3.94	3.43	3.0
0.5	2.78	2.34	2.5

[a] Run buffer, QA-β-CD in 10 mM glycine, pH 2.4; separation voltage, +20 kV; UV detection, 200 nm; enantiomer concentrations, 5 μM **I** and 5 μM **II**.

Table 2
Figures of merit for CE separation of **I** and **II**

R.S.D. of $m_{\mathbf{I}}$[a,b]	0.3% ($n = 10$)
R.S.D. of $m_{\mathbf{II}}$[a,b]	0.4% ($n = 10$)
Resolution, R_s[a,b]	2.5
Selectivity factor, α[a,b]	1.2
Limit of detection, S/N = 3[b]	∼0.3 ng/μl for **I** and **II**
Linearity, R^2[c]	**I**, 0.998 (1.0–70.0 μM); **II**, 0.992 (1.0–97.0 μM)

[a] Injections of 5 μM **I** and 5 μM **II**.
[b] Run buffer, 0.5 mM QA-β-CD in 10 mM glycine, pH 2.4.
[c] Run buffer, 5 mM QA-β-CD in 10 mM glycine, pH 2.4.

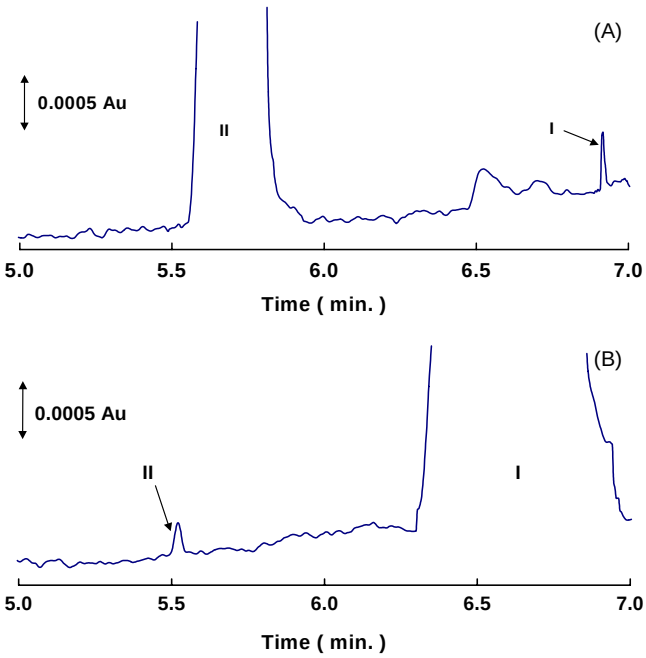

Fig. 3. Evaluation of enantiomeric purity by CE. Electrophereogram of: (A) a mixture of 1.0 μM **I** and 194 μM **II** and (B) a mixture of 210 μM **I** and 1.0 μM **II** in 10 mM glycine buffer, pH 2.4 with 5 mM QA-β-CD. Separation conditions as in Fig. 2.

QA-β-CD and the migration times were highly reproducible, migration times were lengthened, peaks broadened, and the baseline noise increased. The lengthening of the migration time with increasing concentration of QA-β-CD indicates a corresponding decrease in the electroosmotic flow, which is likely due to reversible adsorption of QA-β-CD to the capillary walls.

Knowledge of isomeric purity is an important quality control measurement for chiral drugs. In many cases, determination of isomeric purity is difficult because the major isomer can interfere with detection of the minor isomer. The excellent resolution for **I** and **II** at the higher concentrations of QA-β-CD permits a convenient approach for determination of isomeric purity. Fig. 3 gives the electropherograms of standard mixtures of **I** and **II** in a run buffer of 10 mM glycine pH 2.4 with 5 mM QA-β-CD and demonstrates the ease of detection of the minor enantiomer at approximately 0.5% of the concentration of the major enantiomer. It has been reported that detection of as little as 0.1–0.2% isomeric impurity can be achieved by standard spiking methods, which is well within the accepted range for drug impurities [4].

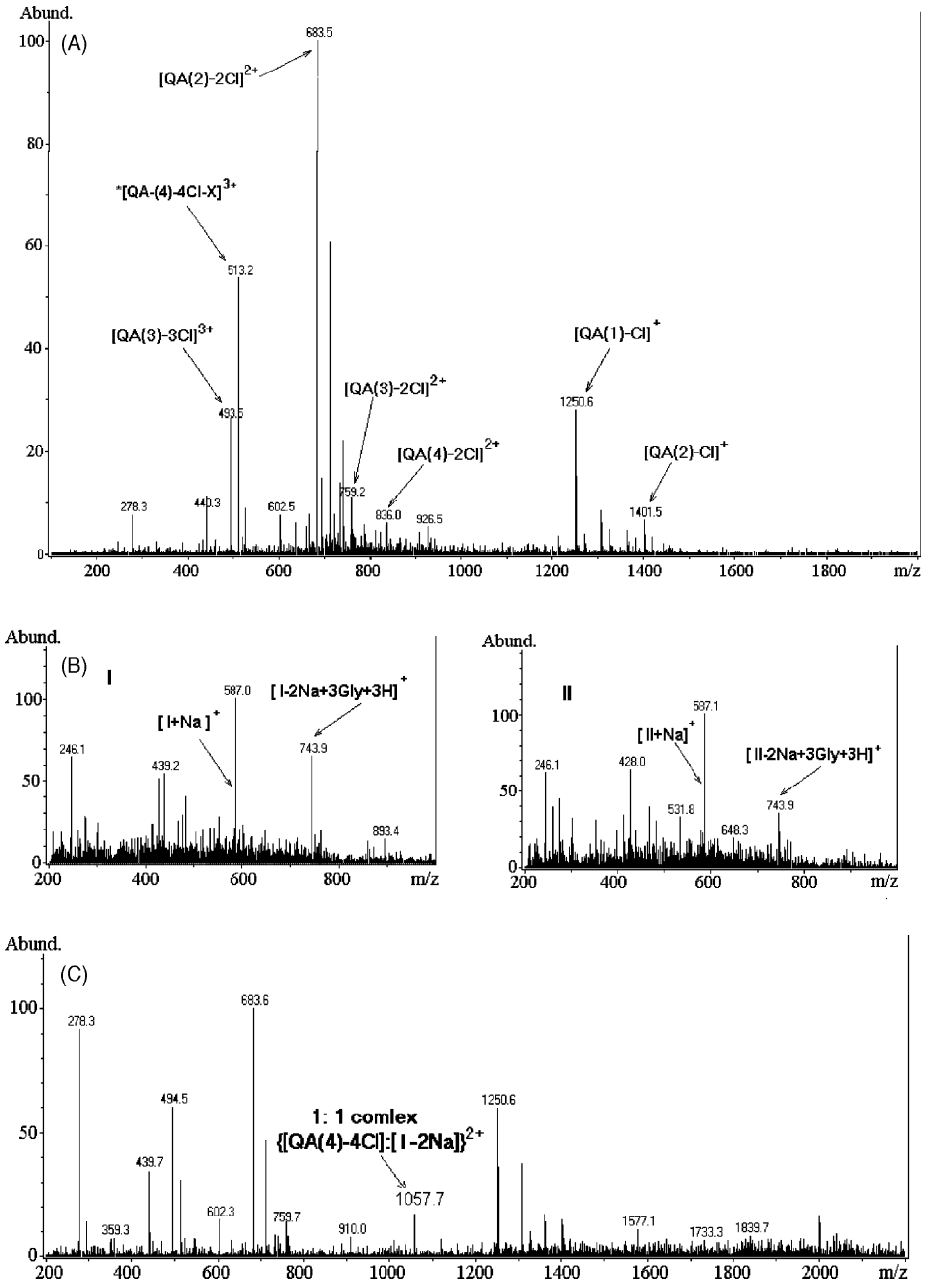

Fig. 4. ESI-MS for solutions of: (A) 50 μM QA-β-CD in water, (B) 5 μM **I** or **II** in 100 μM glycine buffer pH 2.4, (C) 5 μM **I** and 50 μM QA-β-CD in water, (D) 5 μM **II** and 50 μM QA-β-CD in 100 μM glycine buffer pH 2.4, (E) 5 μM **I** and 50 μM β-CD in water, and (F) 5 μM **II** and 50 μM β-CD in 100 μM glycine buffer pH 2.4.

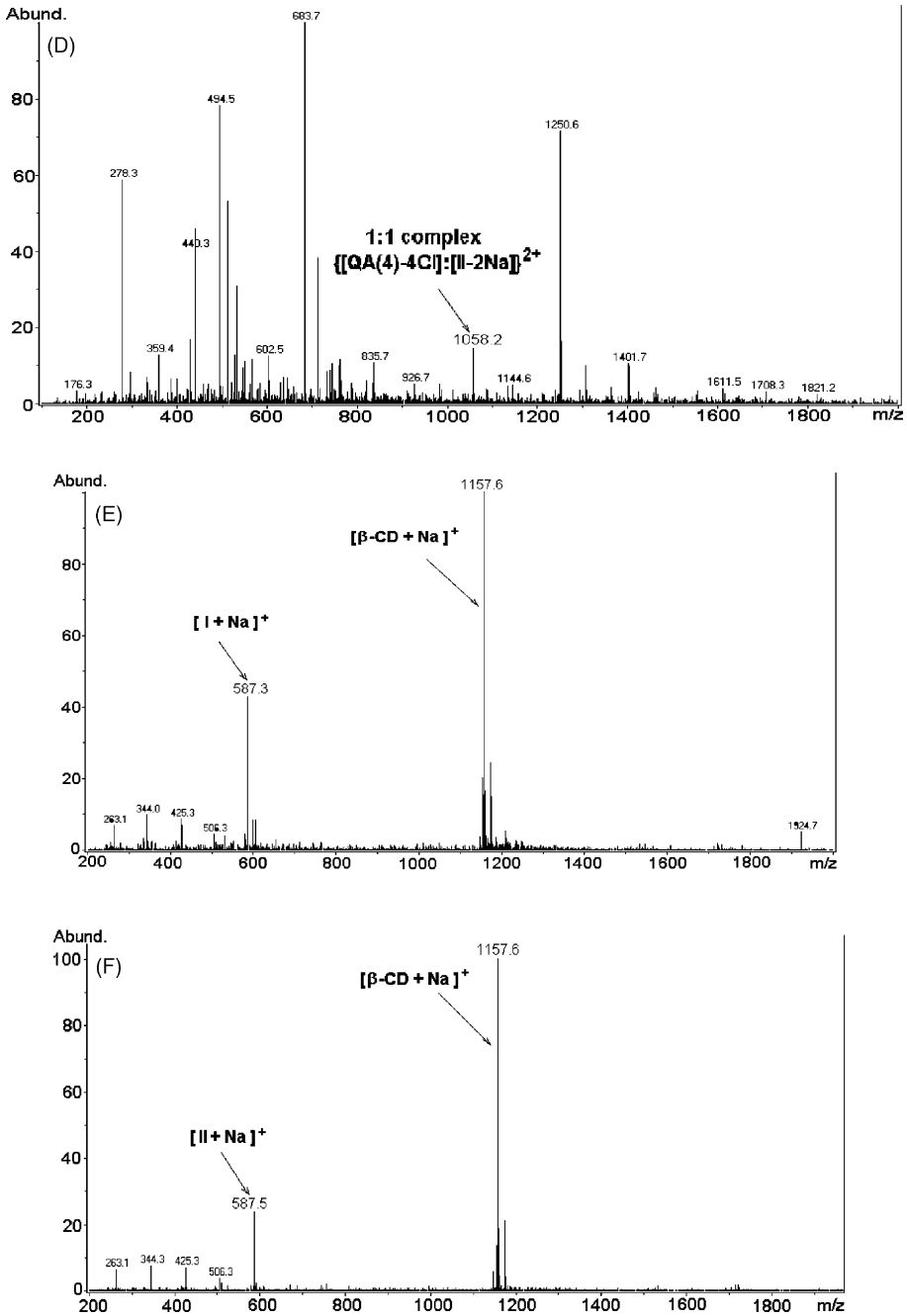

Fig. 4. (*Continued*).

3.3. Binding stoichiometry of *I* and *II* with QA-β-CD

QA-β-CD was successfully employed as a chiral selector to separate **I** and **II**, indicating **I** and **II** must either bind to QA-β-CD with different affinities or by different binding mechanisms. A Job's method [35] was designed to investigate the binding stoichiometry by monitoring the change in absorbance in pH 2.4 glycine buffer for **I** and **II** with increasing amounts of QA-β-CD. For both enantiomers, a stoichiometry of 1:1 was determined.

The assignment of the binding stoichiometry as 1:1 was further supported by observation of the molecular ion peaks from ESI-MS analysis in 100 μM glycine buffer, pH 2.4 (Fig. 4). The ESI mass spectrum of 50 μM QA-β-CD is given in Fig. 4A. The mass spectrum indicates the QA-β-CD used in these experiments is primarily a mixture of mono-, di-, tri-, and tetra-(2-hydroxyl-3-trimethylammonium)-propyl substituted β-CD. Based on ESI-MS results for QA-β-CD reported in the literature [36,37], albeit from another manufacturer, it is likely that more highly substituted derivatives of QA-β-CD are present, but below detectable levels. The molecular ion for both **I** and **II** is observed at *m/z* 587 [**I** or **II** + Na^+]$^+$ (Fig. 4B). When 50 μM QA-β-CD was added

to a solution of 5 μM **I** or **II**, only a single new stable peak at *m/z* 1058 (Fig. 4C and D), which was not observed in Fig. 4A or B, appeared corresponding to [**I** or **II** − $2Na^+$ + QA (4)-β-CD-$4Cl^-$]$^{2+}$. In addition, a concomitant loss of the molecular ion peak for **I** or **II** at *m/z* 587 was observed. In contrast when 50 μM β-CD was added, only strong molecular ion peaks for free **I** or **II** and β-CD were present (Fig. 4E and F). These results demonstrate that **I** and **II** have a very weak affinity for β-CD as compared to QA-β-CD. Although a binding stoichiometry of 1:2 or 2:1 is possible and would form a spectrally silent neutral complex in the mass spectrum, the combination of spectrophotometric and mass spectral evidence strongly suggests a stoichiometry of 1:1 dominates the interaction of **I** and **II** with QA-β-CD. It is important to note that changing either the concentration or pH of the glycine buffer had no significant effect on the peak positions or intensity in the ESI mass spectra for **I**, **II**, QA-β-CD, β-CD, and their mixtures. Therefore, the influence of the acidic glycine buffer had little or no effect on the selector–enantiomer interaction.

3.4. Determination of the selector–enantiomer apparent stability constants

Apparent stability constants, *K*, for **I** and **II** with QA-β-CD and β-CD were evaluated by the method described by Irwin et al. [34] for 1:1 inclusion complexes of β-CD. The method is based on monitoring the shift in wavelength maximum ($\Delta\lambda_{max}$) in the UV-Vis spectrum as the concentration of CD is increased in a solution of constant analyte or guest concentration. Values for *K* for **I** and **II** with QA-β-CD were determined to be 0.068 (±0.002) and 0.073 (±0.002) μM^{-1}, respectively, while the value for *K* for **II** with β-CD was 0.0002 (±0.0002) μM^{-1}. Although the magnitudes of the *K* values for **I** and **II** with QA-β-CD are small, the interaction, and more importantly, the differential interaction ($K_{\mathbf{II}}/K_{\mathbf{I}} \approx 1.1$) is sufficient to promote chiral discrimination and baseline separation of enantiomers **I** and **II** by CE. The comparative values for *K* with QA-β-CD and β-CD are also consistent with the ESI-MS results described above and clearly indicate the importance of the cationic QA group in promoting the selector interaction with enantiomers **I** and **II**. The ion-pairing effect of the QA group presumably acts to anchor the negatively charged molecules in the cavity to form a relatively more stable inclusion complex.

4. Conclusions

Capillary electrophoresis with UV detection was effectively used to separate the enantiomers (**I** and **II**) of the drug candidate, disodium 3-(*p*-isothiocyanatophenoxy)-3-(*p*-isothiocyanatophenyl)propane-1,2-disulfate. Chiral discrimination and baseline resolution were achieved by addition of the cationic chiral selector QA-β-CD to the run buffer. The use of QA-β-CD resulted in a reproducible, baseline resolved chiral separation of **I** and **II**, which was sufficient for quantitative measurements and determination of enantiomeric purity. The additional ion pairing interaction of the highly negatively charged enantiomers with the positively charged QA-β-CD as compared with the neutral selector, β-CD, where formation of an inclusion complex is the primary mode of interaction, was critical to chiral discrimination and separation. UV-Vis spectrophotometry and ESI-MS demonstrated that the interaction between QA-β-CD and the enantiomers occurred preferentially with QA(4)-β-CD in a 1:1 stoichiometry and that chiral discrimination results from a slightly stronger interaction between QA-β-CD and **II** compared to **I**. Finally, it should be noted that CE with QA-β-CD as a chiral run buffer modifier provided a rapid baseline separation of these highly charged enantiomers containing two chiral centers, whereas HPLC could not resolve **I** and **II** in our experiments under a variety of chromatographic conditions.

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