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Capillary electrophoresis of highly sulfated flavanoids and flavonoids

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Abstract

Flavanoids and flavonoids are natural products present in our diet and known to possess multiple biological activities. Sulfated species of these natural products represent highly charged water-soluble organic molecules that possess unique biochemical properties. We describe here the first studies on capillary electrophoresis of these highly charged molecules. Fully sulfated flavanoids and flavonoids can be electrophoresed and resolved under reverse polarity at pH 3.5 using 5–10 kV in less than 20 min. In contrast, at high pH under normal polarity these species can be electrophoresed only if a pressurized capillary is employed. (±)-Catechin sulfate, a racemic sulfated flavanoid, was resolved into its enantiomers using 15% β-cyclodextrin, a chiral selector, but not with α- or γ-cyclodextrins. Yet, the high charge density of these molecules challenges the resolving capability of capillary electrophoresis as diastereomers (−)-epicatechin sulfate and (+)-catechin sulfate do not resolve, even in the presence of cyclodextrins or chiral positively charged amino acids. Overall, capillary electrophoresis of highly sulfated flavanoids and flavonoids is expected to be useful in rapid structure analysis of sulfated flavonoids, either synthetic or natural.

Flavanoids and flavonoids are natural products present in our diet and known to possess a number of biological activities including anti-cancer and anti-HIV infection [1–7]. Flavanoids and flavonoids are polyphenolic structures with a bicyclic–unicyclic scaffold. While flavonoids possess a 2-ene-4-keto function in their heterocyclic ring, flavanoids are devoid of this functionality [8]. Utilizing this bicyclic–unicyclic scaffold, we recently designed some highly sulfated molecules that bind and activate antithrombin, a plasma glycoprotein, for accelerated inhibition of factor Xa, a key enzyme of the blood coagulation cascade. These molecules are the first rationally designed organic multisulfates with potential anticoagulant activity [9–13]. The initial promise of these sulfated flavanoids and flavonoids, organic sulfated molecules, gave rise to a need for their rapid quantitative analyses.

Water-soluble organic polysulfates are difficult to analyze. Traditional adsorption chromatography methods fail because of their high charge density, while methods based on size exclusion suffer from poor resolving capability, especially because these are relatively small molecules with nearly equivalent molecular weights. In contrast, anion exchange chromatography and electrophoresis are expected to be most useful. Capillary electrophoresis (CE),[1] with its high resolving power, ease of analysis, and small sample requirement, is likely to be better suited for these small organic polyanions

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[1] *Abbreviations used:* CE, capillary electrophoresis; CZE, capillary zone electrophoresis; MEKC, micellar electrokinetic capillary chromatography; GAG, glycosaminoglycan; ECS, (−)-epicatechin sulfate; CS, (+)-catechin sulfate; RCS or (±)-CS, (±)-catechin sulfate; QS, quercetin sulfate; AS, apigenin sulfate; 6,2′,3′-FS, 6,2′,3′-flavonoid sulfate; Hdi, heparin disaccharide; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin, γ-CD, γ-cyclodextrin; Mes, 2-(*N*-morpholino)ethanesulfonic acid, hemisodium salt; Tris, Tris(hydroxymethyl) aminomethane.

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[11,12]. Yet, a survey of the literature suggests that organic molecules decorated with multiple sulfate groups have not been investigated at all using CE. In fact, although sulfation is an important phase II conjugation reaction, few sulfate metabolites have been analyzed with CE. The metabolites that have been studied are all monosulfates including (+)-cicletanine sulfate [14,15], hydroquinone sulfate [16], paracetamol sulfate [17], serotonin-*O*-sulfate [18], and vitamin C sulfate [19].

Despite the observation that organic multisulfates have not been studied, sulfated glycosaminoglycans (GAG), nitrogen-containing polysulfated polysaccharides, have been extensively investigated [20–24]. A gamut of CE techniques has been devised with and without chemical derivatization of samples to study heparin, low-molecular-weight heparin, heparin oligosaccharides, and heparin disaccharide [20–24]. Likewise, chondroitin sulfate disaccharides have been resolved with and without precolumn derivatization in the presence or absence of additives under a variety of conditions [22,24–26].

We describe here the first studies on capillary electrophoresis of persulfated flavonoids (Fig. 1). We find that these sulfated flavonoids are relatively more challenging to analyze than sulfated glycosaminoglycan-based oligosaccharides. The results demonstrate that sulfated flavonoids can be electrophoresed at high pH under normal polarity conditions only if a pressurized capillary is employed, while such a limitation is not imposed under reverse polarity. We expect the protocol to be useful for rapid analyses of water-soluble organic polysulfates obtained from either synthetic or natural product procedures.

Materials and methods

(−)-Epicatechin sulfate ((−)-ECS), (+)-catechin sulfate ((+)-CS), and (±)-catechin sulfate (RCS) were obtained through chemical synthesis as described [11–13]. (−)-Epicatechin, (+)-catechin, and (±)-catechin were purchased from Indofine Chemicals (Hillsborough, NJ) and used as such. Triethyl amine–sulfur trioxide complex, α-, β-, and γ-cyclodextrins, L-arginine, L-alanine, boric acid (electrophoresis grade), Tris, sodium phosphate (monobasic and dibasic), glycine, Mes, and other chemicals were reagent grade and obtained from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Sodium hydroxide pellets, hydrochloric acid solution (1 N), and phosphoric acid (85%) were from Fischer Scientific (Fair Lawn, NJ).

Capillary electrophoresis was performed using a Beckmann PACE/MDQ unit with an integrated high-voltage power supply. Uncoated fused silica capillaries were from Beckmann. Ultraviolet spectroscopy was performed using a Shimadzu UV1601PC spectrophotometer at 25 °C. The pH measurements were obtained on an Orion 330 pH meter. The pH meter was calibrated

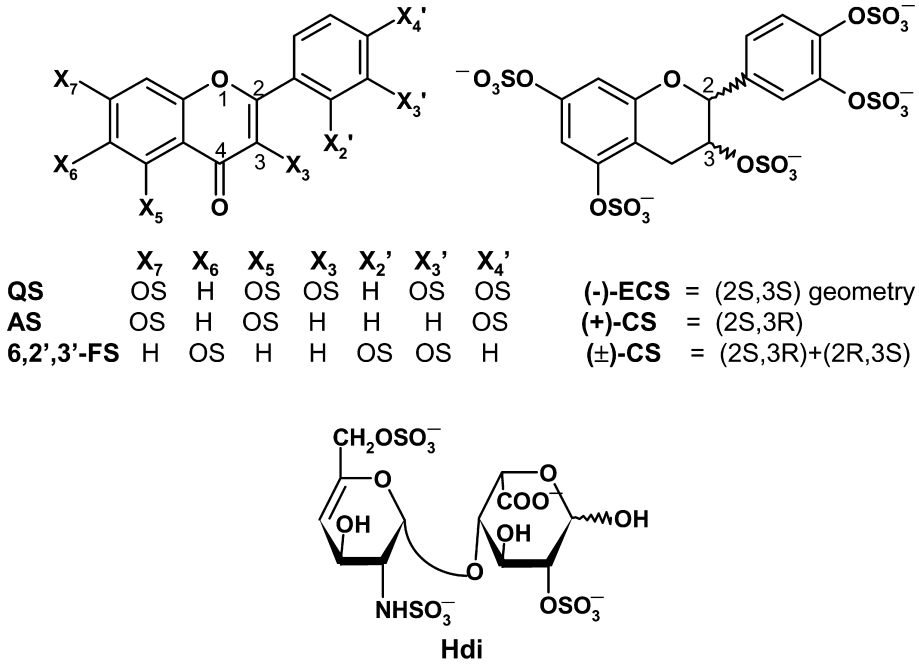

Fig. 1. Structures of sulfated flavanoids ((−)-ECS, (+)-CS, and (±)-RCS), flavonoids (QS, AS, and 6,2′,3′-FS), and heparin disaccharide (Hdi). Numbers show nuclear positions on flavanoid and flavonoid structures. Xs represent substituents OS or H on the bicyclic–unicyclic scaffold, where OS represents the OSO_3^- group.

at room temperature using standard buffer solutions (pH 4.0, 7.0, and 10.0) from Fisher Scientific.

Preparation of sulfated flavanoid and flavonoid standard solution

(−)-ECS, (+)-CS, RCS, QS, AS, and 6,2′,3′-FS were synthesized using commercially available flavanoids and flavonoids ((−)-epicatechin, (+)-catechin, (±)-catechin, quercetin, apigenin, and 6,2′,3′-flavonoid) and triethylamine–sulfur trioxide reagent, purified using a combination of precipitation and chromatographic techniques, and characterized using ^{1}H NMR spectroscopy and elemental analysis [12,13]. Salt present in each sample was removed with size-exclusion chromatography using Sepharose G10 matrix (Sigma). The solid obtained following lyophilization was weighed and 1- to 10-mg/mL stock solutions were prepared in distilled, deionized water. The stock concentration was confirmed using a previously determined ε_{280} of 7700 M^{-1} cm^{-1} for (−)-ECS, (+)-CS, and RCS, while weight concentration was used for QS, AS, and 6,2′,3′-FS.

Preparation of electrophoresis buffers

Several electrophoresis buffers were prepared. These include 20–100 mM sodium phosphate, pH 3.5, 7.0, or 8.5, containing 20–100 mM sodium chloride; 20 mM Tris–HCl, pH 7.0–9.0; 20 mM Mes–HCl, pH 8.5; 50 mM sodium borate containing 50 mM boric acid, pH 8.5; and 50 mM sodium borate containing 50 mM SDS, pH 8.5. The pH was adjusted using either 1 N NaOH or 1 N HCl and the buffer filtered through a 0.22-μm filter. Additives including 1–15% (w/v) α-CD, 1–15% (w/v) β-CD, 1–15% (w/v) γ-CD, 10–100 mM L-Arg, 10–100 mM L-Ala, or 1–5 mM poly-L-Arg were added to above prepared buffers and the pH readjusted, if necessary.

CE analysis of sulfated flavanoids and flavonoids

Electrophoresis of sulfated flavanoids was carried out in a fused silica capillary of 50 μm internal diameter and 32.5 cm effective length to the detector window. A new capillary was activated by washing at 20 psi for 2 min each with 0.1 M phosphoric acid, 0.5 M sodium hydroxide, water, and the run buffer. The sample was injected under pressure of 1 psi for 10–20 s, giving an injection volume of 3–5% of total capillary volume. Electrophoresis was performed at a constant voltage of 2–20 kV in the presence (0.2–1 psi) or absence of pressure. The capillary was maintained at a constant temperature of either 15, 25, or 35 °C using a cooled liquid flow surrounding the capillary. The detection system consisted of a filter-based UV detector operating with either 230- or 280-nm filters. Electrophoretic runs displaying unsteady current profile were removed from analysis. The inter-run variation in migration time was found to be about ± 2%, while the interday variation in migration time was about ± 10%.

Results and discussion

Optimization of electrophoresis buffer

CE of sulfated disaccharides of the heparin and chondroitin sulfate series has been studied at high pH in several buffers since the first reports by Linhardt and co-workers [27,28] and Carney and Osborne [29]. These include combinations of sodium borate–boric acid or sodium borate–SDS with and without additives [30–32]. For both heparin and chondroitin sulfate disaccharides, which differed in the number and position of sulfate groups, separation was typically achieved at high pH within 30 min under normal polarity.

We initiated our CE study of sulfated flavanoids, (−)-ECS, (+)-CS, and RCS, in 50 mM sodium borate–50 mM boric acid buffer, pH 8.0, under normal polarity following the sulfated disaccharide work. However, no peak could be seen in a time span nearly two to three times more than that for disaccharides. Numerous other conditions, including CZE and MEKC conditions [23], at high pH were explored (see Materials and methods), yet no peak was observed within a reasonable time, in striking contrast to the sulfated GAG disaccharides. Two explanations were possible. It was likely that our sulfated flavanoids were not migrating to the detection window because of their high charge density or that they were chemically degrading during electrophoresis.

A simple charge (z) to molecular mass (m) calculation reveals that our sulfated flavanoids ($z/m = 6.3 \times 10^{-3}$) do not differ much from Hdi ($z/m = 6.7 \times 10^{-3}$), a heparin disaccharide. Yet, the overall shape of the molecules may be different. To understand whether sulfated flavanoids were likely to exhibit higher charge density, molecular volume was determined using the Connolly surface algorithm in Sybyl (St. Louis, MO). Connolly surface was generated by rolling a sphere of 1.4 Å radius, simulating a water molecule, on the flavanoid structure. The calculation suggests that, whereas Hdi, with a z value of 4, has a molecular volume of 430.0 Å^3, (−)-ECS, (+)-CS, and (−)-CS ($z = 5$ each) have volumes between 471 and 482 Å^3 (not shown). This suggests that, whereas nearly 108 Å^3 is available for every charge in Hdi, only 94–96 Å^3 is available in our sulfated flavanoids. Thus, our sulfated flavanoids appear to pack greater charge in smaller volume, resulting in higher charge density.

To test whether this prediction is true, we employed a constant positive pressure during electrophoresis at high

pH to force migration toward the cathode. Under a constant pressure of 0.5 psi, peaks corresponding to sulfated flavanoids were observed within 30 min in several buffer systems (not shown). However, the peaks were either broad, or skewed, or distorted in most buffers. For example, pronounced fronting was observed in 20 mM Tris–HCl buffer, pH 8.0, or pronounced tailing was noted in 20 mM Mes buffer, pH 8.0 (Fig. 2). In contrast, 20–100 mM sodium phosphate, pH 8.0, with and without added sodium chloride showed more uniform peak shapes (Fig. 2). These profiles are interesting and suggest pronounced ability of sulfated flavanoids to interact with capillary wall and buffer ingredients, especially ammonium species, or the possibility of buffer ions moving faster or slower than analyte [33]. Such interactions appear to decrease with increasing ionic strength of the buffer, e.g., in the presence of sodium chloride (not shown).

To better understand the migration profile of our sulfated flavanoids under nonpressurized conditions, we studied the pressure dependence of (−)-ECS migration in three buffers, 50 mM sodium phosphate containing 50 mM sodium chloride, pH 8.0, 20 mM sodium phosphate containing 20 mM sodium chloride, pH 8.0, and 20 mM Tris–HCl, pH 8.0. At 1 psi and 10 kV, the migration time to the detector window was essentially equivalent for all three buffers and matched to that predicted on the basis of capillary volume only (Fig. 3). As the pressure was lowered to 0.5 psi, the migration time of (−)-ECS gradually increased from ∼4.5 min to ∼10–18 min. At pressures less than 0.5 psi, a dramatic increase in migration time was seen for all three buffers. The migration time of (−)-ECS was found to increase as the ionic strength of the buffer increased. Fitting the migration time versus the pressure profile to an inverse square root function allows an estimation of the migration time (y intercept) in absence of pressure.[2] The estimated migration times of (−)-ECS under normal polarity at 10 kV in absence of pressurized capillary were 704, 237, and 99 min for the three buffers studied (see Fig. 3). These migration times are at least 10-fold more than that observed for heparin disaccharide (Fig. 1) and show a particularly high charge density for our sulfated flavanoids. This unusual high charge density of (−)-ECS explains its ability to bind in the extended heparin-binding domain of antithrombin, thereby inducing factor Xa inhibition [12].

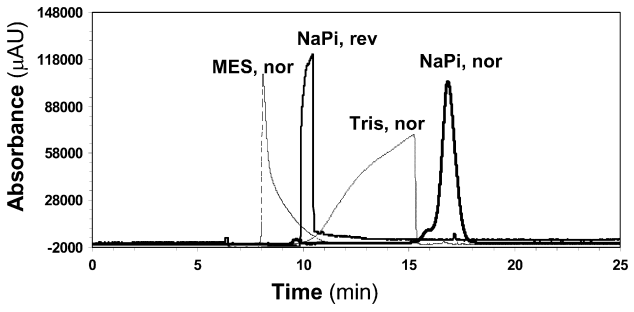

Fig. 2. Representative electrophoresis profiles of (−)-ECS. Electrophoresis was performed under normal polarity at 10 kV and a constant positive capillary pressure of 0.5 psi in 20 mM Mes, pH 8.0 (Mes, nor), or 20 mM Tris–HCl, pH 8.0 (Tris, nor), or 50 mM sodium phosphate, pH 8.0, containing 50 mM NaCl (NaPi, nor). Under reverse polarity, electrophoresis was performed without any capillary pressure in 20 mM sodium phosphate buffer, pH 3.5 (NaPi, rev). (−)-ECS was monitored at 230 nm.

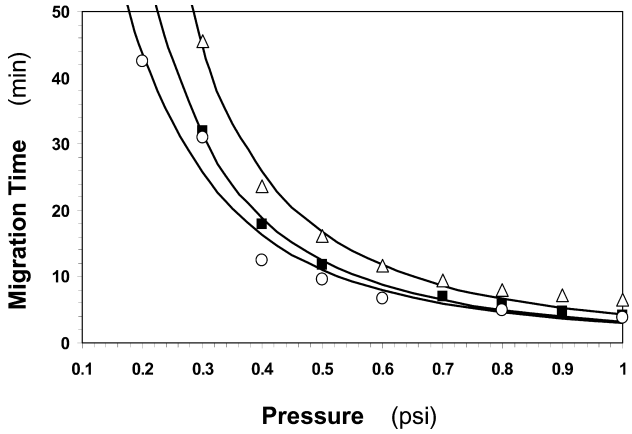

Fig. 3. Profile of migration time of (−)-ECS as a function of the pressure applied on capillary during electrophoresis. The electrophoresis of (−)-ECS was performed at 10 kV with a positive capillary pressure (0.2–1 psi) in either 20 mM Tris–HCl buffer, pH 8.0 (○), 20 mM sodium phosphate buffer, pH 8.0, containing 20 mM NaCl (■), or 50 mM sodium phosphate buffer, pH 8.0, containing 50 mM NaCl (Δ). Solid lines represent nonlinear regression of the data to time vs. pressure relationship (see Footnote 2).

The high charge density of sulfated flavanoids was exploited in analysis under reverse polarity conditions. As expected, (−)-ECS, (+)-CS, and RCS could be readily analyzed under low pH conditions in several buffers including 20 mM sodium phosphate, pH 3.5 (Fig. 2). The peak shape under reverse polarity is essentially Lorentzian, suggesting minimal diffusion of migrating band and absence of wall effects. At 230 nm, the limit of detection was found to be 1 pmol, while the limit of quantitation was approximately threefold higher (not shown).

Separation of mixture of flavanoids and flavonoids

Because flavanoids and flavonoids are widely distributed in the edible plant kingdom, it was of interest to assess whether differently functionalized sulfated species

[2] The relationship was derived as follows. Pressure is force applied per surface area ($\pi \times r^2$), while force is acceleration achieved by object of mass m. Acceleration is velocity, which is distance covered (d) per unit time (min), per time (min). Thus pressure is proportional to $1/(\text{time})^2$ with a proportionality constant of ($m \times d/\pi \times r^2$).

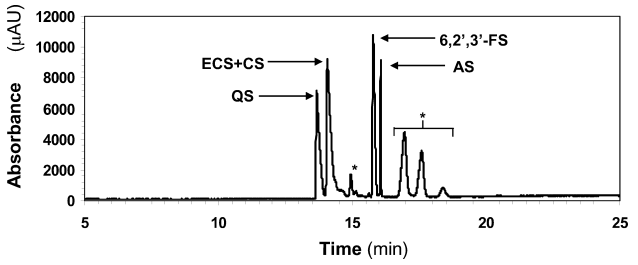

Fig. 4. Separation of a mixture of sulfated flavanoids and flavonoids, (−)-ECS, (+)-CS, QS, AS, and 6,2′,3′-FS. Mixture of 5- and 3-sulfated species resolve well under reverse polarity at 5 kV in 20 mM sodium phosphate buffer, pH 3.5, although diastereomers ECS and CS comigrate. Individual peaks were identified by injecting purified single entities. Peaks marked with asterisk (*) are unidentified decomposition products of sulfated flavonoids QS and 6,2′,3′-FS. The acid stability of these highly charged species is not known.

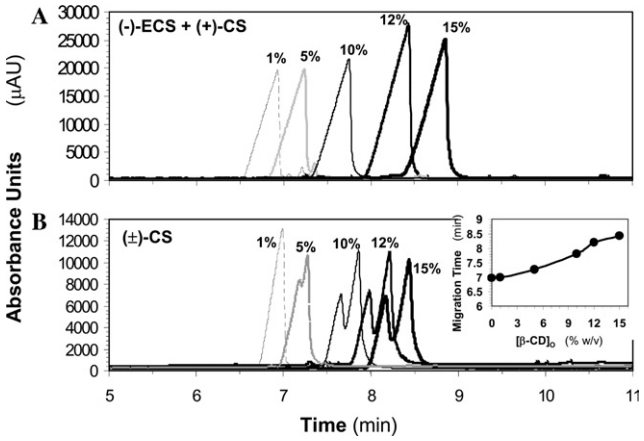

Fig. 5. Electrophoretic profile of diastereomers (−)-ECS and (+)-CS (A) and enantiomers (±)-CS (B) in presence of chiral selector β-cyclodextrin. Electrophoresis was performed in 20 mM sodium phosphate buffer, pH 3.5, under reverse polarity at 10 kV with 1–15% (w/v) β-CD. Inset shows the change in migration time of the first peak, due to (−)-CS, as a function of the concentration of β-CD.

could be resolved using CE. Flavonoids, quercetin sulfate, apigenin sulfate, and 6,2′,3′-flavonoid sulfate were synthesized and characterized according to literature procedures [11,12]. Together with (−)-ECS, (+)-CS, and RCS, these flavonoid sulfates span a range of sulfation pattern and degree of sulfation (Fig. 1). A mixture of these compounds, at approximately equivalent concentrations, could be separated readily under reverse polarity in 20 mM sodium phosphate buffer, pH 3.5 (Fig. 4).

The pentasulfated structures ((−)-ECS, (+)-CS, RCS and QS) migrated nearly 3–4 min faster than trisulfated species (6,2′,3′-FS and AS) at 5 kV. Among the pentasulfated species, QS migrated earlier than (−)-ECS, (+)-CS, and RCS, although the sulfation pattern on the bicyclic–unicyclic flavonoid ring structure is similar, suggesting that small differences in the conformation of the heterocyclic ring affects mobility. In contrast, diastereomers (−)-ECS and (+)-CS comigrate at 5 kV (see Fig. 4). Likewise, 20 mM glycine–HCl or 20 mM acetic acid–sodium acetate buffers, pH 2–4, with or without added NaCl, did not help resolve these diastereomers.

Enantiomeric resolution

The problem of diastereomeric comigration and the growing awareness of understanding the individual enantiomers of a chiral pharmaceutical agent [34,35] led us to investigate CE resolution of (−)-ECS, (+)-CS, and RCS. The enantiomers of a chiral drug generally differ in their biochemical and biophysical properties, and as a consequence techniques that differentiate these properties are greatly helpful. Chiral selectors, α-, β-, and γ-cyclodextrins, and their chemically modified derivatives, have been most often exploited for enantiomeric resolution [34,35].

We were particularly interested in resolving racemate (±)-RCS because of our earlier work with antithrombin, which suggested that the two enantiomers, (±)-RCS, differed greatly in their binding affinities [12]. To resolve RCS we studied its electrophoretic profile in the presence of native α-, β-, and γ-cyclodextrins under reverse polarity conditions. The three cyclodextrins primarily differ from each other in the diameter of their central channel. The diameter ranges from 15 to 20 Å with α-CD having the smallest pore diameter. Modeling predicts that enantiomers of RCS are ellipsoidal with diameters of ∼7 Å along the short axis and ∼10 Å along the long axis.

Electrophoresis of RCS in 20 mM sodium phosphate buffer, pH 3.5, containing 1–15% α-CD under reverse polarity did not resolve the racemic mixture (not shown). In contrast, β-CD was found to resolve RCS into two peaks at 5–15 kV. The resolution improved steadily as the concentration increased to 15% (Fig. 5B), although baseline resolution was not reached at the highest β-CD concentration tested. Unfortunately, solubility problems did not allow higher concentrations to be tested. A plot of dependence of migration time of one of the components on the concentration of β-CD shows most change between 1 and 10% β-CD, with a plateau starting at about 12% (inset to Fig. 5B). γ-CD, on the other hand, could split the RCS peak into two at 10 and 15% concentration (not shown), yet the resolution was marginal in comparison to that of β-CD.

Resolving diastereomers

(−)-ECS and (+)-CS are diastereomers and, therefore, are expected to resolve under achiral conditions. Yet, the two species consistently comigrated in several normal and reverse-polarity conditions. To test whether diastereomers (−)-ECS and (+)-CS could be resolved using cyclodextrins, electrophoresis was performed in

20 mM sodium phosphate buffer, pH 3.5, under reverse polarity at a constant voltage between 2 and 20 kV in the presence of 1–15% α-, β- (Fig. 5A), and γ-CD. Unexpectedly, the diastereomers comigrated under all conditions studied. Although the diastereomers did not resolve, it was interesting to note that, as the concentration of the chiral selector increased, especially that of β-CD, the migration time increased in conjunction (Fig. 5A). Comparing the electrophoresis profile of the diastereomeric mixture ((−)-ECS + (+)-CS) versus the racemic mixture ((±)-RCS) suggests that (−)-CS interacts with β-CD, while (−)-ECS and (+)-CS do not. Thus, the interaction of sulfated flavanoids with β-CD is specific, resulting in enantiomeric resolution.

To test whether diastereomeric resolution could be achieved with other chiral selectors, amino acids including 20–100 mM L-Arg, 20–100 mM L-Ala, or 1–5 mM poly-L-Arg were studied. It was expected that specific differential interaction of sulfate groups with chiral positively charged amino acids will aid diastereomeric resolution. Resolution was attempted under reverse-polarity conditions in 20 mM sodium phosphate buffer, pH 3.5. Although the migration time of (−)-ECS and (+)-CS co-peak increases as the concentration of the chiral selector increases, no resolution was observed (not shown). This is extremely interesting because it suggests that diastereomeric complexes formed with chiral amino acids by flavanoid diastereomers still possess the same electrophoretic mobility. We reason that the high charge to size ratio negates the structural differences between diastereomers (−)-ECS and (+)-CS or that the 3-OSO_3^- moiety, which differs in orientation in the diastereomers, is minimally involved in interacting with chiral selectors. This result supports our earlier biochemical data which show a small approx threefold difference in the antithrombin binding affinity of (−)-ECS and (+)-CS [12]. Finally, we predict that it should be possible to resolve diastereomers (−)-ECS and (+)-CS using covalently modified positively charged chiral cyclodextrins, e.g., aminopropyl- or histamine-modified β-CD [36,37].

In summary, capillary electrophoresis of highly sulfated flavanoids and flavonoids can be readily performed in reverse polarity. Unlike heparin disaccharide and its derivatives, sulfated flavanoids and flavonoids can be analyzed under normal polarity conditions only if pressurized capillary is used. Further, a specific racemic mixture, RCS, can be resolved using β-cyclodextrin as chiral selector. Yet, sulfated flavanoids may be difficult to analyze as exemplified by diastereomers ((−)-ECS and (+)-CS) that are not resolved either in the absence or in the presence of chiral selectors. It is likely that advanced chiral selectors, such as covalently modified cyclodextrins, may perform better enantiomeric and diastereomeric resolution of these highly sulfated species. These first results will greatly aid rapid structure analysis of sulfated flavonoids, either synthetic or natural. Additional work is needed to show the ability of CE to identify sulfation pattern and degree of sulfation in this class of compounds.

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