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Application of Classical Gel Electrophoresis to the Chiral Separation of Milligram Quantities of Terbutaline

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Although the last couple of decades has seen tremendous progress in the ability to do chiral separations, the ability to do larger scale chiral separations has lagged somewhat behind the current analytical chiral separation state of the art. The potential of classical gel electrophoresis for chiral separation of milligram quantities of chiral material is examined. A protocol for the chiral separation of milligram quantities of terbutaline using sulfated cyclodextrin as a chiral additive is demonstrated. The possible advantages of the approach are discussed.

Separation of enantiomers is an important topic to the pharmaceutical industry. Many of the drugs marketed in the United States have at least one chiral center. Examples of chiral drugs currently on the market include ibuprofen and propranolol. Of the 528 synthetically derived chiral drugs, 88% are sold as the racemic mixture.[1]

Despite the commercialization of a large number of different types of high-performance liquid chromatographic chiral stationary phases in the last decade, including the cyclodextrin phases[2,3] the macrocyclic antibiotic phases,[4−6] the $\pi-\pi$ interaction phases,[7,8] and the protein phases,[9−12] as well as the cellulosic and amylosic phases[13,14] and the chiral crown ether phases,[15,16] the problem of large-scale chiral separations remains largely unexplored. Preparative liquid chromatography (prep-LC)[17] and countercurrent[18] and centrifugal partition chromatography[19] have all been used to effect larger than analytical-scale chiral separations.

Prep-LC is often the method of choice for preparative chiral separations, but as with any technique, several factors must be considered. Prep-LC requires the availability of a suitable chiral stationary phase, optimization of chromatographic conditions (e.g., mobile phase and temperature), determination of the adsorption isotherms of both enantiomers on the stationary phase, and evaluation of the robustness of the method. In addition, the mode of chromatography (e.g., batch vs displacement vs recycling vs simulated moving bed) must be chosen. Ultimately, the sample capacity of chiral stationary phases may be circumscribed by the absolute amount of chiral selector in the chiral stationary phases (e.g., typically 0.2−0.3 μmol/m^2 for cyclodextrins)[20] or the density of chirally selective sites of immobilized chiral ligands such as proteins. Further, the low density of chirally selective sites in some chiral stationary phases diminishes the likelihood of cooperativity between adjacent ligands for enhanced chiral recognition.

Historically, many of the chiral selectors currently available as chiral stationary phases for HPLC originated as chiral mobile phase additives, particularly in thin-layer chromatography (TLC).[21] More recently, chiral additives have been shown to be effective for chiral separations by capillary electrophoresis (CE).[22] Chiral additives in CE have several advantages, some of which are highlighted in Table 1. Chiral additives in free solution also offer the potential for multiple complexation,[23] thus possibly reinforcing chiral recognition. Recently, we reported[24,25] the application of sulfated cyclodextrins (ds ∼7−10) as chiral additives in CE for the enantioseparation of terbutaline and over 70 other compounds of pharmaceutical interest. In this work, the general approach was to use a background electrolyte at low pH, to minimize the electroosmotic flow and to operate the system with the anode at the detector end of the column. Under these conditions, neutral and cationic species did not reach the detector in the absence of the polyanionic sulfated cyclodextrin. The structural diversity

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(1) Lin, B.; Zhu, X.; Koppenhoefer, B.; Epperlein, U. *LC-GC* **1997**, *15*, 40.
(2) Ward, T. J.; Armstrong, D. W. In *Chromatographic Chiral Separations*; Zief, M., Crane, L. J., Eds.; Marcel Dekker, Inc.: New York, 1988; pp 131−163.
(3) Armstrong, D. W.; DeMond, W. *J. Chromatogr. Sci.* **1984**, *22*, 411−415.
(4) Armstrong, D. W.; Tang, Y.; Chen, S. *Anal. Chem.* **1994**, *66*, 1473.
(5) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. *J. Chromatogr.* **1996**, *731*, 123.
(6) Armstrong, D. W.; Liu, Y.; Ekborgott, K. H. *Chirality* **1995**, *7*, 474.
(7) Pirkle, W. H.; Welch, C. J. *Tetrahedron: Asymmetry* **1994**, *5*, 777−780.
(8) Pirkle, W. H.; Murray, P. G. *J. Liq. Chromatogr.* **1990**, *13*, 2123−2134.
(9) Hermansson, J.; Eriksson, M. *J. Liq. Chromatogr.* **1986**, *9*, 621−639.
(10) Schill, G.; Wainer, I. W.; Barkin, S. A. *J. Liq. Chromatogr.* **1986**, *9*, 641−666.
(11) Allenmark, S. *J. Liq. Chromatogr.* **1986**, *9*, 425−442.
(12) Okamoto, M.; Nakazawa, H. *J. Chromatogr.* **1990**, *508*, 217−219.
(13) Isaksson, R.; Erlandsson, P.; Hansson, L.; Holmberg, A.; Berner, S. *J. Chromatogr.* **1990**, *498*, 257−280.
(14) Yashima, E.; Okamoto, Y. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 3289−3307.
(15) Shinbo, T.; Yamaguchi, T.; Nishimura, K.; Sugiura, M. *J. Chromatogr.* **1987**, *405*, 145−153.
(16) Hilton, M.; Armstrong, D. W. *J. Liq. Chromatogr.* **1991**, *14*, 9−28.
(17) Küsters, E. *Chim. Oggi* **1996**, *14*, 39−43.
(18) Ma, Y.; Ito, Y.; Foucault, A. *J. Chromatogr.* **1995**, *704*, 75−81.
(19) Oliveros, L.; Puertolas, P. F.; Minguillon, C.; Camacho-Frias, E.; Foucault, A.; Le Goffic, F. *J. Liq. Chromatogr.* **1994**, *17*, 2301−2318.
(20) Stalcup, A. M.; Williams, K. L. *J. Liq. Chromatogr.* **1992**, *15*, 29−37.
(21) Armstrong, D. W.; Faulkner, J. R., Jr.; Han, S. M. *J. Chromatogr.* **1988**, *452*, 323−330.
(22) Ward, T. J. *Anal. Chem.* **1994**, *66*, 632A−640A.
(23) Armstrong, D. W. ; Nome, F.; Spino, L. A.; Golden, T. D. *J. Am. Chem. Soc.* **1986**, *108*, 1418−1421.
(24) Stalcup, A. M.; Gahm, K. H. *Anal. Chem.* **1996**, *68*, 1360−1368.
(25) Gahm, G. H.; Stalcup, A. M. *Chirality* **1996**, *8*, 316−324.

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Table 1. Advantages of Chiral Additives in CE

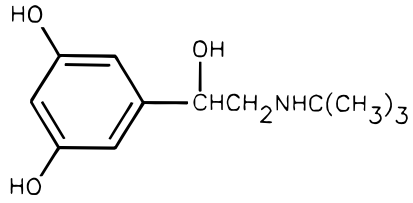
additive can be readily changed
variety of chiral selectors available
rapid screening of chiral selectors
small amounts of BGE, chiral additive required
rapid screening of conditions
no pre-equilibration
multiple complexation possible
rapid screening of analytes

present in solutes successfully enantioresolved and the high resolution (e.g., R_s ~26 for 5-cyclobutyl-5-phenylhydantoin) easily achieved under nonoptimized conditions for some compounds established the versatility of this chiral additive. Unfortunately, CE is generally more suited to analytical separations than to preparative- or semipreparative-scale separations.

Classical gel electrophoresis, a mature separation method used extensively for protein and nucleic acid purification and characterization,[26,27] may complement CE and offer a viable alternative to chromatographic methods for preparative- or semipreparative-scale chiral separations. However, its application to small molecule separations, other than for polypeptides, has been somewhat limited, presumably because the solute begins to diffuse away from the band center as soon as the applied voltage is removed. Although detection is usually accomplished off-line in electrophoretic and thin-layer chromatographic methods, the affinity of the analyte for the chromatographic bed and the immediate removal of the mobile phase following the chromatographic run minimizes the problem of solute diffusivity in TLC. In contrast, in gel electrophoresis, the gel matrix serves primarily as an anticonvective and/or molecular sieving medium designed to minimize interactions with the solute. Hence, there is no mechanism to localize the analyte immediately postrun unless the gel is "fixed". The presence of bulk liquid in the postrun gel no doubt contributes to the solute diffusivity problem which degrades the separation and complicates detection. However, complexation between the solute and a bulky additive (e.g., sulfated cyclodextrin) with reasonably large binding constants (~10^3 M^{-1})[28] might effectively localize the solute.

The use of gel electrophoresis for preparative-scale chiral separations may offer some of the same advantages listed in Table 1 for CE (e.g., choice and concentration of additive). Another advantage of gel electrophoresis is the use of aqueous solvents that are less hazardous than the hydroorganic or organic solvents typically used in most preparative chromatographic-based separations.[29] Also, the absolute volume of solvent required for a classical electrophoretic separation may be much less than that required for the analogous HPLC-, centrifugal partition-, or countercurrent chromatographic-based methods, if one considers the amount required to pre-equilibrate the chromatographic systems. The chiral selector should be readily retrieved subsequent to the electrophoretic separation, an important consideration for costly chiral additives. The presence of the chiral selector in free solution in the electrophoresis system may allow for multiple complexation as well as increased capacity compared to the analogous chromatographic bed where, as noted previously, the absolute amount of available chiral selector is limited by the physical and chemical properties of the substrate and ligand.

Terbutaline, which was readily enantioresolved in our earlier work, is a short-acting β_2-adrenergic agonist which may be of



interest because of its growth-promoting properties and potential illegal use in livestock.[30] Terbutaline is also approved in the United States for the treatment of asthma[31] and for the inhibition of premature labor. The symptoms of asthma are thought to be the result of an imbalance between the bronchodilator and bronchoconstrictive regulatory systems. Of particular concern in the treatment of asthma with β_2-adrenergic agonists like terbutaline, administered to mediate bronchodilation and exclusively marketed as the racemate, is that one enantiomer may actually function as a bronchodilator while the other acts as a bronchoconstrictor.

The purpose of this work was to explore the potential of classical gel electrophoresis using sulfated cyclodextrin as an additive for the chiral separation of milligram quantities of terbutaline. To the best of our knowledge, this is the first report of classical gel electrophoresis used for the chiral separation of a racemic compound. Preliminary work using classical gel electrophoresis for chiral separations employed a horizontal column electrophoresis configuration. Subsequent work was adapted to a vertical Bio-Rad Mini-prep continuous-elution electrophoresis apparatus.

EXPERIMENTAL SECTION

Materials. Hydroxypropyl-β-cyclodextrin used in the original gel work was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ) and functionalized in-house.[32] The sulfated β-cyclodextrin used in the Mini-prep system (13 sulfates/cyclodextrin) was generously donated by Cerestar, Inc. (Hammond, IN). The terbutaline hemisulfate salt was obtained from Sigma Chemical Co. (St. Louis, MO). The agarose (medium EEO, electrophoretic grade) and all other buffer components were obtained from Fisher Scientific (St. Louis, MO).

Apparatus. The instrument used for the CE experiments was either a Quanta 4000 capillary zone electrophoresis (CZE) system equipped with a UV detector (214 nm) interfaced to a Shimadzu Chromatopac CR-501 data station or a Bio-Rad 3000 capillary electrophoresis system equipped with a variable-wavelength UV

(26) Allen, R. C.; Budowle, B. *Gel electrophoresis of proteins and nucleic acids: selected techniques*; Walter de Gruyter: Berlin, 1994.
(27) Bruno, T. J. *Chromatographic and electrophoretic methods*; Prentice Hall: Englewood Cliffs, NJ, 1991; pp 141−169.
(28) Agyei, N. M. Heparin and dextran sulfate as novel chiral selectors for the direct enantioseparation of racemic pharmaceutical drugs by capillary zone electrophoresis. Master's Thesis, University of Hawaii, 1994.
(29) Armstrong, D. W.; Bui, K. H.; Barry; R. M. *J. Chem. Educ.* **1984**, *61*, 457−458.
(30) Doerge, D. R.; Churchwell, M. I.; Holder, C. L.; Rowe, L.; Bajic, S. *Anal. Chem.* **1996**, *68*, 1918−1923.
(31) Stinson, S. C. *Chem. Eng. News* **1997**, *75* (Jan 6), 25.
(32) Stalcup, A. M.; Gahm. K. H. *Anal. Chem.* **1996**, *68*, 1369−1374.

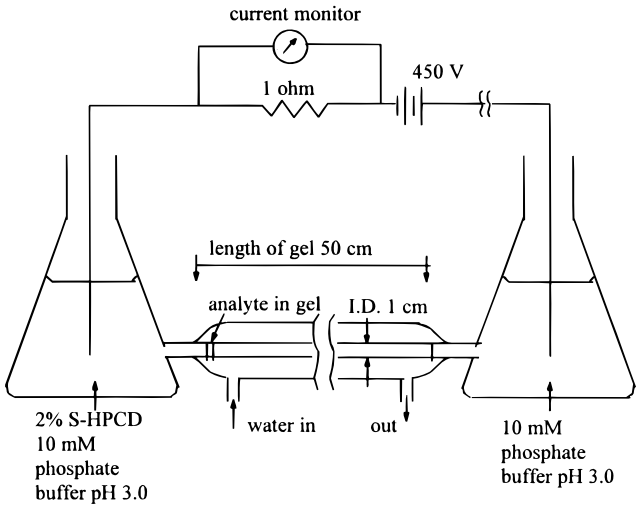


Figure 1. Schematic of original apparatus used for classical gel electrophoresis.

detector set at 211 nm interfaced to a Gateway 2000 PC. Both CZE systems were operated with the cathode at the injector end of the capillary. The fused-silica capillary columns were either 75 μm i.d. with a column length of 60 cm (52.4 cm to detector window) or 50 μm i.d. with a column length of 25 cm (20.4 cm to detector window).

The apparatus used for the original gel electrophoretic work is illustrated in Figure 1. A West condenser was used to supply mechanical support for the electrophoretic bed and provided a convenient method for cooling. Subsequent gel electrophoretic work employed a Bio-Rad Mini-prep cell. The power supply for both systems was a Bio-Rad PowerPac 3000 power supply.

In the Mini-prep cell, the eluent flushing the bottom end of the electrophoretic bed (~10 cm × 7 mm i.d.) was delivered by a Bio-Rad Econo peristaltic pump to a Shimadzu SPD-6A UV variable-wavelength detector interfaced to a Shimadzu Chromatopac CR-501 data station and then to an Isco Retriever II fraction collector.

Methods. The buffer pH, ionic strength, and sulfated cyclodextrin concentration for the gel electrophoretic run was derived from the CE conditions.

To prepare the electrophoretic bed in the West condenser, the condenser was positioned in an upright position, with the bottom end capped with a ground glass stopper, and filled with a 2% agarose gel (medium EEO) aqueous buffer solution (10 mM phosphate, pH 3.2) incorporating 1−2% sulfated hydroxypropyl-β-cyclodextrin. The gel bed length was ~50 cm with a diameter of ~1 cm. A similar procedure was used for the Mini-prep system using the sulfated β-cyclodextrin. In the Mini-prep cell, the gel bed was ~10 cm × 7 mm i.d.

Sample introduction in both systems required that the sample be dissolved in a small amount of the pregelled agarose and introduced to the top end of the column after the gel bed had solidified (~1 h). Standard electrophoretic methods of layering the sample on top of the gel using ethylene glycol or glycerin could not be used in the horizontal configuration and because of competition between the solvent and terbutaline for the cyclodextrin cavity.

In the investigation using the gel immobilized in the West condenser, the slab gel was extruded from the column support after ~12 h of applied voltage (~450 V). The gel was sliced and extracted with methanol, and the individual extracts were subjected to chiral CE analysis using 2% sulfated β-cyclodextrin, 10 mM phosphate buffer, pH 3.2. Individual slices were extracted and analyzed by chiral CE to validate the separation. In the case of the Mini-prep cell, the applied voltage was ~70 V and run times were ~4−5 h. Fractions collected from the Mini-prep cell identified from the UV trace as containing terbutaline were subjected to chiral CE analysis to determine the enantiomeric composition of the fractions. Most of the results have been obtained loading ~3−4 mg of terbutaline.

RESULTS AND DISCUSSION

Thus far, the classical electrophoretic experiments have focused on disc electrophoresis using a sulfated cyclodextrin-impregnated agarose-based gel. The polarity of the applied voltage in the gel is directly analogous to the polarity in the CE experiment, and the mobility of the terbutaline and the cyclodextrin are directly analogous to the CE experiment. Thus, optimization of the CE separation has a direct relationship to optimization of the gel protocol. For instance, as in the CE case, terbutaline does not reach the end of the gel unless complexed with the polyanionic cyclodextrin. Terbutaline and the cyclodextrin proceed in a "countercurrent" process through the gel, thus amplifying the chiral discrimination inherent in the complex.

In this system, the agarose serves primarily as an anticonvective media. The decision to work with agarose was based on its ready availability in high purity and low cost. Although agarose gels are generally not used in a rod configuration because of poor elasticity, in the present application, the gel seemed to be stabilized by the presence of the additive and gel fragility was not encountered in the work performed thus far.

A typical electropherogram of the individual slices of the gel used in the West condenser can be seen in Figure 2. CE analysis of the individual slices was used to generate a histogram (Figure 3) to show how far the individual enantiomers had migrated in the gel and how well the system worked for resolving the enantiomers. It should be noted that while the CE was used to optimize conditions for the gel (maximum resolution), the CE conditions to analyze the individual fractions were selected only on the basis of baseline resolution within the shortest possible analysis time. As can be seen from Figure 3, in some fractions, near enantiomeric purity was achieved. Although there appears to be some inconsistencies in the distribution of the enantiomers in the different fractions, this may well be an artifact of the crudity of the solute recovery process. In any case, the binding between terbutaline and the sulfated cyclodextrin to some extent seemed to mediate the expected solute diffusivity problems associated with classical electrophoretic methods.

The decision to adapt the overall approach to a Mini-prep continuous-elution electrophoretic cell was predicated on reducing the labor-intensive process required for solute recovery, which also introduced potential opportunities for sample remixing. In the Mini-prep system, the eluent is sent to an HPLC UV detector and then to a fraction collector. Figure 4 illustrates a trace obtained from the UV detector monitoring the eluent coming from the end of the electrophoretic bed. It is important to note that the blunted shape of the peaks probably arises from the fact that the sample concentration is well beyond the linear range of the

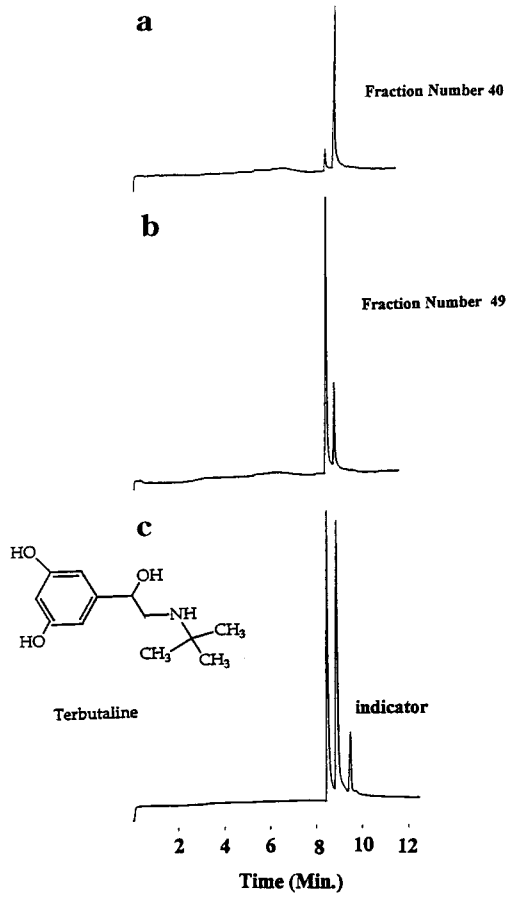


Figure 2. Typical electropherograms obtained for two individual fractions (a, b) collected from the gel used in Figure 1 and the racemic terbutaline (c) using 10 mM phosphate buffer (pH 3.0) with 2% sulfated β-cyclodextrin.

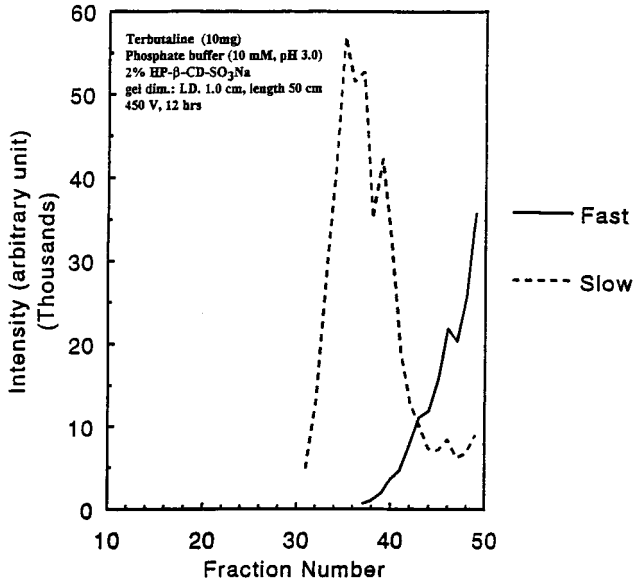


Figure 3. Histogram for terbutaline enantiomers generated from CE analysis of the individual gel slices.

detector. Figure 5 illustrates a typical CE analysis of two terbutaline fractions obtained from the Mini-prep cell. It should be noted that the lower electropherogram in Figure 5 does not represent the most complete separation achieved for the second

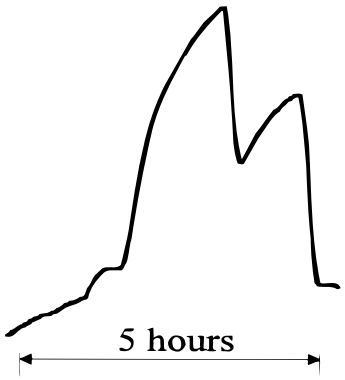


Figure 4. UV trace for ~3 mg of terbutaline eluting from a 2% sulfated cyclodextrin-impregnated agarose gel using 2% sulfated cyclodextrin, 10 mM phosphate buffer (pH 3) as the elution buffer.

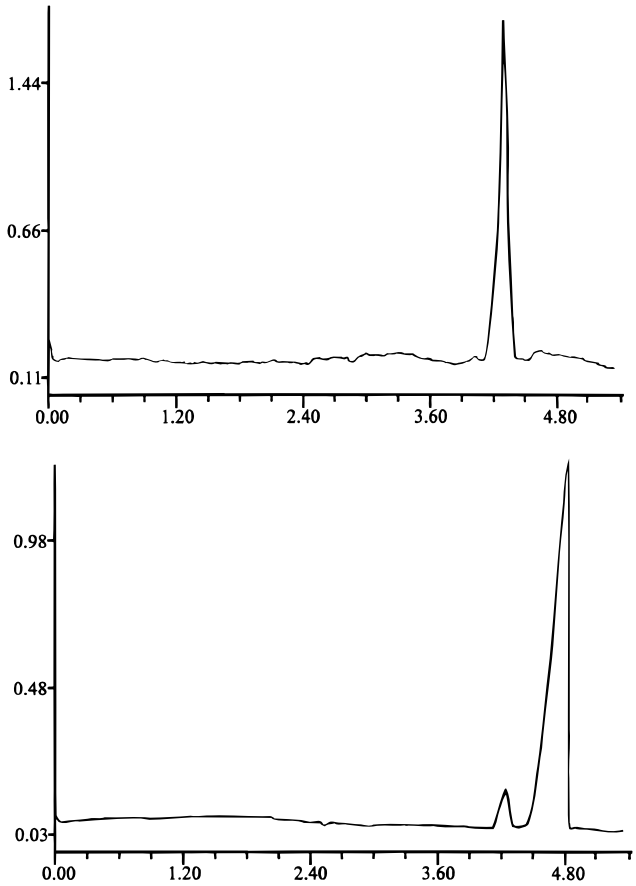


Figure 5. Electropherograms of two individual fractions collected from the Mini-prep electrophoresis cell. CE conditions: 2% sulfated cyclodextrin, 10 mM phosphate buffer (pH 3).

enantiomer but is only included to illustrate the migration times for both enantiomers. Figure 6 illustrates a typical histogram showing the distribution of enantiomers in the individual fractions. As can be seen from the histogram, there is a more uniform distribution of the enantiomers in the fractions, thus supporting the supposition that the inconsistencies in enantiomer distribution in the original gel slices may have been an artifact of the recovery process.

Routine applications of gel electrophoresis typically involve separations of fairly complex, biologically derived samples. As a result, the gel matrix is usually contaminated by denatured

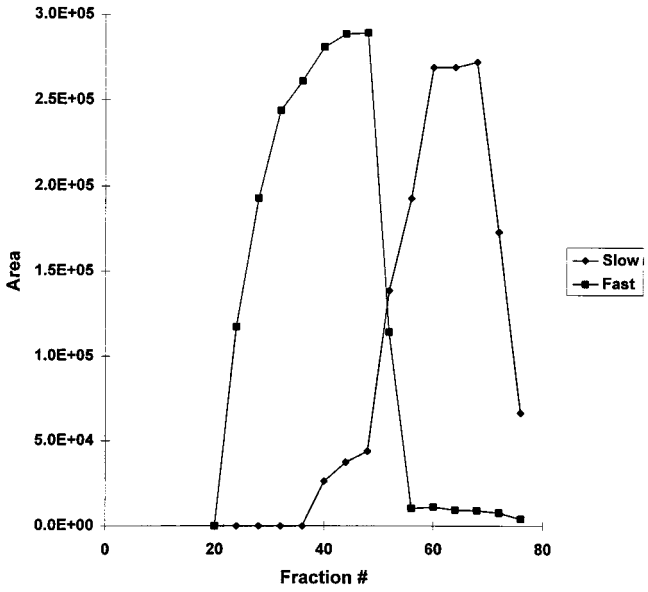


Figure 6. Histogram for terbutaline enantiomers generated from CE analysis of the individual fractions.

proteins or other sample components that fail to elute from the gel, thereby compromising future experiments. However, our samples are generally comprised only of the enantiomeric mixture to be separated. This feature of the separations coupled with the elimination of the need for slicing and extracting the gel by using the continuous-elution cells allows the gel to be used repeatedly. Indeed, gels have been used five times without any noticeable deterioration (e.g., gel integrity, ability to maintain applied voltage). The ability to use the same gel with different analytes or under slightly different experimental conditions (e.g., sample load, run buffer, pH, ionic strength) allows for more meaningful comparisons of experimental results because the bed-to-bed variability is minimized. Typical electrophoretic runs, with an applied voltage of 70 V, take ~4−5 h. Sulfated cyclodextrin is recovered by simply adjusting the run buffer to pH 8, using NaOH solution, adding ethanol.

CONCLUSIONS

The work presented here demonstrates that classical gel electrophoresis is indeed viable as a method for the separation of milligram quantities of chiral compounds although many questions remain to be answered. It should be noted that the separations obtained thus far in the gel are based on nonoptimized conditions. In our initial investigation, it seemed likely that solutes most amenable to this classical gel electrophoresis method should contain an ionizable moiety that complemented the charge on the chiral selector. It was thought that significant binding between the analyte and the cyclodextrin was critical to circumvent the solute diffusivity problems associated with classical electrophoretic methods particularly when the gel had to be extensively manipulated to recover the analyte. However, implementation of the continuous-elution electrophoretic cell should, in principle, allow even neutrals to be chirally resolved because the enantiomers are recovered during instead of after the electrophoretic run. Thus, this approach should be generally applicable to any of the over 90 structurally diverse compounds successfully enantioresolved using the sulfated cyclodextrins and no doubt to other chiral selector/chiral analyte combinations as well.

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