# Use of capillary electrophoresis as a method development tool for classical gel electrophoresis

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**Capillary electrophoresis (CE) was used to optimize the buffer pH, ionic strength and sulfated cyclodextrin concentrations for enantiomeric separation of piperoxan. These enantioseparation conditions were then applied to a classical gel electrophoresis system. Binding constants of the sulfated β-cyclodextrin–piperoxan couple were approximated using CE and the effects of organic solvents on the system were also investigated.**

***Keywords:*** *Capillary electrophoresis; piperoxan; enantiomers; gel electrophoresis; chiral separation*

In recent years, capillary electrophoresis (CE) has been used to perform a number of separations, previously attempted using high-performance liquid chromatography (HPLC).[1] One of the reasons for the technique's gain in popularity is due to its extremely high separation efficiency and high resolution capabilities. A number of advantages are immediately apparent with CE in comparison with HPLC, including simplicity, rapid method development and optimization, fast analysis times, a variety of separation modes and low cost. Other advantages of using CE are that relatively expensive reagents can be used, since they require minimal quantities of reagent and separation media. Therefore, large volumes of organic waste, associated with HPLC methods, are avoided.

An area in which CE has become extremely important is the pharmaceutical industry. In the case of chiral drugs, the analytes possess identical physico-chemical properties in an achiral environment. The development of CE has enabled enantiomeric separations to be performed with relative ease in comparison with HPLC. There is no longer a requirement to investigate the separating capabilities of relatively expensive chiral columns, since most chiral separations can be achieved by the simple addition of a chiral additive to the run buffer.[2] Enantiomeric separations occur if two conditions are met. The binding constants of the chiral additive and the enantiomer couples are different, and there is a difference in mobility of the free and complexed states.

There are a number of chiral separation mechanisms available using CE. These include inclusion complexation, metal–chiral ligand complexation, micellar solubilization, affinity interactions and ion-pairing interactions.[2,3] Of particular interest in this study is the use of sulfated β-cyclodextrin (SCD) as a chiral additive. The first sulfonated cyclodextrin was introduced for this purpose by Tait *et al.*[4] Since then, directly sulfated cyclodextrins have been effectively demonstrated for the enantiomeric separation of a wide number of chiral drugs.[5]

At present, analytical separations are readily achieved using CE, but it is difficult to scale up separations to yield preparative amounts. Stalcup *et al.*,[6] however, recently demonstrated that it is possible to perform enantiomeric separations using a larger scale electrophoresis system operating under similar conditions to those used in CE. Enantiomerically pure chiral drugs can be obtained in quantities which may be suitable for preliminary pharmacokinetic and pharmacodynamic studies in a research and development environment.[7] In addition, the application of a gel system with continuous buffer elution from one end, enables analytes to be collected in a similar fashion to preparative HPLC.

A chiral compound which has exhibited good enantioseparation using SCD as a chiral additive[5] and which is suitable for this study is piperoxan (Fig. 1). Piperoxan is an adrenergic blocker which is used to block or inhibit the release or activity of norepinephrine in the human body. This action dilates blood vessels (thus lowering blood pressure) and slows the heart rate. Piperoxan is used to treat hypertension and is also used as a diagnostic aid for pheochromocytoma.[8]

This report details the investigation of various buffer systems used with a SCD chiral additive for the enantioseparation of D,L-piperoxan. After determination of suitable separation conditions using CE, baseline enantioseparation performed using a classical gel electrophoresis system enables usable quantities of enantiomerically pure piperoxan to be obtained. Enantiomeric purity of piperoxan fractions can be determined using CE. In order to further evaluate the system, determination of binding constants for the piperoxan–SCD couple and the effect of organic modifiers on the CE separation are described.

## Experimental

### *Instrumentation*

Capillary electrophoresis experiments were performed using a Bio-Rad BioFocus 2000 system (Bio-Rad, Hercules, CA, USA). Fused silica capillary (25 μm id, 17 cm total length, 12.4 cm to the detector) coated with polyacrylamide was also obtained from Bio-Rad. Detection of piperoxan was achieved at the anodic end of the capillary at a wavelength of 270 nm. All experiments were performed between −3.0 and −8.0 kV and thermostatted at 15 °C. Hydrodynamic injections of 4 mM piperoxan were made at 5 psi for 0.4 s (2 psi $s^{-1}$). The neutral marker was 20 mM nitromethane.

Classical gel electrophoresis investigations were performed using a 'Mini Prep Cell' (Bio-Rad; (Fig. 2). The power supply for the system was a Bio-Rad PowerPac 3000. In the Mini Prep Cell, the elution buffer, which flushed the bottom of the electrophoretic bed (≈100 × 7 mm), was delivered by a Bio-Rad Econo peristaltic pump to a Shimadzu (New York, USA) SPD-6A UV variable-wavelength detector, interfaced to a Shimadzu Chromatopac CR-501 data station. The eluate was fractionated by an Isco Retriever II fraction collector.

Viscosity measurements were made using a Cannon–Ubbelohde type viscometer. The relative viscosity, $\eta_r$, was calculated using the equation, $\eta_r \approx (t/t_o)$, where $t_o$ is the efflux time of the solvent (water) and $t$ is the efflux time of the buffer solution.[9]

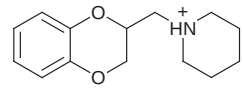


**Fig. 1** Piperoxan.

### *Reagents*

Sulfated β-cyclodextrin (nominal 13 sulfates/cyclodextrin) was donated by Cerestar, Inc. (Hammond, IN, USA). The D,L-piperoxan hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The agarose (medium EEO, electrophoretic grade) and all other buffer components {4-morpholineethanesulfonic acid (MES), 2-[tris(hydroxymethyl)-methylamino]-1-ethanesulfonic acid (TES), glycine, citric acid, formic acid, acetonitrile, methanol and *N*,*N*′-dimethylformamide} were obtained from Fisher Scientific (St. Louis, MO, USA).

### *Methods*

The buffer solutions for CE prepared from MES, TES and glycine were 100 mM each containing 2% SCD. The concentration of citric acid used for investigations with changing SCD concentration was kept constant (150 mM). For other CE investigations, citric acid was used at concentrations required to adjust the respective SCD concentration to pH 3.

The conditions used for CE were applied to the classical gel electrophoresis system. The gel was prepared by heating 2% agarose solution, prepared from buffer containing 1% SCD adjusted to pH 3 using ≈54 mM citric acid. The gel, heated using a microwave oven, was reheated and cooled three times to remove air bubbles. Finally, the hot gel solution was carefully poured into the gel chamber. The gel bed was ≈100 × 7 mm diameter. Run times using the Mini Prep Cell were about 4–5 h with an applied potential of 120 V and current of ≈10 mA.

## Results and discussion

CE served two functions in the investigations. Primarily, optimization of the buffer pH, ionic strength and sulfated cyclodextrin concentrations was performed using the CE system. Secondly, fractions collected from the Mini Prep Cell identified from the UV trace as containing piperoxan were subjected to chiral CE analysis to determine the enantiomeric composition of the fractions.

The highly sulfated β-cyclodextrin (SCD) used in the investigations was designated as having 13 sulfate groups per cyclodextrin, and thus negatively charged at pH 3. The mechanism of enantioseparation is therefore thought to occur by a process of electrostatic attraction and by inclusion of piperoxan in the hydrophobic cavity of the cyclodextrin.

Earlier work[6] with SCD as a chiral additive in CE used a phosphate buffer system. It was believed that a buffer system with a lower mobility than phosphate could enable the system to be run at higher voltages and enhance the separation. In addition, it was felt that an organic buffer might be easier to remove from the fractions collected from the Mini Prep Cell, which would facilitate recovery of both the analytes and additive.

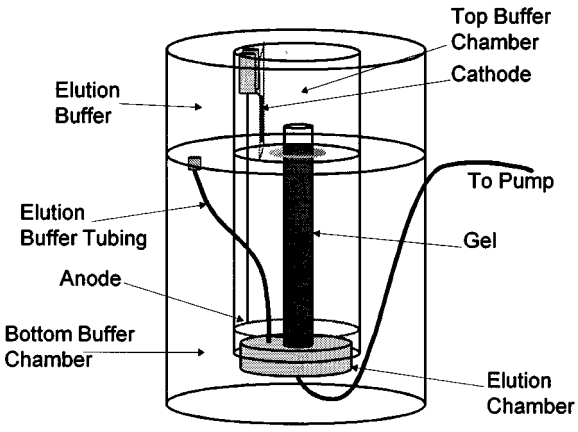


**Fig. 2** The Bio-Rad Prep Cell.

In preliminary investigations, MES, TES and glycine were chosen as buffer components since they are zwitterions at the pHs of interest and should therefore have relatively lower mobilities than positively or negatively charged compounds of the same size. The pHs of the aforementioned buffer solutions were between 4 and 6 after addition to SCD at various concentrations. Although separation of piperoxan enantiomers was achieved using the prepared buffers in a CE system with a coated capillary, the pH of the buffers was considered too high for use in the gel electrophoresis system, where the EEO could be significant at pH > 3. A buffer system prepared using citric acid appeared to be suitable for the enantioseparation of piperoxan, and displayed better resolution than the other buffer systems investigated (Table 1).

A study of mobility of the piperoxan enantiomers was performed, in which the concentration of citric acid was kept constant (150 mM), but the concentration of SCD was increased. The buffer pH was adjusted with sodium hydroxide to pH 3. At this high concentration of citric acid, all of the buffer solutions had the same citric acid concentration since this was the concentration required to adjust the 3% SCD solution to pH 3. As expected, the apparent mobility of the piperoxan enantiomers increased with increasing SCD concentration over the range investigated. This may be explained by the higher concentration of SCDs, forcing the piperoxan to spend a larger amount of time in the complexed form, and thus migrate towards the detector. Current was found to increase with increasing SCD concentration. Plot of mobility *versus* SCD concentration for the individual enantiomers were essentially parallel. Thus, little or no benefit was obtained going to higher SCD concentrations.

The measured viscosities in the range of 0.75–3.00% SCD were essentially constant ($\eta_r \approx 1.10 \pm 0.01$). It is probable that the high concentration of citric acid ensured that viscosity differences between solutions was minimal.

One aim was to determine binding constants of the piperoxan enantiomers with SCD and these were obtained from an equation for binding constants for lectin sugar systems[10] adapted by Tanaka *et al.*[11] for use with cyclodextrins:

$$\frac{1}{\mu - \mu_f} = \frac{1}{(\mu_{SCD} - \mu_f)K} \times \frac{1}{[SCD]} + \frac{1}{\mu_{SCD} - \mu_f} \quad (1)$$

where $\mu$ is the apparent mobility of the analyte, $\mu_f$ is the apparent mobility of the analyte with no SCD present, $\mu_{SCD}$ is the apparent mobility of the analyte complexed with SCD and $K$ is the binding constant. The apparent electrophoretic mobilities were determined using the equation:[12]

$$\mu_{eph} - \mu_{eo} = \frac{lL}{Vt} \quad (2)$$

where $\mu_{eph}$ and $\mu_{eo}$ are the electrophoretic and electroosmotic mobilities, $l$ is the length of capillary to the detector, $L$ is the total capillary length, $V$ is the operating voltage and $t$ is the migration time. Although a coated column was used, injection

**Table 1** A comparison of migration times and resolution of the enantiomers of piperoxan in different buffer systems

| Reagent (100 mM with 2% SCD) | Migration times/min | Resolution |
|---|---|---|
| TES | 2.69/4.41 | 3.22 |
| MES | 3.47/5.52 | 3.25 |
| Glycine | 4.20/6.43 | 3.11 |
| Citric acid | 4.10/7.53 | 4.34 |

of a neutral marker (nitromethane), revealed that there was significant electroosmotic flow (EOF) ($\mu_{eof}$ = 1.92 $\times 10^{-5}$ cm$^2$ V s$^{-1}$), but it was fairly constant over the SCD concentration range investigated ($\pm 7.11 \times 10^{-8}$ cm$^2$ V s$^{-1}$). A stable coating of SCD on the inside of the capillary resulting in a negative surface charge might account for this EOF. The diffusion of the analytes led to 'fronted' peak shapes. The migration times were therefore taken at the time where the peak is split into two portions of equal area. It should be noted that this does not compensate for the slight variation in response from the leading to the tailing edge of the peak. Fig. 3 displays the graph used to determine the binding constants using the intercept/slope. The values of the binding constants were $K_1$ = 1150 ± 130 and $K_2$ = 722 ± 50 for the first and second eluting enantiomers, respectively.

The effect of the addition of methanol and acetonitrile to the run buffer is displayed in Fig. 4. SCD (2%) was used with 150 mM citric acid adjusted to pH 3 using sodium hydroxide. It was found that migration times of the piperoxan enantiomers increased with addition of the organic modifiers to the run buffer. The difference in migration times observed with the organic modifiers is believed to be a result of several factors. There could be competition of the organic modifiers and piperoxan for the hydrophobic SCD cavity. In addition, it is possible that the piperoxan enantiomers could have a greater affinity for the background electrolyte which would decrease the free energy driving the complexation. As can be seen from Fig. 4, at concentrations up to approximately 5% modifier, there is no significant difference between methanol and acetonitrile. After additions above 5%, however, there is increased resolution between the enantiomers using acetonitrile in comparison with methanol. It is therefore possible to enhance enantioseparation using acetonitrile. It is likely, however, that run times could become impractical using the gel electrophoresis system. Conversely, no enantioseparation was found in the presence of *N*,*N*′dimethylformamide (DMF). At a 10% concentration of DMF, the migration time of the single peak (containing both unseparated enantiomers) was greater than the migration time of the first eluting enantiomer, but lower than that of the second

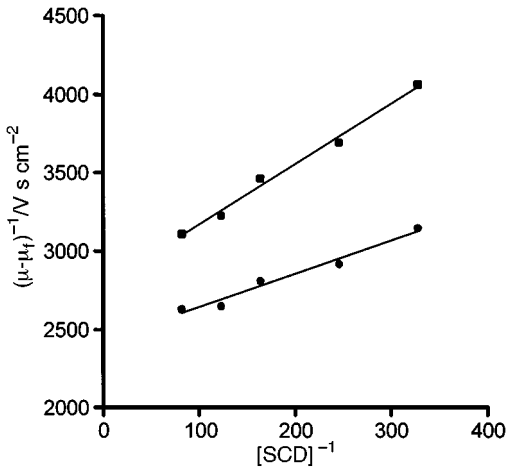


**Fig. 3** Plot of $(\mu-\mu_f)^{-1}$ *versus* $[SCD]^{-1}$ for the piperoxan enantiomers. 150 mM citric acid was used with varying concentrations of SCD, the buffer pH was adjusted with sodium hydroxide to pH 3.

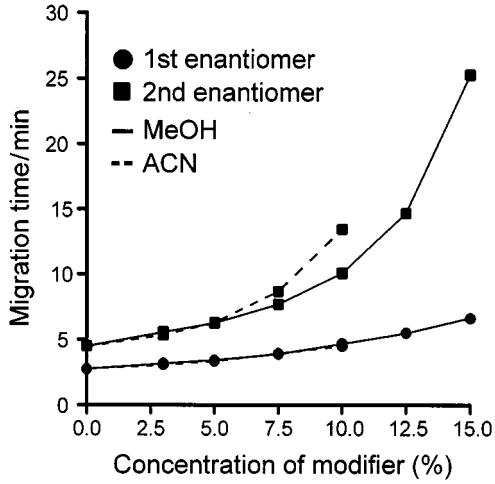


**Fig. 4** Addition of organic modifiers [methanol (MeOH) and acetonitrile (ACN)] to the SCD system. The buffer was 150 mM citric acid, 2% SCD and the respective concentration of organic modifier. The buffer pH was adjusted with sodium hydroxide to pH 3 before addition of modifier.

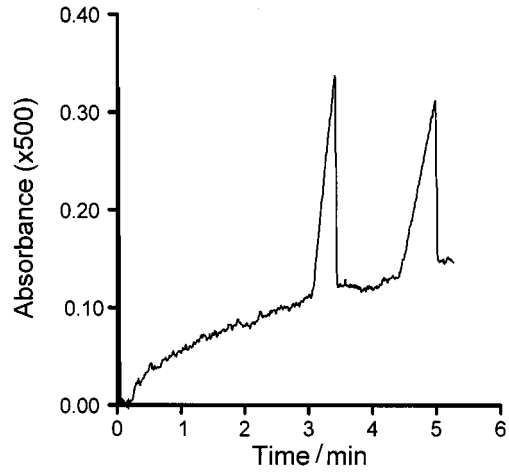


**Fig. 5** Enantiomeric separation of piperoxan using CE. The buffer was 1% SCD adjusted to pH 3 with ≈ 54 mM citric acid.

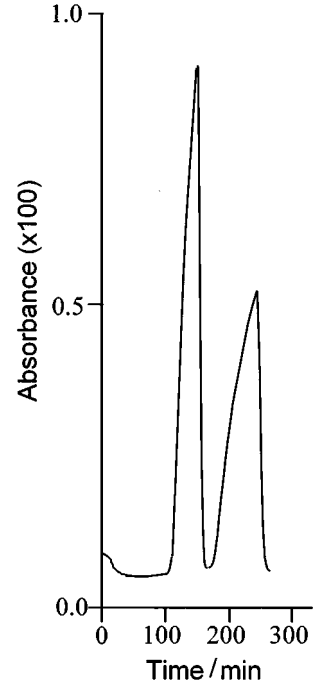


**Fig. 6** Enantioseparation of piperoxan using the Prep Cell. The top and elution buffers were 1% SCD adjusted to pH 3 with ≈ 54 mM citric acid. The bottom buffer was ≈ 54 mM citric acid adjusted with sodium hydroxide to pH 3.

eluting enantiomer of piperoxan, for the corresponding buffers containing 10% methanol and acetonitrile, respectively.

The buffer conditions for separation of the piperoxan enantiomers using CE were adapted to a Bio-Rad Prep Cell. The agarose gel served as an anticonvective medium and was not considered to play a significant part in the enantioseparation of the piperoxan. Agarose gels were chosen in preference to polyacrylamide gels, owing to ease of preparation, ready availability and because agarose is less toxic than the polymerizing agents used to prepare the polyacrylamide gel. The agarose gels were found to be stable over the voltage range used and were mechanically stable when removed from the gel cylinder. Running the system at higher voltages, however, could cause the agarose to melt as a result of joule heating (agarose gelling temperature 34–35 °C).

The enantioseparation of piperoxan using CE is displayed in Fig. 5. Owing to the lack of enantiomerically pure piperoxan standards, it was not known which enantiomer eluted first. Fig. 6 displays an electropherogram obtained from the Prep Cell for 0.5 mg sample loading of piperoxan. Fronted peak shapes were obtained, in accordance with CE, with a resolution of 1.34 ($N$ = 137 ± 8). It was found that by running with the entire system placed in a bowl of ice water (*i.e.*, the bottom buffer solution was cooled), the current was reduced and the separation could be performed at a higher voltage (170 V). A separation of similar resolution was achieved, but in a shorter run time (< 4 h). Fig. 7 displays the electropherograms of fractions collected from the Prep Cell run, indicating that the fractions were enantiomerically pure. Prior work with terbutaline failed to achieve the level of separation obtained with the gel system in this study.

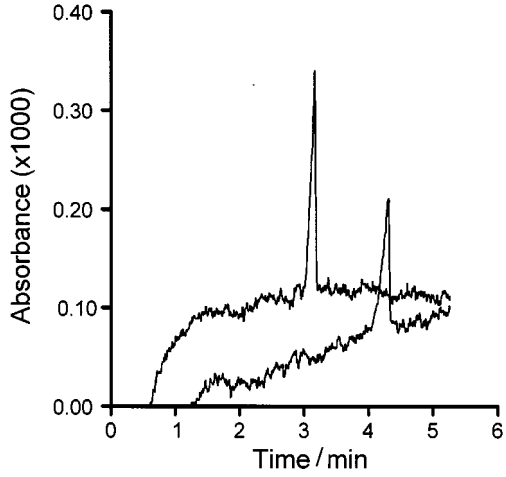


**Fig. 7** Enantiomerically pure fractions of piperoxan run on CE. The buffer was 1% SCD adjusted to pH 3 with ≈ 54 mM citric acid.

In conclusion, CE was used to optimize parameters for scale-up of a chiral separation to preparative gel electrophoresis. Although more work is required to assess the general applicability and limits of this approach, preparative chiral gel electrophoresis may offer a viable alternative to more traditional methods.

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