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Practical aspects in chiral separation of pharmaceuticals by capillary electrophoresis
II. Quantitative separation of naproxen enantiomers☆

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Abstract

In recent years, there has been considerable activity in the separation and characterization of optically active molecules. In this paper we report a new, improved and automated electrophoretic method for the separation of enantiomers in the form of high-performance capillary gel electrophoresis using hydroxypropyl-β-cyclodextrin as a chiral selector. Rapid, efficient separation of naproxen enantiomers is shown with very low detection limits and excellent detection linearity. The intra- and inter-day as well as intra- and inter-column migration time reproducibility was less than 2% R.S.D. Trace level enantiomeric contamination determination is also shown.

1. Introduction

Recently, there has been a great deal of interest in the separation and characterization of optically active compounds [1]. The problem is a challenging one since optical isomers possess identical physical characteristics and differ only slightly from another in their spatial orientation [2]. Conventionally, chiral separations were achieved by gas chromatography [3] and more recently by high-performance liquid chromatography [4]. The potential advantages of using capillary gel electrophoresis over HPLC are the higher peak efficiency attained that results in higher resolution with similar selectivities and low buffer and chiral selector consumption [5]. It has been shown that inclusion complex equilibria using different native, α-cyclodextrin [6–9], β-cyclodextrin [6–12], γ-cyclodextrin [7,8,13,14], and chemically modified cyclodextrins such as dimethyl-β-cyclodextrin, trimethyl-β-cyclodextrin [8,13–17], hydroxypropyl-β-cyclodextrin (HP-β-CD) [18–20] as chiral selectors is very powerful in the separation of enantiomers. These natural and derivatized cyclodextrins were implemented as chiral selectors in different capillary electrophoresis (CE) separation modes such as capillary zone electrophoresis [6,13,15], micellar electrokinetic chromatography [21–23], isotachophoresis [14–17] and capillary gel electrophoresis (CGE) [7,24].

Early attempts of CGE separations employed cross-linked polyacrylamide gel filled capillaries with various native cyclodextrins (α, β and γ) that were physically entrapped into the small

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pore size polymer matrix [7]. D and L forms of dansylated amino acids were easily separated within 10 min with these gels. The major disadvantage of cross-linked (chemical gels) compared to non-cross-linked linear polymer networks (physical gels) is that chemical gels are non-replaceable, therefore if the gel becomes contaminated or develops bubbles the gel-filled capillary must be replaced [25].

In this paper we report the use of a non-cross-linked, hydrophilic linear polymer network in conjunction with HP-β-CD for CGE of naproxen enantiomers. This polymer network is advantageous in that suppressing the ζ potential on the inside surface of the capillary substantially. Polymer networks containing chiral selectors are easily prepared and are easily replaceable in the capillary column, if necessary. Examples are shown with high resolutions under optimized separation conditions as well as the usefulness of this system in the demonstration of very low level ($<1\%$) enantiomeric contamination determination.

2. Materials and methods

2.1. Apparatus

In all these studies, the P/ACE system 2100 CE apparatus (Beckman Instruments, Fullerton, CA, USA) was used in reversed-polarity mode (cathode on the injection side). The separations were monitored on-column at 230 nm. The temperature of the cartridge containing the polymer network-filled capillary column was thermostated at 20 ± 0.1°C by the liquid cooling system of the P/ACE instrument. The electropherograms were acquired and stored on an IBM 486/66 MHz computer and were evaluated with the System Gold software package (Beckman Instruments).

2.2. Procedures

In all the CE experiments a 20 cm effective length (27 cm total length) $\times$ 375 μm O.D. $\times$ 25 μm I.D. bare fused-silica capillary tubing was used. The use of low-viscosity polymer network permitted replacement of the gel–buffer system in the capillary column by means of the pressure-rinse operation mode of the P/ACE apparatus (i.e., replaceable gel). It is important to note that the inside surface of the capillary column was deactivated by means of 1 *M* HCl rinse prior to each run in these experiments to decrease ζ potential of the inside capillary wall, thus causing further decrease in electroosmotic flow in the polymer network-filled capillary column. The samples were injected by pressure (typically: 3–20 s, 0.5 p.s.i. = 3447.4 Pa) into the replaceable gel-filled capillary column.

2.3. Chemicals

Ultrapure-grade 200 m*M* 2-(N-morpholino)-ethanesulfonic acid (MES) buffer adjusted to pH 5.0 by tetrabutylammonium hydroxide (TBAH) was used in the experiments (ICN, Costa Mesa, CA, USA). The exact composition of all the buffers used during the pH optimization experiments (pH 3, 4, 5, 6, 7, 8 and 9) were described elsewhere [26]. The chiral selector, HP-β-CD with the average substitution rate of 4.9, was purchased from American Maise Products (Hammond, IN, USA). The racemate and *S* forms of Naproxen (Syntex, Palo Alto, CA, USA) were dissolved in deionized water containing 10^{-5} *M* TBAH, in 0.1 and 1 mg/ml concentrations, respectively. Prior to injection this mixture was diluted ten times by the running buffer, 200 m*M* MES/TBAH, 10 m*M* HP-β-CD, 0.4% polymeric additive, pH 5.0. The samples were stored at -20°C or freshly used. The internal standard of *p*-toluenesulfonic acid (pTSA) was purchased from Sigma (St.Louis, MO, USA). All buffer and gel solutions were filtered through a 0.45-μm pore size filter (Schleicher and Schuell, Keene, NH, USA) and carefully vacuum degassed before use.

3. Results and discussion

3.1. Separation optimization

CGE with chiral selectors has proven to be a powerful tool for the separation and purity

assessment of optically active molecules [7,24]. As we have shown earlier [26] in accordance to the theory of Vigh and co-workers [27,28], chiral separation method development should follow the following scheme: (1) optimization of the separation pH, (2) optimization of the chiral selector concentration, (3) optimization of the applied electric field strength and (4) optimization of the separation temperature. Following this optimization scheme [26] the subsequent separation parameters were found to be as best for the separation of the naproxen enantiomers using HP-β-CD as a chiral selector. (1) Evaluating the pH range of 3–9 by one pH unit increments [26], maximum resolution was attained employing the pH 5.0 buffer. (2) Varying the chiral selector concentration between 2 and 100 mM a concentration of 10 mM was found to be optimal for the separation of the naproxen enantiomers. (3) A maximum in enantiomeric resolution was found at 700 V/cm when the applied electric field was varied from 100 to 1000 V/cm by 100 V/cm increments. (4) Checking the effect of the temperature between 20 and 50°C on the separation of the R and S forms of naproxen, 20°C was found to give the highest resolution. As the result of this optimization, Fig. 1 shows the complete baseline separation

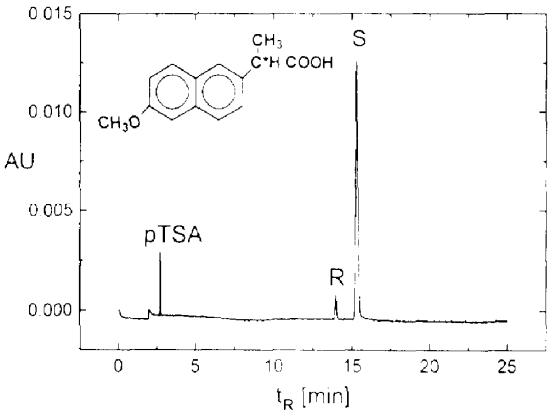

Fig. 1. CGE separation of 1:10 mixture of R- and S-naproxen. Peaks: pTSA = p-toluenesulfonic acid, anionic marker; R = R-naproxen; S = S-naproxen. Conditions: gel–buffer: 200 mM MES/TBAH, 10 mM HP-β-CD, 0.4% polymeric additive, pH 5.0, Injected amount: 0.1 ng R-naproxen and 1 ng S-naproxen. Effective capillary length: 20 cm, internal diameter: 25 μm; detection: 230 nm; run temperature: 20°C; applied electric field strength: 700 V/cm; current: 3 μA.

($R_s = 3.54$) of the 1:10 mixture of R- and S-naproxen in 15 min using these separation parameters.

3.2. Reproducibility

Initial characterization of CGE of enantiomers using HP-β-CD as a chiral selector in the gel–buffer system involved the determination of reproducibility for migration time. The uncorrected and corrected migration times and chiral selectivities (α) for the first and 100th runs of the mixture of 0.01 mg/ml R- and 0.1 mg/ml S-naproxen accompanied by the relative standard deviations are shown in Table 1. The migration times of the pTSA were used for the calculation of the corrected migration times of the naproxen enantiomers, i.e.:

$$t_M'^{R\text{-naproxen}} = t_M^{R\text{-naproxen}} / t_M^{pTSA} \quad (1)$$

This data represents two batches of capillaries and buffers that were cycled for 100 runs (intra- and inter-day variability) with a 1 M HCl wash in between each run. The good reproducibility of the results (R.S.D. < 1%) may be attributed to the ability to wash the capillary between runs and the replacement of the polymer network by means of a rinsing step after each run. If a sample containing particulates is applied to the gel, or a sample containing a contaminant too large to be analyzed in the standard run time, the replacement of the low-viscosity gel alleviates any damage to this non-cross-linked linear polymer matrix.

3.3. Detection limit and detection linearity

Employing the pressure injection mode, the injection flow was found to be 1 nl/s for this particular capillary gel–buffer system. This was calculated based on the migration velocity of the pTSA internal standard using the low-continuous-rinse mode with injection pressure of 0.5 p.s.i. (3447.4 Pa) [29]. The minimum amount detected on column with peak-to-noise ratio of 4 for R-naproxen was $8 \cdot 10^{-14}$ g ($0.75 \cdot 10^{-15}$ mol) that corresponds to the minimal detectable sample concentration of 300 nM. Detection mass

Table 1
Reproducibility data of the enantiomeric separation of *R*- and *S*-naproxen

	Migration time (min)			Corrected migration time		α
	R	*S*	pTSA	*R'*	*S'*	
Batch I						
Run 1	15.25	13.26	2.73	5.586	4.857	1.092
Run 100	16.07	14.71	2.87	5.599	5.125	1.092
R.S.D. (1–100) (5)	6	9.8	4.87	0.42	0.92	
Batch II						
Run 1	15.9	14.5	2.788	5.703	5.204	1.096
Run 100	16.31	14.87	2.88	5.663	5.163	1.096
R.S.D. (1–100) (%)	3.6	2.42	3.47	0.7	0.78	
Batch-to-batch reproducibility (% change in migration time)						
Run 1	4.09	3.7	2.08	2.05	1.75	
Run 100	1.47	1.07	0.35	1.13	0.73	

Separation conditions as in Fig. 1.

linearity was evaluated by pressure injection increasing amounts of solute in equal volumes onto the capillary column. The peak area was found to be a linear function of the injected amount in the sample concentration range of 1 μg/ml ($4.3 \cdot 10^{-7}$ *M*) to 10 mg/ml ($4.3 \cdot 10^{-3}$ *M*), with 0.998 correlation coefficient:

$$\text{Peak area} = 1.3314 + 0.9754 C_{\text{naproxen}} \quad (2)$$

where C_{naproxen} is the concentration of the naproxen in mg/ml. Peak area normalization to migration times and limit of detection (LOD) was calculated according to Altria et al. [30].

3.4. Enantiomeric purity assessment

For trace analysis, i.e. 1% enantiomeric contamination or below, analytical criteria are very rigorous [31]. Some of the reasons for this are unequal contributions of peak overlap, peak asymmetry, etc. For example when the trace component migrates closely, faster or slower, problems associated with mobility mismatch that causes peak asymmetry (fronting or tailing, respectively) making quantitative trace analysis difficult [32]. Since the "pure" *S*-naproxen contained 1.26% *R* form, the only way to evaluate the ability to detect 0.1% excess impurity was to spike the *S* form with 0.1% *R* form (0.2% racemate). As shown in Fig. 2, the detected enantiomeric contamination level for the *R* form, by the addition of 0.2% racemate to the *S* form, increased from 1.26% to 1.37%, so that a 0.11% difference was detected in this way.

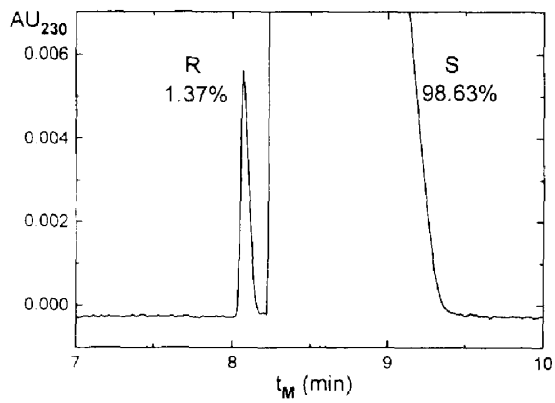

Fig. 2. Detection of 1.37% *R*-naproxen in the presence of *S*-naproxen. The "pure" *S* enantiomer containing 1.26% *R* form was spiked by 0.1% *R* form (0.2% racemate) thus 0.11% enantiomeric contamination is shown. Separation conditions as in Fig 1.

3.5. System suitability

Doyle [31] suggests that a minimal resolution value of 2.0 is necessary for racemic mixtures as an appropriate criterion for quantitative applications. The required separation efficiency can then be easily calculated based on the resolution equation Karger and Foret [33] derived for capillaries with no electroosmotic flow:

$$R_s = 0.25N^{1/2} \cdot \frac{\alpha - 1}{\alpha} \quad (3)$$

where R_s is resolution, N is the number of theoretical plates and α is chiral selectivity. Thus, with a given chiral selectivity of the separation system a minimum separation efficiency (N_{min}) is required to see the separation. If this minimal efficiency is not achieved trace analysis is not viable. In the instance of the naproxen example (using conditions of Fig. 1) to fulfill the above criterion of $R_s = 2$, separation efficiency should be at least $N_{min} = 8500$ with the chiral selectivity of $\alpha = 1.095$. This becomes very important in trace analysis where the main component is usually heavily overloaded, in order to observe a detectable amount of the enantiomeric impurity. However, sample overloading in most instances causes severe peak asymmetry (peak asymmetry at 10% of the peak height, $A_{10\%} > 10$). Therefore, peak efficiency needs be large enough in enantiomeric purity analysis to enable the detection of impurities at very low ($< 1\%$) level.

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