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Enantiomeric separation of enzymatic hydrolysis products of dihydropyrimidinone methyl ester with cationic cyclodextrin by capillary electrophoresis

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Abstract

The achiral separation of dihydropyrimidinone (DHP) methyl ester and its corresponding carboxylic acid and the chiral separation of their respective enantiomers were achieved in a single analysis using capillary electrophoresis (CE) with quaternary ammonium-β-cyclodextrin (QA-β-CD) as a chiral buffer additive. Separation of the DHP methyl ester from the corresponding carboxylic acid was achieved because the acid was negatively charged at pH 8.3 of the running buffer and the ester is neutral. Upon the addition of QA-β-CD, the enantiomers of the acid and ester were well resolved before and after the electroosmotic flow, respectively. In addition, the minor DHP methyl ester enantiomer (*R* isomer) was well separated from several impurities. This CE system was used to monitor the progress of a bioresolution reaction that utilizes an enzyme to convert the *R* isomer of the ester to its corresponding acid. The quantities of all four enantiomers can be determined using a single set of CE conditions. In addition, it is demonstrated that samples can be directly injected into the capillary without sample pretreatment due to the fact that the coating of the cationic CD on the capillary surface prevents adsorption of the positively charged enzyme. The effects of other experimental parameters such as type of CDs, concentration of CDs, pH, temperature, and the preconditioning of capillary were also studied.

Keywords: Enantiomer separation; Cyclodextrins; Dihydropyrimidinone methyl ester

1. Introduction

Cyclodextrins (CDs) have been one of the most successful types of chiral selectors used in capillary electrophoresis (CE) for separation of enantiomers [1–3]. Recently, charged CDs have been used as chiral selectors in CE and have expanded the range of applicability [4]. Charged CDs have been applied to separate neutral compounds [5–8] as well as compounds that have the opposite charges of the CDs [5–9]. Most importantly, charged CDs can also be used to manipulate the electroosmotic flow (EOF) [8] and enhance the separation resolution [10–13]. It has been demonstrated that an anionic CD can be used to resolve a large number of neutral and basic racemates at a single CD concentration under a counter-EOF condition [10]. Another group of researchers has shown that single-isomer anionic CDs can be used to separate basic, acidic and neutral racemates due to their unique separation selectivity

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[14–16]. The ability of charged CDs to separate enantiomers with the same charges of the CDs has also been demonstrated [11–13,17–19]. Furthermore, both neutral and charged CDs can also be used for nonaqueous CE chiral separation of hydrophobic enantiomers [20,21].

Dihydropyrimidinone (DHP) methyl ester (see Fig. 1) is a backbone for drugs from several therapeutic categories, such as calcium channel blockers, and antihypertensive agents. In this study, the desired enantiomer for the drug product is the *S* isomer. The *R* enantiomer is removed from the racemic mixture by selective enzymatic hydrolysis of the *R* ester to its corresponding *R* acid. The acid is then removed through extraction. Other side products are also generated during the hydrolysis.

Initially, two chromatographic methods were needed to monitor the enzymatic hydrolysis process. A reversed-phase high-performance liquid chromatography (HPLC) method was used to separate and quantify the acid and ester, and a chiral supercritical fluid chromatography (SFC) (or normal-phase LC) method with Chiralcel OD column was used to monitor the level of the enantiomers of the ester. Both methods required sample pre-treatment and the enantiomers of the acid could not be quantified.

In this paper, we report both the achiral and chiral separation of enzymatic hydrolysis products of DHP methyl ester racemate under a single set of CE conditions. The enzyme used to perform the hydrolysis of the DHP ester was positively charged at the pH of Tris running buffer (pH 8.3). In order to avoid the potential problems caused by this positively charged enzyme, quaternary ammonium-β-CD (QA-β-CD) was initially chosen to coat the surface of the capillary dynamically. This allows samples to be directly injected into the capillary without prior removal of the enzyme through sample pretreatment. Separation integrity is maintained even after injection of hydrolysis reaction profile samples that have undergone only a simple dilution. This single method simplifies the procedure for monitoring the progress of the enzymatic hydrolysis of the DHP ester by providing quantitative information for the enantiomers of the DHP ester and acid in a single run. Sample preparation time was also reduced. This method is shown to be precise, accurate and rugged.

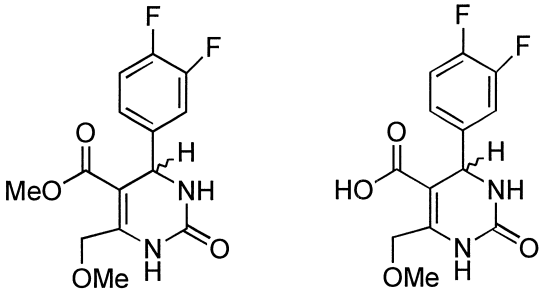

Fig. 1. Structure of DHP ester and DHP acid.

2. Experimental

2.1. Apparatus

All experiments were carried out on a Hewlett-Packard HP ^{3D}CE system with a photodiode array detector (Hewlett-Packard, Santa Clarita, CA, USA). The detector was operated at 210 nm with a 4 nm bandwidth. Untreated regular and bubble cell fused-silica capillaries (Hewlett-Packard) of 50 μm I.D. were used. The total length of the capillary was 64.5 cm, and the length to the detector was 56 cm. The capillary temperature was maintained at 25°C for method development while it was 15°C during the validation and samples analysis. Samples were injected into the capillary by pressure (5 mbar for 4 s).

2.2. Chemicals

DHP methyl ester and acid racemates were prepared by the Process Chemistry Department, Merck Research Labs. (Rahway, NJ, USA). Structures are shown in Fig. 1. QA-β-CD (Lot. G8029, degree of substitution of 3.5), and QA-γ-CD (Lot. F 8045, degree of substitution of 3.6) were purchased from Cerestar USA (Hammond, IN, USA). Cyclodextrin concentrations were calculated as weight/volume percent (w/v, %) because the charged CDs were mixtures with different degrees of substitution. Electrophoresis grade tris(hydroxymethyl)aminomethane (Tris) was obtained from Bio-Rad Labs. (Richmond, CA, USA). Pyridine was purchased from Aldrich

(Milwaukee, WI, USA). Acetic acid was purchased from Fisher Scientific (Springfield, NJ, USA).

2.3. Procedures

The background electrolyte was prepared by mixing 0.5 *M* acetic acid and Tris stock solutions, and diluting to desired concentrations with Milli-Q deionized water. Running buffer was prepared by dissolving CDs in the background electrolyte to give the desired concentration. The buffer was filtered through a 0.45-μm membrane before use. New uncoated capillaries were conditioned with water for 10 min, followed by 1 *M* and 0.1 *M* sodium hydroxide for 30 min each, followed by 10 min of water before use. Capillaries were preconditioned with running buffer for 4 min before injection and flushed with water for 3 min after each run.

2.4. Sample preparation

Two sample preparation procedures were utilized for samples from the enzymatic resolution reaction mixture. The first involved the precipitation of the enzyme by adding 9 ml of acetonitrile to 1 ml of sample. The samples were centrifuged at 500 rpm for 5 min and the clear acetonitrile supernatant was pipetted into sample vials. The solvent was evaporated under a stream of nitrogen gas. The samples were reconstituted with 30% methanol in background electrolyte. The second procedure was a simple dilution of reaction sample with 30% methanol in water. All samples were filtered through a 0.45-μm membrane before injection.

3. Results and discussion

The achiral separation of the DHP ester and acid was achieved relatively easily using a running buffer of Tris acetate at pH 8.3 with no chiral buffer additive. Under these conditions, the EOF was positive as expected and the ester co-migrated with the EOF while the acid migrated after the EOF. Once the achiral separation of the acid and ester was achieved, the separation of the enantiomers of the ester and acid through the addition of CDs to the running buffer was investigated.

3.1. CD type

There are a variety of CDs available and the factor having the greatest effect on the separation selectivity is the type of CDs used. Since the DHP ester is neutral at the running buffer pH, both cationic and anionic CDs could be used for the separation of its enantiomers. In this study, our goal was to seek the possibility of both chiral and achiral separations without sample preparation. Therefore, the effect of the sample matrix must be considered when choosing a chiral selector and other separation conditions. The samples contain a relatively large concentration of the enzyme (subtilisin) used for the bioresolution. At pH 8.3, the enzyme is positively charged, and could interact with the capillary wall or chiral selectors depending on the conditions used. These types of interactions could affect separation efficiency, selectivity, and method ruggedness. Choosing the appropriate pH and chiral selector may help to reduce or eliminate these problems.

Upon the addition of the cationic CD, the EOF decreased with increasing concentration and became negative when the CD concentration exceeded 0.005% in the aqueous buffer solution [8,21]. Separation of the enantiomers of both the ester and acid was achieved in the presence of 2.5% of QA-β-CD (Fig. 2). The first two peaks (migrating before the EOF) were *R*- and *S*-DHP acid, respectively; while the peaks migrating after the EOF were *R*- and *S*-DHP ester, respectively. This indicates that the mobility of the acid–QA-β-CD complexes was negative while that of the ester–QA-β-CD complexes was positive.

QA-γ-CD was also evaluated for the separation of the DHP ester. The ester could be baseline separated using QA-γ-CD in less than 30 min. However, other impurities typically present in actual samples were not well separated from the *R* enantiomer. Based on these results, QA-β-CD was chosen for more detailed evaluation.

3.2. Effects of CD concentration

3.2.1. Electrophoretic mobility

Fig. 3a shows the effect of QA-β-CD concentration on the electrophoretic mobility of ester and acid enantiomers. At zero CD concentration, the

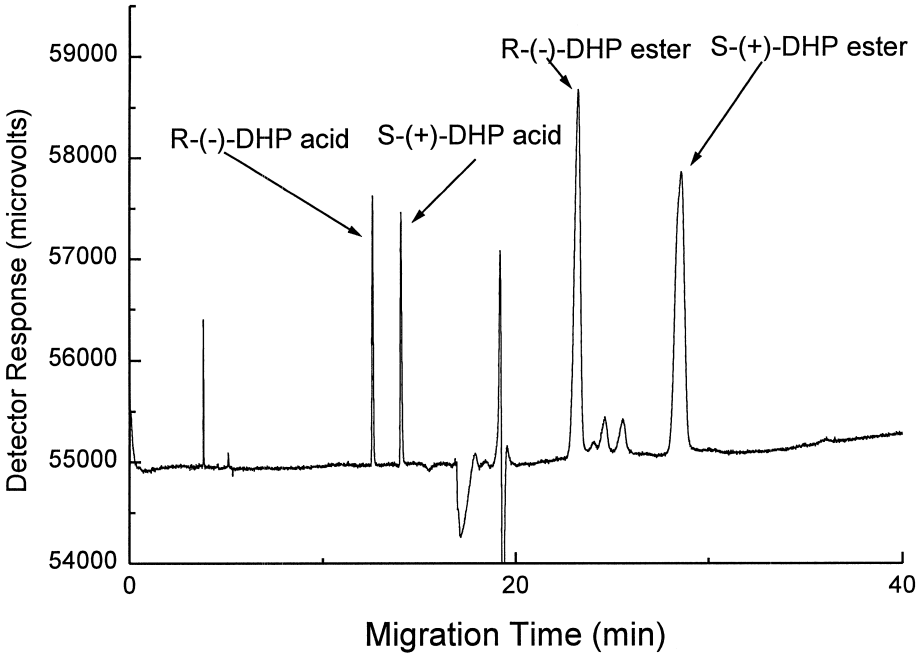

Fig. 2. Separation of DHP methyl ester and acid with QA-β-CD. Running buffer: 25 m*M* Tris acetate (pH 8.30) at 2.5% QA-β-CD. Field strength: +515 V/cm.

ester co-migrated with the EOF. With the addition of the charged CD, the electrophoretic mobility of ester increased with increasing CD concentration. This is due to the complexation between the CD and the ester.

For the acid, the electrophoretic mobility also increased with increasing CD concentration. However, the mobility of the CD–acid complexes did not change from negative to positive. This is due to the fact that the complexation between the acid and the cationic CD is still relatively weak.

3.2.2. Selectivity

For the ester enantiomers, increasing the CD concentration decreased the selectivity (Fig. 3b) as predicted by Vigh and co-workers [14–16,22].

For the acid enantiomers, the separation selectivity increased with increasing QA-β-CD concentration. No selectivity maximum was observed in the CD concentration range studied (Fig. 3b) due to the weak interactions between the CD and the acid enantiomers [22].

3.2.3. Resolution

For the DHP ester enantiomers, the resolution increased with increasing CD concentration (Fig. 3c) as predicted by Vigh and co-workers [14–16,22]. According to their simulation, the binding constants between the ester enantiomers and QA-β-CD are relatively small since no resolution maximum was observed [22]. It is understandable from the compound structure because both rings have side chains to prevent the molecule from being deeply included into the CD cavity.

For the acid enantiomers, the resolution also increased with CD concentration from 0–2.0% and leveled off above 2.0% QA-β-CD (Fig. 3c). An increasing CD concentration also resulted in an increase in ionic strength and therefore a decrease in the EOF. The decreased EOF also contributes to increased resolution.

3.3. Effect of pH

In CE chiral separations, changes in running buffer pH may cause changes in the charges of

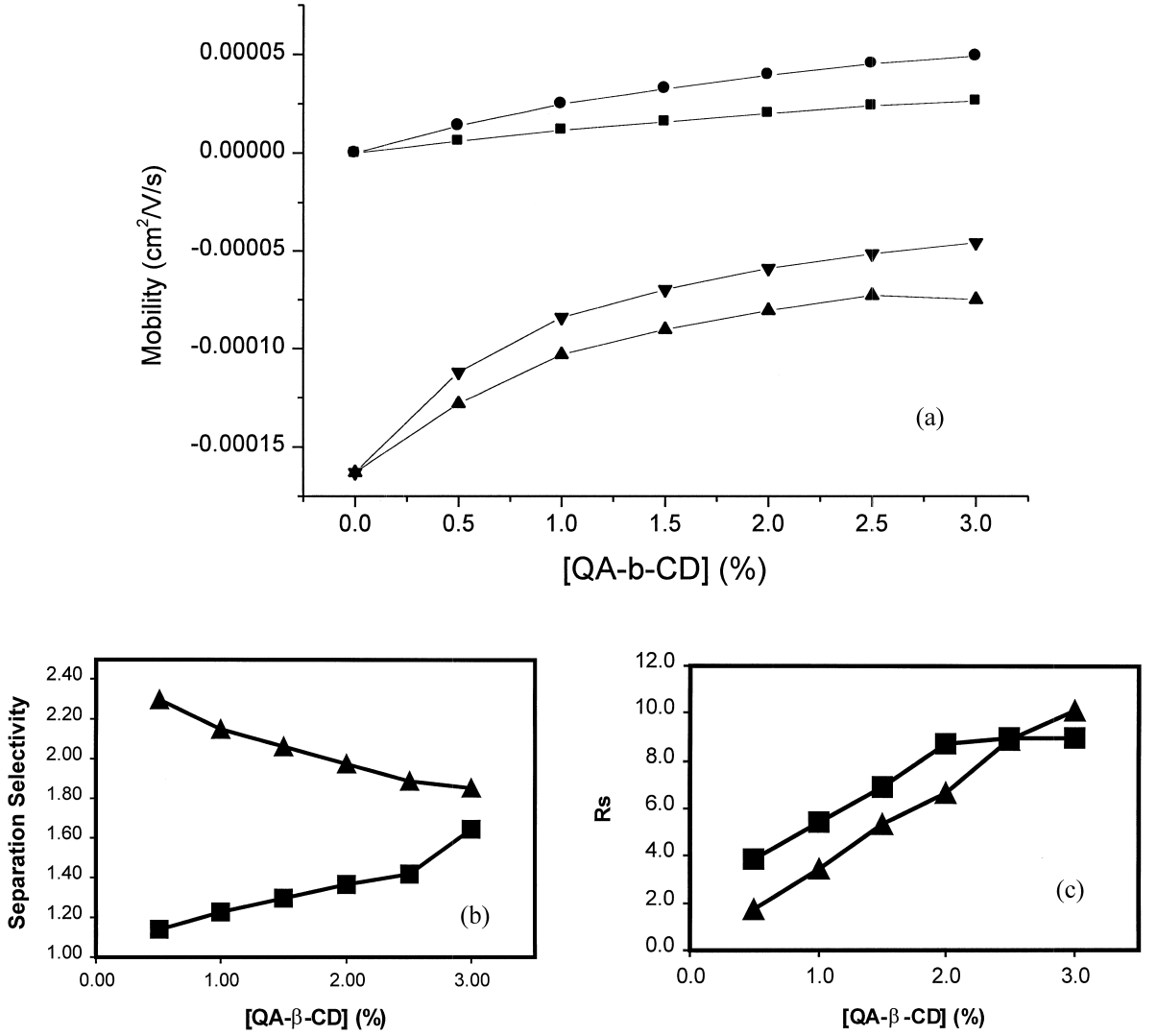

Fig. 3. Effect of QA-β-CD concentration on separation of DHP ester and acid. Running buffer: 25 m*M* Tris acetate (pH 8.30). Field strength: −326 V/cm. Analytes: (a) *R*-(−) (filled up triangle), *S*-(+) (filled down triangle) DHP methyl acid, *R*-(−) (filled square), and *S*-(+) (filled circle) DHP ester; (b) and (c) DHP acid racemate (filled square), and DHP methyl ester (filled triangle). (a) Effect of QA-β-CD concentration on electrophoretic mobilities of DHP ester and acid enantiomers. (b) Effect of QA-β-CD concentration on separation selectivity of DHP ester and acid enantiomers. (c) Effect of QA-β-CD concentration on resolution of DHP ester and acid enantiomers.

analytes and/or chiral selectors. This will lead to changes in separation selectivity [23]. Changes in pH can also affect the EOF and thus resolution [23].

In our separation system, QA-β-CD is a strong electrolyte and remains positively charged in running buffer below pH 12.1. Therefore, below pH 12.1, changes in pH only affect the charge on the acid (the ester remains neutral in the pH range studied). Two buffers were used to study the effect of pH. Tris acetate buffer was used for pH from 3.5 to 5.7. Tris phosphate buffer was used to cover pH from 6 to 10.5. The mobility, separation selectivity, and resolution of both the ester and acid enantiomers remained essentially unchanged in a phosphate buf-

fer from pH 6.0 to 10.5 because the charges of the acid, ester and the CD did not change with pH in this range.

For the ester enantiomers, pH had little effect on electrophoretic mobility and separation selectivity (Fig. 4a and b). Below pH 3.9, the resolution increased with increasing pH. It reached a peak value when the pH was 3.9. Above pH 4.5, no change in resolution was observed for the ester (Fig. 4c).

For the acid, changes in pH between 3.5 and 5.7 had a significant effect on the separation. At pH 3.9 and below, the mobility of the acid–CD complexes was close to zero. Little interaction between the acid and the CD occurred and no separation of the acid enantiomers was achieved. Above pH 3.9, the enantiomers migrated before the EOF when a negative voltage was applied and chiral separation of the acid was observed. This shows that the mobility of the

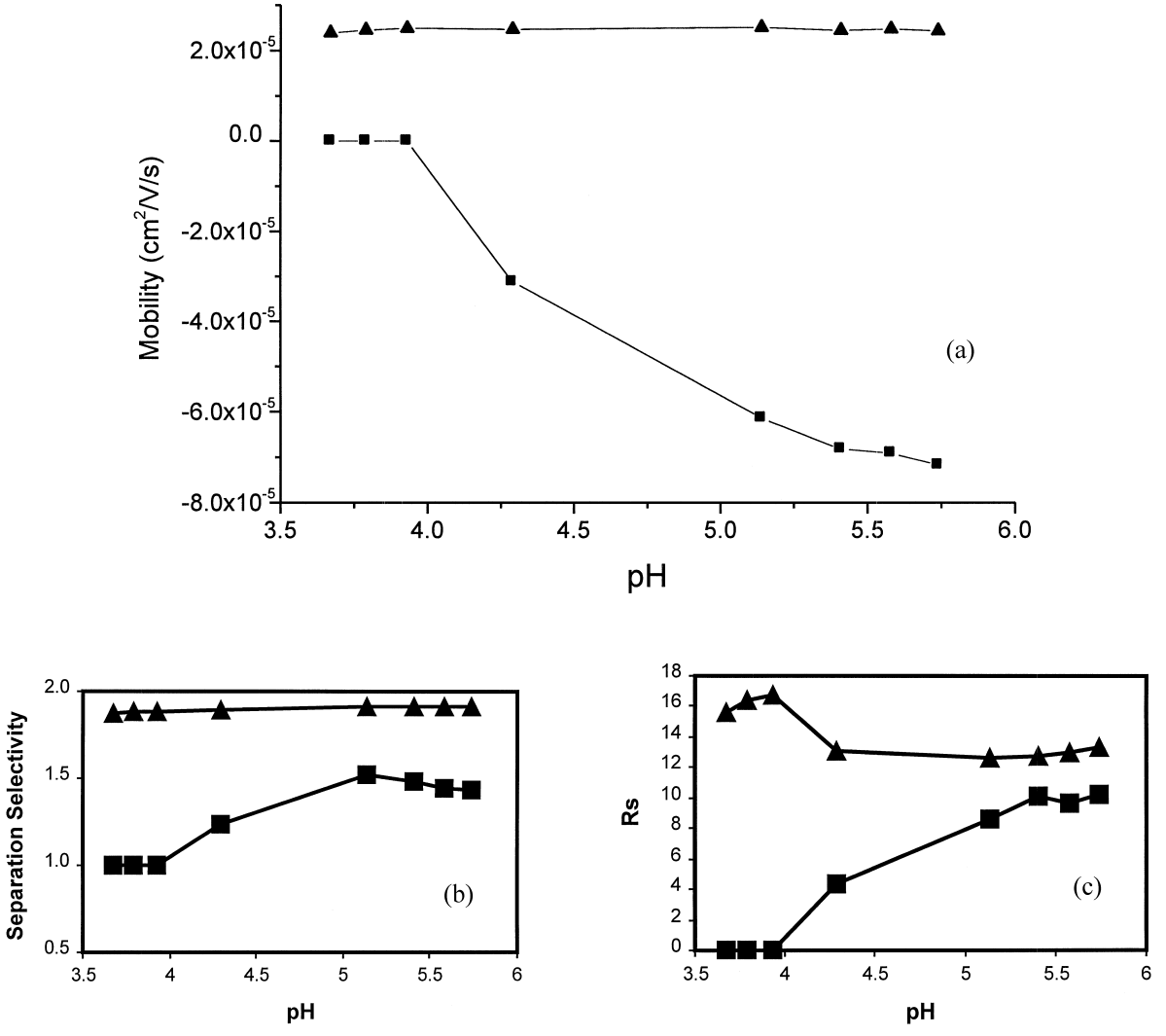

Fig. 4. Effect of pH on chiral separation. Running electrolyte: 2.5% QA-β-CD in Tris acetate. Field strength: −326 V/cm. Analytes: DHP ester (filled triangle), and DHP acid (filled square). (a) Effect of pH on electrophoretic mobility of *R* enantiomers of DHP ester and acid enantiomers. (b) Effect of pH on separation selectivity. (c) Effect of pH on resolution.

CD–acid complexes was negative. The negative mobility was caused by the deprotonation of the acid (Fig. 4a). Chiral separation of the DHP acid was achieved only when the acid was negatively charged. From pH>4.3 to 5.5, resolution increased with increasing pH. Resolution leveled off when pH≥5.5 (Fig. 4c) because above this pH the acid was fully deprotonated.

3.4. Temperature effect

Although the original conditions provided enough resolution of the enantiomers for both the ester and acid (Fig. 2), it was found that impurities generated during the bioresolution process co-migrated with the *R* enantiomer of the ester. In order to separate the *R* enantiomer from the impurities, the temperature of the capillary was changed. Fig. 5 shows the effect of capillary temperature on chiral separation of a bioresolution sample after enzyme precipitation. As the temperature of the capillary was lowered, separation selectivity increased with decreasing temperature.

3.5. Sample analysis

Another goal of our project was to be able to inject samples without time-consuming pre-treatment to remove the enzyme. In our initial work, removal of the enzyme was performed to avoid several potential problems. First, enzymes can adsorb to the capillary wall causing changes in migration times of analytes and changes in peak shape [24]. Secondly, it is possible that enzymes can interact with analytes. Thirdly, broad protein peaks may interfere with detection of analytes. In order to avoid these problems, our initial sample preparation involved removal of the enzyme by acetonitrile precipitation.

Since the enzyme is positively charged at the pH of the running buffer, it will interact strongly with a negatively charged capillary surface. If the enzyme adsorbs to the capillary surface, asymmetric peaks

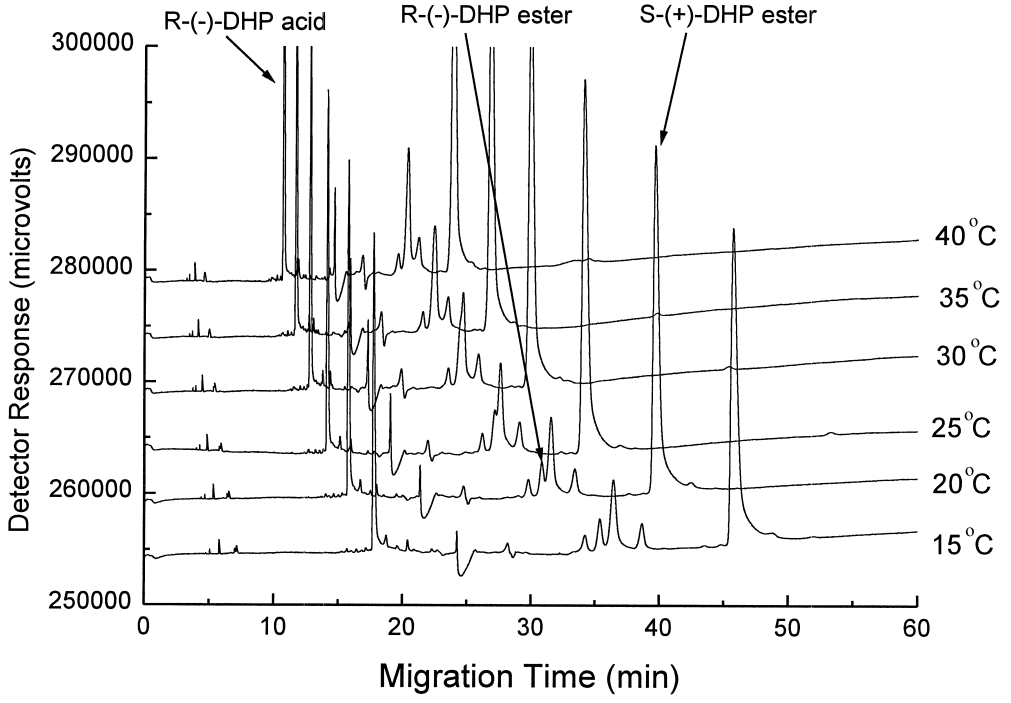

Fig. 5. Effect of temperature on separation. Running buffer: 2.5% QA-β-CD in 25 m*M* Tris acetate (pH 8.30). Field strength: −326 V/cm. Sample: bioresolution sample day 8 after enzyme precipitation. Capillary: bubble capillary.

will be observed. Coating the surface of the capillary is a common solution to this type of problem. At the pH of our running buffer, cationic CDs such as QA-β-CD will dynamically coat the wall of the capillary. This positively charged coating will reduce interaction of the positively charged enzyme with the capillary surface and reduce the need for capillary conditioning between injections and possibly allow direct injection of enzyme-containing samples.

In order to reduce the amount of sample pretreatment and to test the effectiveness of dynamic coating of the capillary wall by the cationic CD, another sample treatment was evaluated. Hydrolysis reaction samples were simply diluted using 30% methanol in water and then injected. To date, most direct injections of biological fluids reported have been in micellar electrokinetic chromatography (MEKC) systems where sodium dodecyl sulfate (SDS) can minimize protein adsorption onto the capillary wall [24–26]. Fig. 6 shows the separation of the DHP ester samples after fourteen days of hydrolysis with and without acetonitrile precipitation. Fig. 6a shows that acetonitrile effectively removed the enzyme as well as several other minor components. However, Fig. 6b illustrates that without acetonitrile treatment, the enzyme migrated as a sharp peak and did not interfere with other components of interest. Reconditioning of the capillary between injections with strong base was not necessary. Simply flushing the capillary for 3 min with water followed by 4 min with running buffer was sufficient to clean the capillary and replenish the dynamic coating of the CD on the capillary wall. This procedure was sufficient to maintain separation integrity between injections. Therefore, the direct injection method was chosen for the sample analysis.

The enzymatic hydrolysis process requires approximately 14 days to go to completion. In order to monitor the reaction progress, samples are taken once per day. Concentrations of the DHP acid and ester that were determined by CE compared well with results obtained by reversed-phase HPLC. The enantiomeric excess of the DHP ester determined by CE compared well with results obtained by chiral SFC and normal-phase HPLC. The enantiomeric excess of the DHP acid was also determined by this CE method. Only a trace amount of the *S* form of the acid was detected indicating that the enzyme was highly selective for the *R* enantiomer of the ester.

3.6. Method validation

The method was validated by evaluating several characteristics of the system including linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, precision and ruggedness. In order to enhance the method sensitivity, a capillary with a ''bubble'' detection cell was used in the validation and sample analysis. During the enzymatic hydrolysis process, the concentration of DHP ester in the aqueous phase was typically about 3–4 mg/ml. Therefore, standard solutions ranging from 0.004 mg/ml to 4 mg/ml, which are equal to 0.1% to 100% of the sample concentration, were injected in duplicate to test the linearity. At 210 nm, the r^2 value was 0.998. The LOD ($S/N=3$) and LOQ ($S/N=10$) for DHP ester were 0.0024 and 0.0076 mg/ml, respectively. For method accuracy, a 0.951 mg/ml standard solution of the *S* form of DHP ester was spiked with 0.01 mg/ml of the *R* enantiomer of the DHP ester. The spiked solution was injected five times. The average recovery of the *R* enantiomer was 110%. The relative standard deviation (RSD) values ($n=5$) were 2.6%, and 1.4% for the *R* and *S* forms of DHP enantiomers, respectively. The ruggedness was evaluated using the same spiked solution. Two different capillaries and two different instruments were used over a 20-day period. The RSD ($n=4$) for the *R* isomer concentration was 6.7%. Based on the day-to-day results, the RSDs for migration times of two enantiomers were $\leq$3.8%.

4. Conclusion

CE with QA-β-CD as a chiral buffer additive has been used to separate the enantiomers of DHP acid and ester in a single run. This method provides quantification of all four enantiomers with good sensitivity, accuracy and ruggedness. Sample preparation time is also significantly reduced. Results compare well with those obtained with reversed-phase HPLC, chiral SFC and normal-phase HPLC.

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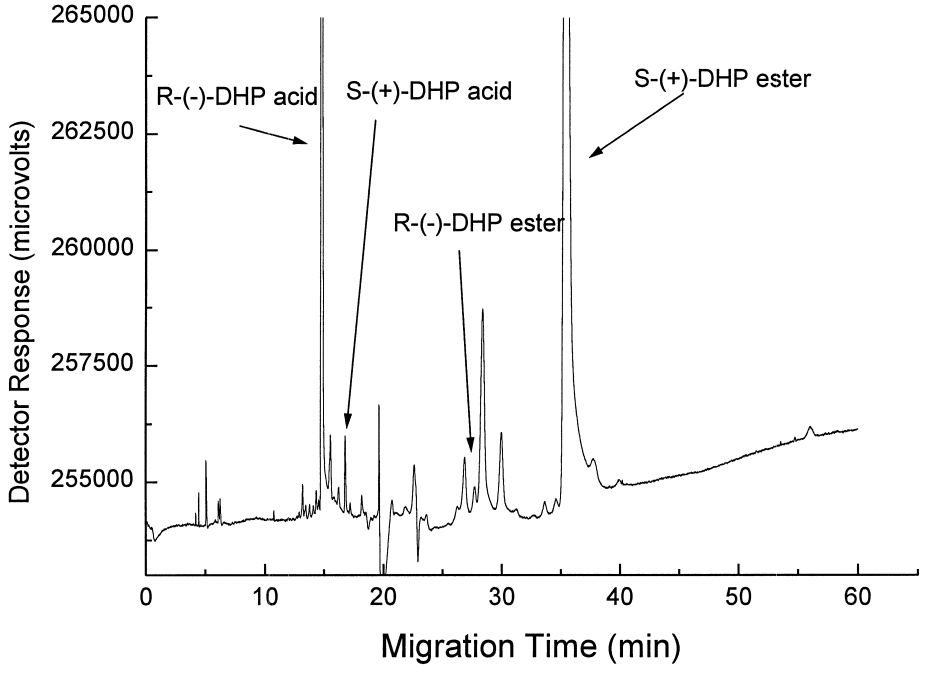
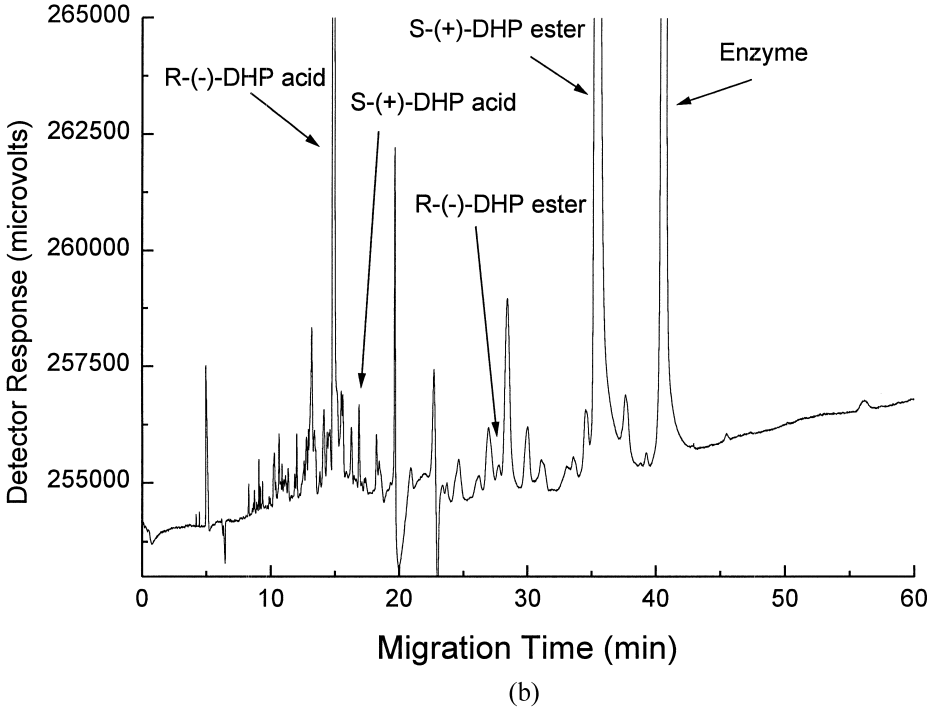


(a)



(b)

Fig. 6. Sample analysis. Running buffer: 2.5% QA-β-CD in 12.5 m*M* Tris acetate (pH 8.30) at 15°C. Field strength: −326 V/cm. Samples: (a) day 14 sample after enzyme precipitation, (b) day 14 sample after 1:10 dilution.

DHP methyl ester and acid racemates and Cerestar USA, Inc. for the charged CDs.

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