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Enantiomeric separation of a tetrapeptide with cyclodextrin Extension of the model for chiral capillary electrophoresis by complex formation of one enantiomer molecule with more than one chiral selector molecules

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Abstract

In this paper the enantiomeric separation by capillary electrophoresis (CE) of the tetrapeptide LEF553 (Tyr–D–Arg–Phe–Phe–HN_2) and its diastereoisomers is presented. The run buffer consisted of 10 mmol l^{-1} heptakis(2,6-di-O-methyl)-β-cyclodextrin in 0.1 mol l^{-1} phosphoric acid adjusted to pH 3.0 with triethanolamine. Large differences were found between the different steric conformations of the peptide. The relationships between the apparent mobility differences of the enantiomers versus the cyclodextrin concentration were investigated. For some of the conformations of the peptide, the curve forms that were obtained could not be explained by Wren's model, which assumes a 1:1 interaction between enantiomer and chiral selector. A possible explanation could be that each peptide molecule could interact with two or three cyclodextrin molecules. An extended model in which the enantiomer complexes with more than one chiral selector molecule is presented. Using this extended model, curves for the mobility difference versus the chiral selector concentration can be obtained that have the same shape as the experimental curves. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Chiral selectors, model; Peptides

1. Introduction

The μ-opioid receptor selective compound LEF553, Tyr–D–Arg–Phe–Phe–NH_2, is a tetrapeptide that contains four chiral centres and thus has sixteen stereoisomers (Fig. 1). As LEF553 has the LDLL conformation, its enantiomer has the DLDD conformation. The other fourteen conformations are diastereoisomers of LEF553 which can be separated from LEF553 by non-chiral separation techniques.

Capillary electrophoresis (CE) methods have been

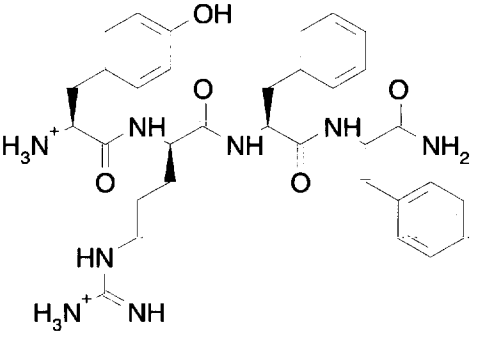

Fig. 1. LEF553, Tyr–D–Arg–Phe–Phe–NH_2.

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successfully established for chiral separations, many reviews have been published [1–11] and several chiral methods have been validated [12–26]. In addition, some papers have dealt with the enantiomeric separation of derivatised peptides [27,28]. Recently, Schmid and Gübitz [29] and Kuhn and coworkers [30,31] presented the enantiomeric separation of underivatised di- and tripeptides, mainly glycyl-di- and tripeptides and some leucyl-dipeptides, containing 1 or 2 chiral centres. They used a chiral crown ether, (+)-18-crown-6-tetracarboxylic acid, as chiral selector. Crown ethers are known to form a complex with an $-NH_3^+$ group [30]. To date no reports on the enantiomeric separations of underivatised tetrapeptides have appeared in literature.

Since LEF553 contains several aromatic groups, cyclodextrins (CDs) might be successful as chiral selectors. The advantages of CDs are that they are easily available, relatively inexpensive and well-known in our laboratory. In this paper we present the enantiomeric separation of the tetrapeptide LEF553 and its diastereoisomers with CE using heptakis(2,6-di-O-methyl)-β-cyclodextrin as the chiral selector.

2. Experimental

2.1. Chemicals

Sodium hydroxide, phosphoric acid and triethanolamine were obtained from Merck (Darmstadt, Germany). Heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) was obtained from Sigma (St. Louis, MO, USA). The tetrapeptides were synthesised by BioChem Immunosystems (Montreal, Canada).

2.2. Equipment

The experiments were performed on an HP^{3D}CE instrument (Hewlett-Packard, Waldbronn, Germany), comprising a diode array detector and ChemStation software for data handling. The capillary (Hewlett-Packard) was 64.5 cm long (56.0 cm effective length)×50 μm I.D.. The applied voltage was 25 kV, with an initial ramping of 500 V s^{-1}. The temperature was 30°C. Injection was performed at 50 mbar over 5 s giving ca. 6 nl injection volume. All injections were performed in duplicate. The UV detection was at 200 nm with a band width of 4 nm. Preconditioning of the capillary was included for each run and comprised 1 min flush with water, 4 min flush with 0.1 mol l^{-1} of NaOH (sodium hydroxide solution for high-performance CE, Fluka, Buchs, Switzerland), 1 min flush with water and 4 min flush with run buffer. New capillaries were first flushed for 1 min with water and then for 30 min with 0.1 mol l^{-1} NaOH. Flushing is performed at a pressure of 1 bar.

The background electrolyte solution (BGE) was made by adjusting a solution of 0.1 mol l^{-1} of phosphoric acid to pH 3.0 with triethanolamine. The run buffer contained various amounts of DM-β-CD in BGE, resulting in concentrations of 0–100 mmol l^{-1} DM-β-CD.

All solutions were freshly prepared using Milli-Q purified water and filtered through PTFE filters, 0.45 μm pore size (Micron Separations, Westboro, USA).

2.3. Resolution

The resolution was calculated according to Eq. (1):

$$R_s = 1.18\frac{t_2 - t_1}{w_{1/2,1} + w_{1/2,2}} \quad (1)$$

where t_1 and t_2 are migration times and $w_{1/2,1}$ and $w_{1/2,2}$ the peak widths at half the peak height of the enantiomers.

2.4. Apparent mobilities

The apparent mobilities were calculated according to Eq. (2):

$$\mu = \frac{lL}{tV} \cdot \frac{I_0}{I_C} - \mu_{eo} \quad (2)$$

where l is the capillary length to the detector cell, L the total capillary length, V the applied voltage, t the migration time of the enantiomer, I_0 the current measured at 0 mmol l^{-1} DM-β-CD and I_C the current at the actual DM-β-CD concentration. The factor I_0/I_C was introduced to correct for the increasing viscosities at increasing DM-β-CD concentrations [32,33]. μ_{eo} is the electroosmotic flow (EOF)

mobility in the buffer system without CD added, i.e., 0 mmol l^{-1} DM-β-CD. Since μ_{eo} is negative in this BGE, it was determined by applying a voltage of −30 kV and injecting ethanol.

3. Results and discussion

LEF553 is a tetrapeptide that contains four chiral centres (Fig. 1). Consequently there are sixteen stereoisomers that can be sorted as eight enantiomer pairs. LEF553 has the LDLL configuration, so its enantiomer has the DLDD conformation. The other seven enantiomer pairs are diastereoisomers. Since the peptide has several aromatic groups which could possibly interact with a CD cavity [34], DM-β-CD was initially tested at a concentration of 10 mmol l^{-1} in a background electrolyte system of 0.1 mol l^{-1} phosphoric acid that was adjusted to pH 3.0 with triethanolamine. This buffer system, that was introduced by Bechet et al. [35], has been used for the chiral separation of local anaesthetics such as ropivacaine, bupivacaine and prilocaine and is known to be very robust [24,26,32].

LEF553 was easily separated from its enantiomer and had a resolution as high as 7.5 (Fig. 2A). The other enantiomer pairs injected in the same buffer system (Fig. 2) exhibited large differences in resolution. LEF553 and its enantiomer DLDD showed the highest resolution, while the DLDL–LDLD pair was not resolved at all. These differences support the fact that it is hard to predict from the molecular structure which chiral selector will be successful for an enantiomeric separation. It is not sufficient that the enantiomers complex with the chiral selector as they must also have a different affinity for the selector to achieve enantiomeric separation [36,37]. It is common practice therefore to test several different chiral selectors before selecting the best one for the enantiomer pair of interest. Since CE instruments are highly automated today, valuable information on many different chiral selectors can be obtained fairly rapidly.

At pH 3.0 the peptide is positively charged at both the arginine residue and the N-terminus and has a positive electrophoretic mobility. A complex of the peptide with an uncharged CD has a lower charge-to-mass ratio and will migrate slower than the free

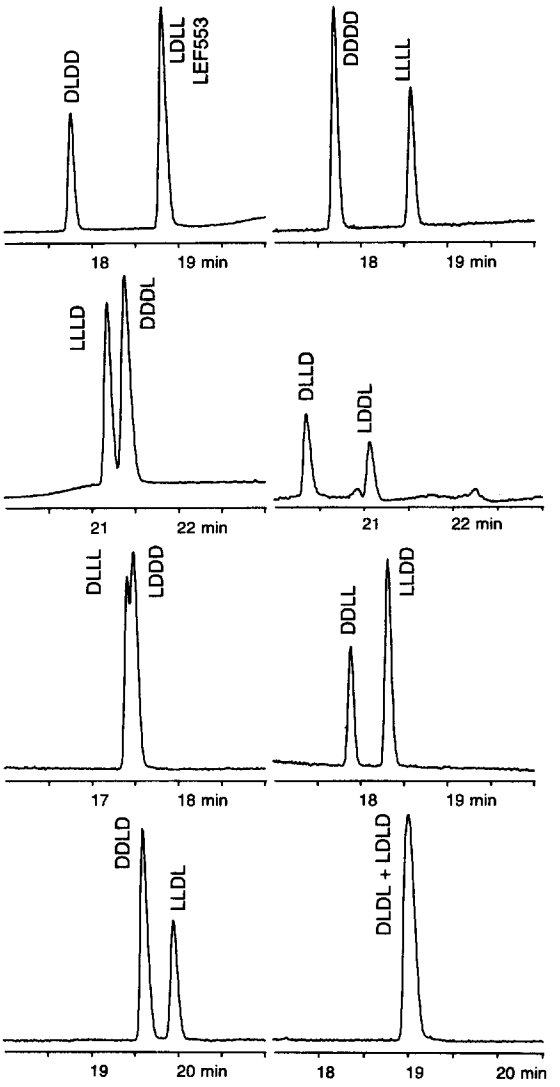

Fig. 2. Electropherograms of the enantiomeric separation of LEF553 and its enantiomer and diastereoisomers. Run buffer: 10 mmol/l DM-β-CD in 0.1 mol/l phosphoric acid, adjusted to pH 3.0 with triethanolamine. Other conditions: see Section 2.

peptide. An analyte with a high affinity for the CD spends more time as a complex and will thus migrate more slowly than a analyte with a low affinity. Peptides with a –DD or –LL conformation at the C-terminus migrated faster than those with a –LD or –DL conformation (Fig. 2). This indicates that the latter group of peptides formed stronger complexes with the CD leading to lower net migration velocities although they did not show better resolutions. Thus

different conformations of the tetrapeptide have different equilibrium constants and consequently different optimum separation conditions.

To investigate the chiral mechanism and to find better conditions for the poorly separated enantiomer pairs, the mobility differences over a concentration range of 0–100 mmol l^{-1} DM-β-CD (Fig. 3) were studied. For each enantiomer pair duplicate determinations were done and freshly prepared buffers were used. No baseline separation was observed for the DLDL–LDLD pair in this concentration range. For the DDLL–LLDD pair and the LLLL–DDDD pair, plots that could be fitted by Wren's model for enantiomeric separations in CE [36–38] were observed. However, for the other enantiomer pairs curve shapes were obtained that could not be explained by Wren's model. Instead of decreasing mobility differences at higher DM-β-CD concentrations, the mobility difference increases after a local minimum.

Wren's model assumes the interaction of a freely soluble analyte with a single chiral selector:

$$R + C \overset{K_R}{\rightleftharpoons} RC \qquad S + C \overset{K_S}{\rightleftharpoons} SC \tag{3}$$

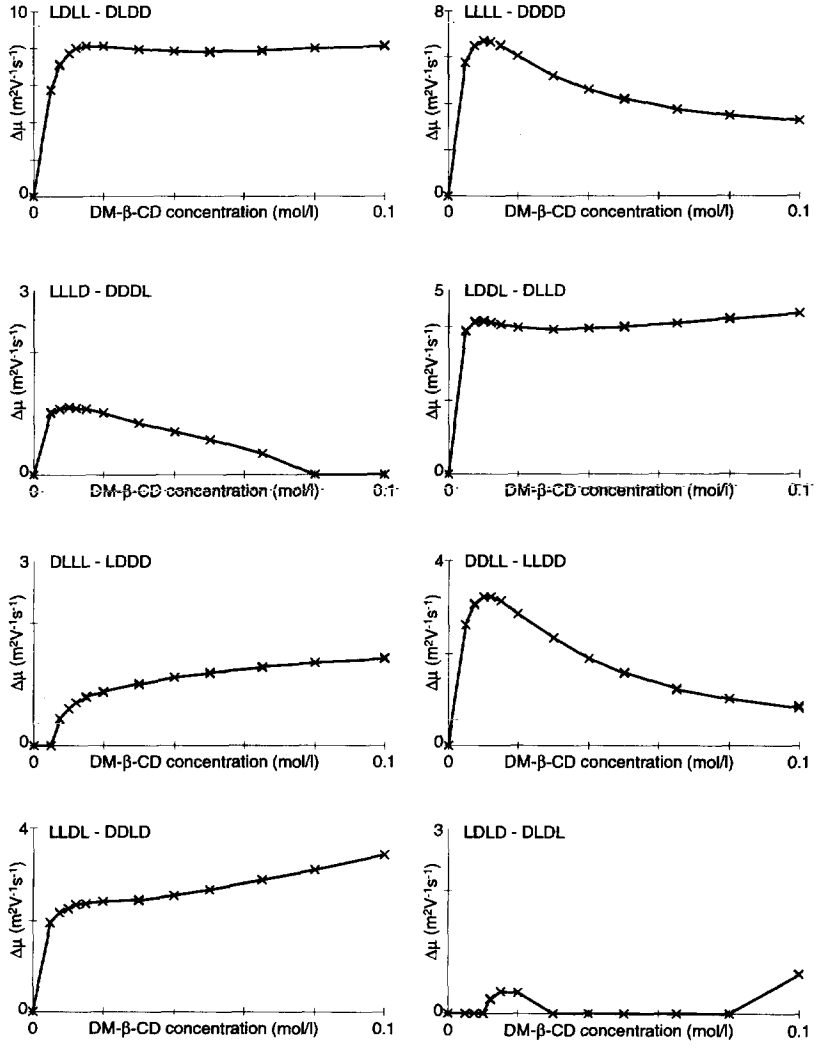

Fig. 3. Electrophoretic mobility differences versus DM-β-CD concentration of the eight enantiomer pairs of Tyr–Arg–Phe–Phe–NH_2. Conditions: see Section 2.

where R and S are a pair of enantiomers and C is the chiral selector. K_R and K_S are the association equilibrium constants. The equilibria between the enantiomers and the chiral selector are assumed to be reached rapidly. The electrophoretic mobilities of R and S in free solution are equal and defined as μ_0. The inclusion complexes RC and SC have the electrophoretic mobilities $\mu_{R,1}$ and $\mu_{S,1}$, respectively. The mobility difference between enantiomers R and S thus becomes [38]:

$$\Delta\mu_{ep} = \frac{\{K_R(\mu_{R,1}-\mu_0) - K_S(\mu_{S,1}-\mu_0) + K_R K_S(\mu_{R,1}-\mu_{S,1})[C]\}[C]}{(1+K_R[C])(1+K_S[C])} \quad (4)$$

When the chiral selector concentration is zero, there is no enantiomeric separation. When the selector concentration becomes very large, the mobility difference converges to $\mu_{R,1}-\mu_{S,1}$. If the electrophoretic mobilities of the complexes are equal, i.e., $\mu_{R,1}=\mu_{S,1}=\mu_1$, then Eq. (4) simplifies to:

$$\Delta\mu_{ep} = \mu_R - \mu_S = \frac{(\mu_0-\mu_1)(K_S-K_R)[C]}{1+[C](K_R+K_S)+K_R K_S[C]^2} \quad (5)$$

Curve fittings of the data obtained for the tetrapeptide enantiomers to Wren's model were unsuccessful (data not shown). Using the equilibrium constants and limiting mobilities obtained by the curve fitting procedures, the experimentally obtained plots could not be reconstructed, although this has been shown to be possible for the local anaesthetics [32]. It is known that DM-β-CD is not pure but consists of a group of β-CDs with different degrees of methylation [24,39–41]. If these different CDs compete for the same complex formation site on the enantiomer, then the curve form of the mobility difference ($\Delta\mu_{ep}$) versus the (total) CD concentration [C] will be similar to the curve form described by Wren's model. Therefore the experimentally obtained plots could not be explained by heterogeneity of the chiral selector.

A possibility is that one peptide molecule may complex with more than one CD molecule. The peptide contains three aromatic groups and it is not unlikely that in certain conformations it can form complexes with two or three DM-β-CD molecules.

3.1. Extended model

Supposing an analyte has two complex formation sites with a chiral selector, then three different complexes could be formed, two when the analyte is complexed with one chiral selector and a third when it is complexed with two chiral selectors. Analogously, with three complex formation sites seven different complexes could be formed.

Two complexation sites would lead to the following equilibria:

$$R + C \overset{K_{R,1}}{\rightleftharpoons} RC_1 \qquad S + C \overset{K_{S,1}}{\rightleftharpoons} SC_1 \quad (6)$$

$$R + C \overset{K_{R,2}}{\rightleftharpoons} RC_2 \qquad S + C \overset{K_{S,2}}{\rightleftharpoons} SC_2 \quad (7)$$

$$R + 2C \overset{K_{R,1-2}}{\rightleftharpoons} RC_{1-2} \qquad S + 2C \overset{K_{S,1-2}}{\rightleftharpoons} SC_{1-2} \quad (8)$$

RC_1 is the first complex of enantiomer R with equilibrium constant $K_{R,1}$, RC_2 is the second complex of enantiomer R with equilibrium constant $K_{R,2}$, RC_{1-2} is the complex of enantiomer R with two chiral selectors with equilibrium constant $K_{R,1-2}$. The annotation for the S enantiomer is the same.

The apparent mobility of enantiomer R reflects the time that the enantiomer is complexed (as RC_1, RC_2, or RC_{1-2}) or is free:

$$\mu_R = \frac{[R]}{\sum R}\cdot\mu_0 + \frac{[RC_1]}{\sum R}\cdot\mu_{R,1} + \frac{[RC_2]}{\sum R}\cdot\mu_{R,2} + \frac{[RC_{1-2}]}{\sum R}\cdot\mu_{R1-2} = \frac{[R]\mu_0 + K_{R,1}[R][C]\mu_{R,1} + K_{R,2}[R][C]\mu_{R,2} + K_{R1-2}[R][C]^2\mu_{R1-2}}{\sum R} \quad (9)$$

$$\sum R = [R] + [RC_1] + [RC_2] + [RC_{1-2}] \quad (10)$$

μ_0 is the mobility of the free enantiomer, $\mu_{R,1}$ is the mobility of the RC_1 complex, $\mu_{R,2}$ is the mobility of the RC_2 complex and $\mu_{R,1-2}$ is the mobility of the RC_{1-2} complex. Rearrangement of the terms results in the apparent mobility of R as:

$$\mu_R = \frac{\mu_0 + (K_{R,1}\mu_{R,1} + K_{R,2}\mu_{R,2})[C] + K_{R,1-2}\mu_{R,1-2}[C]^2}{1 + (K_{R,1}+K_{R,2})[C] + K_{R,1-2}[C]^2} \quad (11)$$

The annotation for the S enantiomer is the same. The mobility difference, $\mu_R - \mu_S$, is a complicated equation (see Appendix A) that has $[C]^4$ terms in both the numerator and the denominator.

Analogously, the model can be extended for three (Appendix B) or more complex formation sites.

This extended model for chiral separation in CE results in more diverse and complex forms of plots of the mobility difference versus chiral selector concentration. If the equilibrium constant for the complex of the analyte with two chiral selectors, $K_{R,1-2}$, is zero, the plot for the mobility difference will have the same form as described by Wren's theory. However, when the equilibrium constant for the double complex is greater than zero, the curves can differ substantially. Fig. 4 shows some calculated plots of mobility difference versus chiral selector concentration according to our extended model. The mobility for the free peptide was determined experimentally at zero DM-β-CD concentration. The mobilities for the complexes were estimated from the free mobility by multiplying by the molecular mass of the free peptide and dividing by the mass of the complex. The values for the equilibrium constants for the single complexes were in the order of magnitude as observed for other chiral separations under similar conditions. If the equilibrium constants for the double complexes were zero (Fig. 4a) or at a maximum (Fig. 4b), the curve for the mobility difference had the same form as described by Wren. However, with other values for the double complexes the curves differed (e.g., Fig. 4c,d), and curves similar to those obtained experimentally for the tetrapeptide could be reconstructed. Also when three complexation sites for the chiral selector were taken into account, curves similar to the experimental plots could be obtained (Fig. 5, Appendix B).

The ability to reconstruct curves similar to the experimental curves does not prove that the tetrapeptide forms complexes with more than one DM-β-CD molecule, but demonstrates that complex forma-

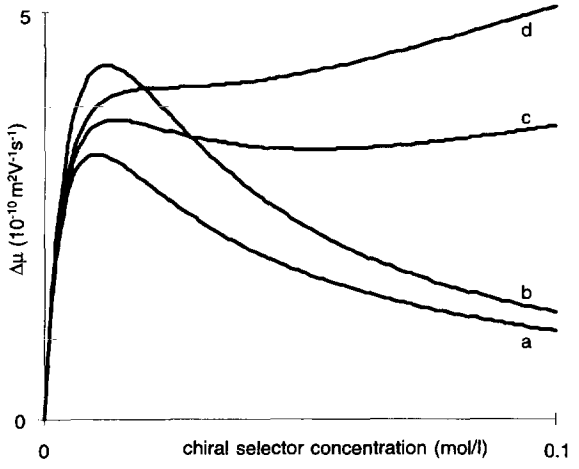

Fig. 4. Calculated mobility difference versus chiral selector concentration according to the model presented in this paper where the analyte has two complex formation sites. $\mu_0 = 2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1} = \mu_{R,2} = \mu_{S,1} = \mu_{S,2} = 0.7 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1-2} = \mu_{S,1-2} = 0.4 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $K_{R,1} = 45$ l mol^{-1}, $K_{R,2} = 50$ l mol^{-1}, $K_{S,1} = 50$ l mol^{-1}, $K_{S,2} = 55$ l mol^{-1}, (a) $K_{R,1-2} = 0$ l^2 mol^{-2}, $K_{S,1-2} = 0$ l^2 mol^{-2}; (b) $K_{R,1-2} = 2250$ l^2 mol^{-2}, $K_{S,1-2} = 2750$ l^2 mol^{-2}; (c) $K_{R,1-2} = 20$ l^2 mol^{-2}, $K_{S,1-2} = 100$ l^2 mol^{-2}; (d) $K_{R,1-2} = 20$ l^2 mol^{-2}, $K_{S,1-2} = 150$ l^2 mol^{-2}.

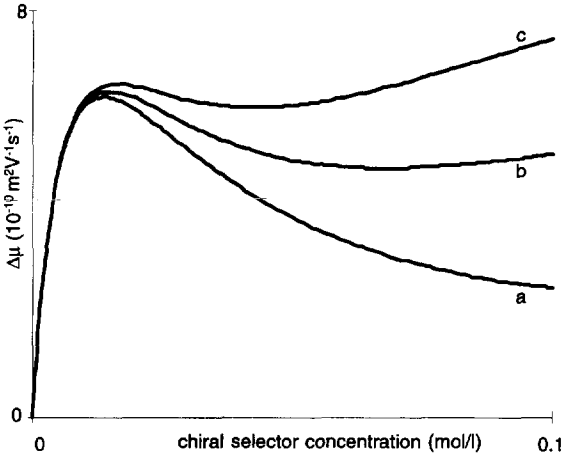

Fig. 5. Calculated mobility difference versus chiral selector concentration according to the model where the analyte has three complex formation sites (see Appendix B). $\mu_0 = 2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1} = \mu_{R,2} = \mu_{R,3} = \mu_{S,1} = \mu_{S,2} = \mu_{S,3} = 0.7 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1-2} = \mu_{S,1-2} = \mu_{R,1-3} = \mu_{S,1-3} = \mu_{R,2-3} = \mu_{S,2-3} = 0.4 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1-2-3} = \mu_{S,1-2-3} = 0.3 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $K_{R,1} = 15$ l mol^{-1}, $K_{R,2} = 20$ l mol^{-1}, $K_{R,3} = 25$ l mol^{-1}, $K_{S,1} = 20$ l mol^{-1}, $K_{S,2} = 25$ l mol^{-1}, $K_{S,3} = 30$ l mol^{-1}, $K_{R,1-2} = 80$ l^2 mol^{-2}, $K_{R,1-3} = 90$ l^2 mol^{-2}, $K_{R,2-3} = 100$ l^2 mol^{-2}, $K_{S,1-2} = 90$ l^2 mol^{-2}, $K_{S,1-3} = 100$ l^2 mol^{-2}, $K_{S,2-3} = 110$ $l^2 mol^{-2}$, (a) $K_{R,1-2-3} = 500$ l^3 mol^{-3}, $K_{S,1-2-3} = 1000$ l^3 mol^{-3}; (b) $K_{R,1-2-3} = 500$ l^3 mol^{-3}, $K_{S,1-2-3} = 2000$ l^3 mol^{-3}; (c) $K_{R,1-2-3} = 500$ l^3 mol^{-3}, $K_{S,1-2-3} = 3000$ l^3 mol^{-3}.

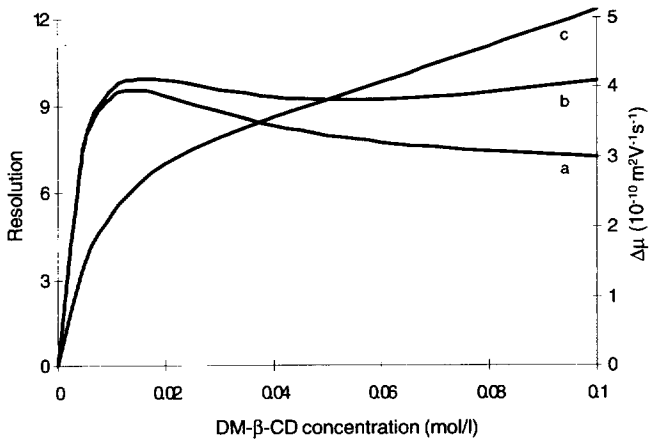

Fig. 6. Calculated mobility difference and resolution calculated from the extended model presented in this paper. $\mu_0 = 2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1} = \mu_{R,2} = \mu_{S,1} = \mu_{S,2} = 0.7 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $\mu_{R,1-2} = \mu_{S,1-2} = 0.4 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}, $K_{R,1} = 45$ l mol^{-1}, $K_{R,2} = 50$ l mol^{-1}, $K_{S,1} = 50$ l mol^{-1}, $K_{S,2} = 55$ l mol^{-1}, $K_{R,1-2} = 20$ l^{2}mol^{-2}, $K_{S,1-2} = 100$ l^2 mol^{-2}, $N = 3 \cdot 10^5$. The viscosity correction factor increases linearly from 1.00 at 0 mol l^{-1} DM-β-CD to 1.37 at 0.1 mol l^{-1} DM-β-CD. (a) $\Delta\mu$, not corrected for changes in viscosity; (b) $\Delta\mu$, corrected for changes in viscosity; (c) Resolution.

tion of one tetrapeptide molecule with two or three CD molecules is a possible explanation for the experimentally obtained data.

Finally, it is important to stress that the mobilities in the model and in the figures shown are corrected for increasing viscosities at increasing DM-β-CD concentrations. The viscosity not only affects the mobility difference between the enantiomers, but also the electrophoretic and electroosmotic mobility and therefore, the resolution [37,42]:

$$R_S = \frac{\sqrt{N}}{4} \cdot \frac{\Delta\mu}{\overline{\mu_{ep} + \mu_{eo}}} \tag{12}$$

This is illustrated by a reconstruction for the resolution (Fig. 6). First, the electrophoretic mobilities were calculated according to Eq. (11) and Fig. 4c and then the electrophoretic and electroosmotic mobilities were corrected for the viscosity increase at increasing DM-β-CD concentrations (see Section 2). The resolutions were calculated from Eq. (12) with $N = 3 \cdot 10^5$, which was the plate number observed for LEF553. Fig. 6 shows that although the mobility difference can decrease at higher DM-β-CD concentrations, the resolution might still increase due to the higher viscosities.

4. Conclusions

The enantiomeric separations with DM-β-CD of a tetrapeptide, LEF553, and the diastereoisomers of the tetrapeptide were presented. The differences in resolution for the different enantiomer pairs demonstrated that it is difficult to predict the results for a certain chiral selector using only the molecular structure of the analyte. The curve forms of the plots of the mobility difference versus chiral selector concentration could not be explained by Wren's model for a 1:1 interaction between analyte and chiral selector. A possible explanation is that the tetrapeptide molecule, which contains three aromatic groups, complexes with two or three DM-β-CD molecules. An extension of Wren's model in which the interaction of an analyte with more than one chiral selector molecule is taken into account is presented. This model could explain the curve forms of the mobility difference versus the chiral selector concentration plots that were experimentally obtained for the eight enantiomer pairs of the tetrapeptide.

These experiments show that in order to understand the chiral mechanism, find the optimum sepa-

ration conditions and obtain robust methods it is important to experimentally determine the relationship between the electrophoretic mobility difference and the chiral selector concentration.

Acknowledgments

We are grateful to Ulla Gasslander and Ingrid Granelli for stimulating discussions.

Appendix A

The mobility difference between two enantiomers when one enantiomer molecule complexes with two chiral selector molecules

$$\Delta\mu = \mu_R - \mu_S$$

$$= \frac{\{(K_{S,1}+K_{S,2}-K_{R,1}-K_{R,2})\mu_0+K_{R,1}\mu_{R,1}+K_{R,2}\mu_{R,2}-K_{S,1}\mu_{S,1}-K_{S,2}\mu_{S,2}\}[C]}{1+(K_{R,1}+K_{R,2}+K_{S,1}+K_{S,2})[C]+\{K_{R,1-2}+K_{S,1-2}+(K_{R,1}+K_{R,2})(K_{S,1}+K_{S,2})\}[C]^2+\{K_{R,1-2}(K_{S,1}+K_{S,2})+K_{S,1-2}(K_{R,1}+K_{R,2})\}[C]^3+K_{R,1-2}K_{S,1-2}[C]^4}$$

$$+\frac{\{(K_{S,1-2}-K_{R,1-2})\mu_0+K_{R,1-2}\mu_{R,1-2}-K_{S,1-2}\mu_{S,1-2}+(K_{R,1}\mu_{R,1}+K_{R,2}\mu_{R,2})(K_{S,1}+K_{S,2})-(K_{S,1}\mu_{S,1}+K_{S,2}\mu_{S,2})(K_{R,1}+K_{R,2})\}[C]^2}{1+(K_{R,1}+K_{R,2}+K_{S,1}+K_{S,2})[C]+\{K_{R,1-2}+K_{S,1-2}+(K_{R,1}+K_{R,2})(K_{S,1}+K_{S,2})\}[C]^2+\{K_{R,1-2}(K_{S,1}+K_{S,2})+K_{S,1-2}(K_{R,1}+K_{R,2})\}[C]^3+K_{R,1-2}K_{S,1-2}[C]^4}$$

$$+\frac{\{K_{R,1-2}(K_{S,1}(\mu_{R,1-2}-\mu_{S,1})+K_{S,2}(\mu_{R,1-2}-\mu_{S,2}))-K_{S,1-2}(K_{R,1}(\mu_{S,1-2}-\mu_{R,1})+K_{R,2}(\mu_{S,1-2}-\mu_{R,2}))\}[C]^3}{1+(K_{R,1}+K_{R,2}+K_{S,1}+K_{S,2})[C]+\{K_{R,1-2}+K_{S,1-2}+(K_{R,1}+K_{R,2})(K_{S,1}+K_{S,2})\}[C]^2+\{K_{R,1-2}(K_{S,1}+K_{S,2})+K_{S,1-2}(K_{R,1}+K_{R,2})\}[C]^3+K_{R,1-2}K_{S,1-2}[C]^4}$$

$$+\frac{(\mu_{R,1-2}-\mu_{S,1-2})K_{R,1-2}K_{S,1-2}[C]^4}{1+(K_{R,1}+K_{R,2}+K_{S,1}+K_{S,2})[C]+\{K_{R,1-2}+K_{S,1-2}+(K_{R,1}+K_{R,2})(K_{S,1}+K_{S,2})\}[C]^2+\{K_{R,1-2}(K_{S,1}+K_{S,2})+K_{S,1-2}(K_{R,1}+K_{R,2})\}[C]^3+K_{R,1-2}K_{S,1-2}[C]^4}$$

Appendix B

Complex formation of one enantiomer molecule with three chiral selector molecules

$$\mu_R = \frac{\mu_0+(K_{R,1}\mu_{R,1}+K_{R,2}\mu_{R,2}+K_{R,3}\mu_{R,3})[C]+(K_{R,1-2}\mu_{R,1-2}+K_{R,1-3}\mu_{R,1-3}+K_{R,2-3}\mu_{R,2-3})[C]^2+K_{R,1-2-3}\mu_{R,1-2-3}[C]^3}{1+(K_{R,1}+K_{R,2}+K_{R,3})[C]+(K_{R,1-2}+K_{R,1-3}+K_{R,2-3})[C]^2+K_{R,1-2-3}[C]^3}$$

μ_R is the apparent mobility of enantiomer R. [C] is the chiral selector concentration. The free enantiomer has mobility μ_0. The complexes of one enantiomer with one chiral selector have equilibrium constants $K_{R,1}$, $K_{R,2}$ and $K_{R,3}$ and mobilities $\mu_{R,1}$, $\mu_{R,2}$ and $\mu_{R,3}$, respectively. The complexes of one enantiomer with two chiral selectors have equilibrium constants $K_{R,1-2}$, $K_{R,1-3}$ and $K_{R,2-3}$ and mobilities $\mu_{R,1-2}$, $\mu_{R,1-3}$ and $\mu_{R,2-3}$. The complex of one enantiomer with three chiral selectors has equilibrium constants $K_{R,1-2-3}$ and mobility $\mu_{R,1-2-3}$. The annotation for the S enantiomer is the same.

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