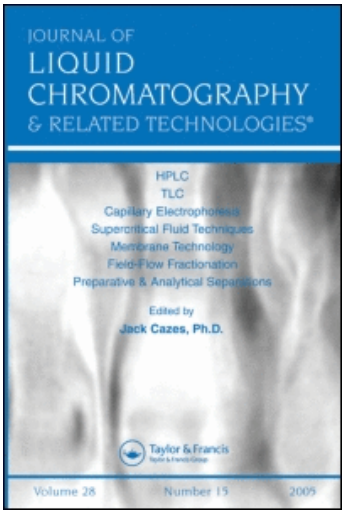

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Chiral Separation of Fluoxetine and Its Analogs with Charged Cyclodextrins by Capillary Electrophoresis

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ABSTRACT

Chiral separation of fluoxetine was performed by capillary electrophoresis (CE) using 11 different cyclodextrins (CDs) including neutral and charged derivatives as chiral selectors. The separation conditions were optimized in terms of the type of the CDs and their concentration, as well as pH of the background electrolyte. The optimized conditions offered usefulness in determination of a trace amount of chiral impurity with a limit of quantitation (LOD) of 0.2% and a limit of detection (LOD) of less than 0.1%. Four fluoxetine analogs that have different substituents on the amine moiety were also analyzed by CE to study the structural effects on chiral recognition. Three of them that have simple alkyl substituents showed similar separation behavior to that of fluoxetine, while addition of a carboxyl group on the amine moiety caused a significant change in chiral

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separation. The charge on both the analytes and the CDs was found to be one of the important factors, which affected chiral separation.

Key Words: Chiral separation; Capillary electrophoresis; Cyclodextrins; Fluoxetine.

INTRODUCTION

The chirality of biologically active compounds has been of great interest, especially in the pharmaceutical industry. Because drug enantiomers may be readily discriminated by biological systems, they may have very different toxicological, pharmacokinetic, pharmacodynamic, and metabolic profiles. This fact led the Food and Drug Administration (FDA) to establish guidelines that recommend the production of chiral drugs as single enantiomers.[1] Therefore, development of suitable analytical methods to separate enantiomers is important in the isolation of single isomers, as well as the control of enantiomeric purity of drugs.

The compounds of interest in this study are fluoxetine (*N*-methyl-γ-[4-(trifluoromethyl)-phenoxy]benzenepropanamine) and some of its analogs. Fluoxetine is known as a selective inhibitor of serotonin reuptake[2] and is used in treating a variety of major psychiatric and metabolic derangements, including depression,[3,4] eating disorders such as bulimia nervosa,[5] and obsessive-compulsive disorder.[6] Fluoxetine has two enantiomers, the *R*- and *S*-form, as shown in Fig. 1. There is a small, but distinguishable stereospecificity in the seretonin reuptake inhibition with the *S*-form being slightly more potent.[7] The metabolite of fluoxetine, norfluoxetine (*N*-demethylated form of fluoxetine), also shows inhibition of serotonin reuptake, allowing a prolonged biological effect of the drug. Surprisingly, the *R*-form of norfluoxetine is about 16 times more potent than its *S*-form.[8] Despite the pharmaceutical importance of the study in chiral separation of these compounds, only several studies have been reported for chiral separation of fluoxetine with both capillary electrophoresis (CE)[9–15] and high performance liquid chromatography (HPLC),[16–18] few of which showed a baseline resolution.

The most commonly used chiral selectors in CE are cyclodextrins (CDs) and their derivatives. Many properties of CDs such as good complexation ability with various analytes, good water solubility, low UV cut-off, commercial availability, low cost, and good stability makes this class of compounds attractive over other chiral selectors.[19] More recently, charged CDs have been introduced[20,21] and found to be very useful for the chiral separation of many compounds including basic drugs.[22–28] In this study, different CDs were used to investigate the chiral

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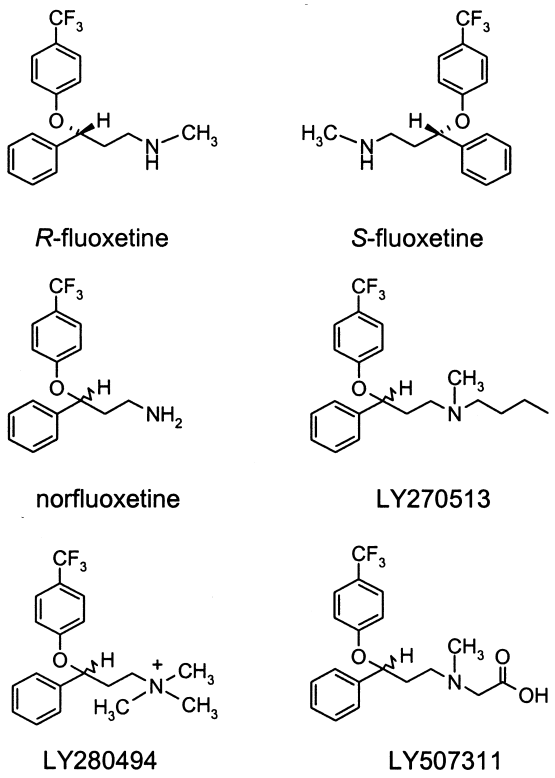

Figure 1. Fluoxetine and its analogs.

separation of fluoxetine and some of its analogs. To optimize the chiral separation condition, we varied the type of CDs and their concentration, as well as pH of the background electrolyte. To understand how chemical structures of substituents on the analyte of interest would affect the chiral recognition by CDs, four fluoxetine analogs with different substituents on the amine moiety (Fig. 1) were analyzed under various conditions. The effects of charge status of both the analytes and CDs on chiral separation are also discussed.

EXPERIMENTAL

Chemicals

β-Cyclodextrin (β-CD) and heptakis-(2,6-di-*o*-methyl)-β-CD (HDM-β-CD) were purchased from Sigma (St. Louis, MO). Heptakis-(2,3-diacetyl-6-sulfato)-

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β-CD (HDAS-β-CD), heptakis-(2,3-dimethyl-6-sulfato)-β-CD (HDMS-β-CD), and heptakis-6-sulfato-β-CD (HS-β-CD) were obtained from Regis (Morton Grove, IL). Hydroxypropyl-α-CD [HP-α-CD, degree of substitution (DS) = 3.5], hydroxypropyl-β-CD (HP-β-CD, DS = 5.3), hydroxypropyl-γ-CD (HP-γ-CD, DS = 6.4), sulfated-α-CD (S-α-CD, DS = 12), and quaternary ammonium-β-CD (QA-β-CD, DS = 3.5) were from Cerestar (Hammond, IN). γ-Cyclodextrin (γ-CD) was from Beckman Instruments (Fullerton, CA). Fluoxetine and its analogs norfluoxetine, LY270513, LY280494, LY507311 were obtained from Lilly Research Laboratories (Indianapolis, IN). All other chemicals were analytical grade and used as received.

Buffer and Sample Preparation

The buffer solutions with desired pHs were prepared by dissolving the appropriate salt and adjusting pH with acid or base. The running buffers were prepared by adding desired amounts of CDs into the buffer solutions. Samples were dissolved into Milli-Q water (0.1 mg mL^{-1}). All the solutions were filtered through 0.2 μm syringe filters (Acrodisc® CR PTFE) from German Sciences (Ann Arbor, MI) before use.

Capillary Electrophoresis

All CE experiments were carried out on a P/ACE MDQ CE instrument (Beckman Instruments, Fullerton, CA) using a 50 μm i.d. × 60 cm (50 cm effective length) fused silica capillary (Polymicro Technologies, Phoenix, AZ) at 20°C. The UV detection wavelength was 214 nm. A separation voltage of 20 kV with normal polarity was applied across the capillary. The newly prepared capillary was treated for 10 min with 0.1 M NaOH, followed by rinsing with water for 10 min, then finally the desired buffer solution for 10 min at 20 psi. Between runs, the capillary was washed with the running buffer for 2 min. Samples were injected by applying pressure of 0.5 psi for 5 s. All the data are given as average values of triplicate CE runs.

RESULTS AND DISCUSSION

Selection of the Selectors

A variety of CDs and their derivatives were used to study the selection of CDs on the separation of fluoxetine enantiomers. Figure 2 shows the typical electropherograms of fluoxetine using some of the CDs at pH 2.5, where fluoxetine is positively charged. In Table 1, the best peak resolution (R_s) values

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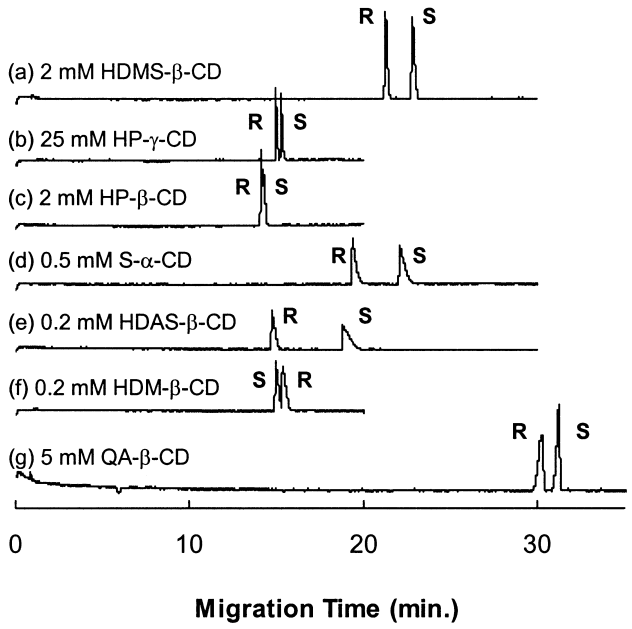

Figure 2. Typical electropherograms of fluoxetine enantiomers using some of the CD chiral selectors. pH 2.5; separation voltage: 20 kV; 50 μm i.d., 50 cm long capillary; detection wavelength: 214 nm. (a) 2 mM HDMS-*β*-CD, $R_s = 5.3$; (b) 25 mM HP-*γ*-CD, $R_s = 1.4$; (c) 2 mM HP-*β*-CD, $R_s = 0.52$; (d) 0.5 mM S-α-CD, $R_s = 6.1$; (e) 0.2 mM HDAS-*β*-CD, $R_s = 7.3$; (f) 0.2 mM HDM-*β*-CD, $R_s = 1.1$; (g) 5 mM QA-*β*-CD, $R_s = 2.3$.

obtained within 30 min of migration time for each selector, along with other data, are listed. From these results, it can be clearly seen that R_s is dependent on the type of the CDs. The negatively charged CDs (HDAS-*β*-CD, HDMS-*β*-CD, and S-α-CD) showed higher R_s than neutral or positively charged CDs. This can be explained by the enhanced binding between the negatively charged CD and cationic fluoxetine due to the electrostatic interaction. This may be one of the important interactions in chiral separation. However, sufficient separation of fluoxetine enantiomers was observed with the neutral or positively charged CDs as well. Thus, more subtle, but stereo–specific interactions such as hydrogen bonding would be the main force in chiral recognition. In the case of QA-*β*-CD, the inclusion of fluoxetine into a cavity of the CD carrying the same net positive charge may be thermodynamically favored. These results indicate that the charges on both the analyte and the selector play important

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Table 1. Migration time of first (t_1) and second (t_2) eluted fluoxetine isomers, resolution (R_s), separation factor (α) and first eluted isomer for each selector at pH 2.5.[a]

Selector	Conc. (mM)	t_1 (min)	t_2 (min)	R_s	α	First isomer
HDAS-β-CD	0.2	14.7	18.8	7.4	1.28	R
S-α-CD	0.5	19.3	22.1	6.1	1.14	R
HDMS-β-CD	2	21.3	22.9	5.3	1.07	R
HDM-β-CD	0.2	14.9	15.3	1.1	1.03	S
HP-β-CD	5	15.9	16.0	0.7	1.01	S
HP-γ-CD	25	15.0	15.3	1.4	1.02	R
QA-β-CD	2	21.0	21.3	1.8	1.02	R
β-CD[b]						
γ-CD[b]						
DM-β-CD[b]						
HP-α-CD[b]						

[a]The data in the table are for the best chiral separation obtained within 30 min of analysis time.
[b]No separation observed.

roles in chiral separation and the resolution is enhanced when the analyte and the selector have opposite charges, but this is not a requirement for chiral separation to be achieved. Another reason for the high R_s with the negatively charged CDs seems to be the high electrophoretic mobility of the CDs in the opposite direction to positively charged fluoxetine enantiomers, which may improve the separation factor α, independent of the selectivity of the fluoxetine/CD complexation itself.[29]

Concentration of the Selectors

In addition to the type of the selectors, their concentration also influences R_s as shown in Fig. 3 for some CDs. The resolution increased as the concentration of the CD increased up to a certain concentration, depending on the type of the CDs. The migration time also increased with the concentration of the CD. Since the enantiomers and the CD are in rapid equilibrium with the enantiomer/selector complex, the higher concentration of the CD would enhance the formation of the complex, which may have lower electrophoretic mobility than the free enantiomer. Consequently the analyte would be expected to remain longer in the capillary at higher concentration of the CD and, thus, improve the resolution. The lower mobility is more apparent when the negatively charged CDs are used, due to the opposite direction of the

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Figure 3. Dependence of R_s of fluoxetine on concentration of the selector at pH 2.5. (a): HDAS-β-CD, (b): HP-γ-CD, (c): QA-β-CD.

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electrophoretic mobility of the complex to the uncomplexed fluoxetine enantiomers, which are positively charged at pH 2.5. As a result, the resolution improvement was larger with the negatively charged CDs.

Effect of pH of the Background Electrolyte

The pH of the background electrolyte has a crucial effect on chiral separation in CE. The electrophoretic mobility of the analyte and the electroosmotic flow (EOF) are substantially pH dependent. In addition, the complexation of the analyte and the selector, as well as the stability of the complex, can be markedly affected by pH. To see the effect of pH on the chiral separation of fluoxetine, the separation was carried out at pH 9.0, and the results were compared with those previously taken at pH 2.5 in Table 2. The concentrations of the CDs were chosen such that a direct comparison of the results was possible. The R_s values were smaller at pH 9.0 than at pH 2.5 for neutral and negatively charged CDs, where other conditions were kept constant. R_s decrease is more remarkable for the negatively charged CDs. One of the reasons for the smaller R_s for the neutral and negatively charged CDs at higher pH may be due to the larger EOF, which carries the entire solution in the capillary to the outlet. Therefore, the resident time of the complex in the capillary becomes shorter at higher pH, leading to the smaller R_s. In contrast, for the positively charged QA-β-CD, R_s was larger at pH 9.0. This is probably due to the adsorption of QA-β-CD on the capillary wall, which is negatively charged at pH 9.0. The adsorption of the CD decreases EOF and, thus, increases the resident time of the analyte, which may cause the better resolution. Another contributor to the resolution may be the charge status of fluoxetine at pH 9.0. Taking into account the fact that the pK_a of fluoxetine is

Table 2. Chiral separation of fluoxetine at pH 2.5 and 9.0.

Selector/ conc. (mM)	pH 2.5				pH 9.0			
	t_1 (min)	t_2 (min)	R_s	α	t_1 (min)	t_2 (min)	R_s	α
HDAS-β-CD (0.1)	12.4	14.0	4.4	1.13	4.1	4.2	1.1	1.02
S-α-CD (0.5)	19.3	22.1	6.1	1.14	4.5	4.6	1.4	1.02
HDMS-β-CD (1)	15.6	16.1	2.9	1.04	4.2	4.3	0.3	1.01
HDM-β-CD (0.1)	13.3	13.6	0.7	1.03	4.2	4.2	0.1	1.01
HP-β-CD (1)	12.7	12.9	0.5	1.01	4.3	4.3	0	1
HP-γ-CD (10)	12.3	12.5	1.1	1.01	4.4	4.5	1.0	1.01
QA-β-CD (0.5)	15.8	15.9	0.2	1.01	78.2	82.1	2.7	1.05

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9.9, fluoxetine is less positively charged at pH 9.0 than at pH 2.5. Thus, the electrostatic interaction between the fluoxetine enantiomers and the negatively charged CD would be weaker at pH 9.0 compared to that at pH 2.5. In contrast, the smaller net positive charge on the analyte should be more favorable for the positively charged CD due to less electrorepulsion.

At pH 9.0, the R_s values improved as the concentrations of the negatively charged CDs = increased (for example, $R_s = 18.0$ and 10.1 for 10 mM HDAS-β-CD and S-α-CD, respectively); however, other CDs did not show significant improvement of R_s at higher concentrations at this pH. These results suggest that the sufficient resolution of fluoxetine enantiomers can be achieved both at pH 2.5 and 9.0 with the negatively charged CDs, although higher concentrations of CDs are required at pH 9.0.

Limit of Detection and Limit of Quantitation

For the analytical control of enantiomeric impurities in drug products in the course of quality control, the analytical method employed should enable accurate determination of enantiomeric impurities in the products with high enantiomeric excess (e.g., $\geq 99.8\%$).[30] In CE, a limit of quantitation (LOQ) of an enantiomeric impurity is not always small enough. This is mainly due to the fact that since UV detection in CE is not very sensitive, a large amount of sample injection is necessary to detect a trace amount of impurity, which causes the peak overlap if R_s is not large enough. Therefore, large R_s values are required for chiral purity control in CE. In the case of fluoxetine, the use of the negatively charged CDs would be a good choice to offer a large R_s value. Figure 4 shows determination of 0.2% *R*-fluoxetine, which is known to have a less potent bioactivity than *S*-fluoxetine. Using 3 mM HDMS-β-CD, it was possible to determine 0.2% of *R*-fluoxetine [relative standard deviation (RSD) = 4%, $n = 5$] without the hindrance from the peak of *S*-fluoxetine. *R*-fluoxetine was detectable to the levels below 0.1%, although RSD became larger ($\sim$10%). This result clearly shows the usefulness of the negatively charged CDs for the chiral purity control of *S*-fluoxetine.

Effects of the Substitution of Amine Moiety

Chiral separation of fluoxetine analogs that have substitutions on the amine moiety (norfluoxetine, LY270513, LY280494, and LY507311) was also conducted to study the effects of substituted groups at this position on chiral recognition. The electropherograms of these compounds obtained with 2 mM HDMS-β-CD at pH 2.5 are shown in Fig. 5. At this pH, all of the five compounds have the net positive charge. The results showed no significant

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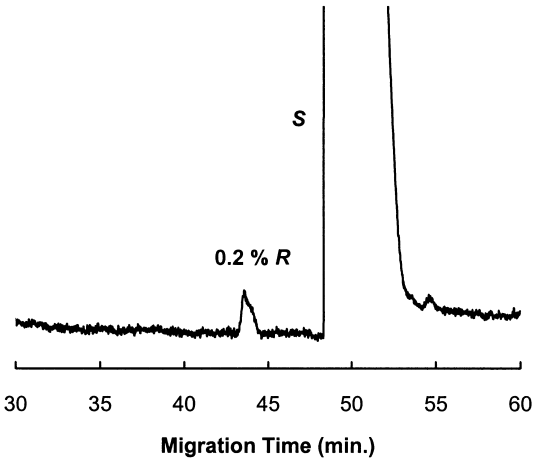

Figure 4. Determination of 0.2% *R*-fluoxetine (chiral impurity). 3 mM HDMS-*β*-CD, pH 2.5. Other experimental conditions are same as in Fig. 2.

difference in chiral recognition of fluoxetine and the analogs, except for LY507311, which gave a longer migration time and smaller R_s value. Similar results were found with other neutral and negatively charged selectors. The longer migration time of LY507311 may indicate a stronger interaction of this pair of enantiomers with the CDs. LY507311 has a carboxyl group that may be able to form an extra hydrogen bond with the selector, which, in turn, may enhance the interaction between LY507311 and the CD. However, as shown in smaller R_s values with neutral and negatively charged CDs, this hydrogen bond may not be chiral specific but have the same effect on both enantiomers and/or may interfere with the chiral recognition occurring at other portions of fluoxetine. The positively charged CD also gave longer migration time for LY507311, but larger R_s (see Table 3). With the positively charged CD, the extra hydrogen bond may have a positive effect on chiral recognition.

These results indicate that simple alkyl substituents on the amine moiety of fluoxetine do not show a significant contribution to chiral recognition under these experimental conditions; however, functional groups such as carboxyl seem to show a much more pronounced effect.

Effects of the Charge

As suggested in the previous sections, the charge status of both the analyte and the selector seems to have a significant influence on chiral separation. In order

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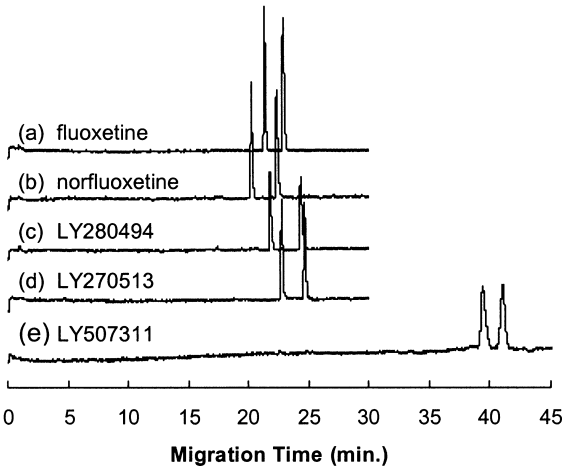

Figure 5. Electropherograms of enantiomers of fluoxetine and its analogs with 2 mM HDMS-β-CD at pH 2.5. Other experimental conditions are same as in Fig. 2. (a) fluoxetine: $R_s = 5.3$, (b) norfluoxetine: $R_s = 7.2$, (c) LY280494: $R_s = 8.8$, (d) LY270513: $R_s = 6.7$, and (e) LY507311: $R_s = 2.3$.

to study this effect in more detail, a series of experiments was carried out at different pHs (pH 2.5, 5.0, 9.0, 11.0) using fluoxetine and two analogs (LY280494 and LY507311) and the charged CDs. These analytes may have different charge status depending on pH. Fluoxetine is positively charged at a pH lower than its pK_a, 9.98 and neutral at a higher pH; LY507311 is positively charged at a lower pH, zwitter ionic around the neutral pH, and negatively charged at a higher pH; LY280494 has a positive charge through the entire pH range.

Table 3. Chiral separation of fluoxetine and the analogs at pH 2.5.

	2 mM HDMS-β-CD (negative)		0.2 mM HDM-β-CD (neutral)		5 mM QA-β-CD (positive)	
Analyte	t_1 (min)	R_s	t_1 (min)	R_s	t_1 (min)	R_s
Fluoxetine	21.3	5.3	14.9	1.1	30.2	2.4
Norfluoxetine	20.2	7.2	13.2	1.0	28.7	2.1
LY270513	22.8	6.7	13.8	0.7	41.5	3.1
LY280494	21.8	8.8	15.5	1.0	31.7	2.8
LY507311	38.8	2.3	27.8	0	78.1	14.0

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Figure 6 shows the electropherograms with 10 mM S-α-CD taken at pH 9.0. The separation data are given in Table 4. At pH 9.0, the separation behavior of the analogs was different from that observed at pH 2.5. At pH 9.0, LY507311 had a slightly shorter migration time than fluoxetine with the negatively charged CDs and the longer migration time with the positively charged CD. In this case, the carboxyl group on LY507311 is deprotonated to the carboxylate anion, causing a repulsion with the negatively charged CDs and an attraction to the positively charged CD. Thus, compared to fluoxetine, LY507311 may have the weaker interaction with negatively charged CDs (shorter migration time) and the stronger interaction with the positively charged CD (longer migration time). The shorter migration time would give the poorer chiral separation, and the longer migration time would give the better chiral separation.

The separation behavior of LY280494 was also very similar to that of fluoxetine at pH 2.5, but quite different at pH 9.0. The longest migration time and largest R_s of three compounds were seen with the negatively charged CDs and the shortest migration time and smallest R_s with the positively charged CD. In contrast to the other compounds, LY280494 is fully positively charged at pH 9.0, yielding the stronger ionic interaction with the negatively charged CDs (longer migration time and larger R_s) and the weaker interaction with the positively charged CD (shorter migration time and smaller R_s).

Figures 7 (a) and (b) show the plots of R_s value vs. pH for each analyte with 1 mM HDMS-β-CD and 0.5 mM QA-β-CD, respectively. As can be seen in (a), R_s decreased as pH increased for all three compounds. In fact, the resolution of LY507311 was completely lost at a pH higher than 5.0 where

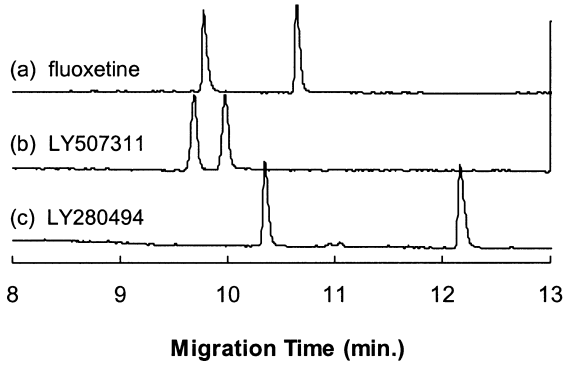

Figure 6. Electropherograms of enantiomers of fluoxetine and its analogs with 10 mM S-α-CD at pH 9.0. Other experimental conditions are same as in Fig. 2. (a) fluoxetine: $R_s = 10.1$, (b) LY507311: $R_s = 2.7$, and (c) LY280494: $R_s = 18.6$.

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Table 4. Chiral separation of fluoxetine and the analogs at pH 9.0.

	10 mM S-α-CD (negative)		0.5 mM QA-β-CD (positive)	
Analyte	t_1 (min)	R_s	t_1 (min)	R_s
Fluoxetine	9.8	10.1	78.2	2.7
LY280494	10.3	18.6	59.1	0
LY507311	9.7	2.7	92.3	23.3

the carboxyl group may exist as the carboxylate anion, which could diminish the interaction with the negatively charged HDMS-β-CD. Although the resolution of LY280494 enantiomers also decreased at higher pH mainly due to the faster EOF, it still showed significant chiral separation even at pH 11.0. Fluoxetine showed intermediate R_s values of these two and lost the resolution at pH 11.0 where it exists as neutral. The opposite phenomena were observed in (b). No significant separation of LY280494 enantiomers was observed with the positively charged QA-β-CD in the entire pH range. In contrast, LY507311 was well separated, especially at a higher pH, indicating the ionic interaction between the negatively charged LY507311 and the positively charged QA-β-CD is one of the important factors in chiral

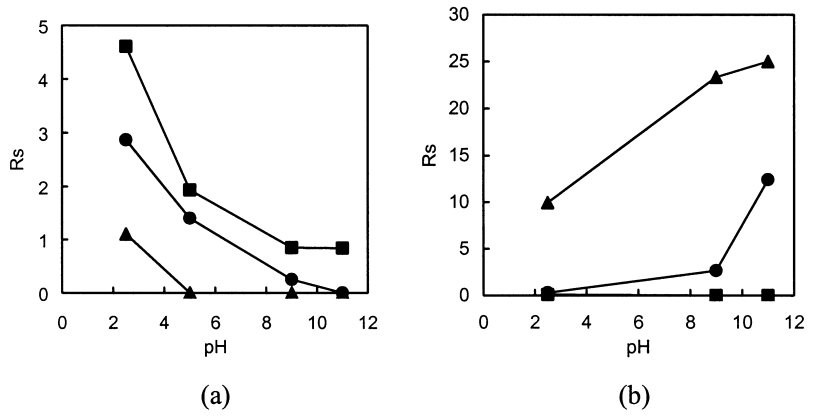

Figure 7. Dependence of R_s on pH. (a) 1 mM HDMS-β-CD and (b) 0.5 mM QA-β-CD were used as chiral selectors. ●: fluoxetine, ▲: LY507311, ■: LY280494. R_s value of LY507311 at pHs 9.0 and 11.0 in (a) was obtained with the reverse polarity (no peaks observed with the normal polarity within 2 hours).

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separation. Fluoxetine showed smaller R_s values at a pH lower than 9 where it is positively charged. However, the resolution was increased at pH 11.0 where it exists as neutral, indicating that chiral recognition can occur without ionic interactions and that additional forces are involved in chiral recognition. From these results, it can be concluded that the ionic interaction between an analyte and a chiral selector is one of the major contributions to chiral separation; although the results also suggested other interactions are necessary as well.

CONCLUSIONS

Chiral separation of fluoxetine by CE was conducted using 11 different CDs as chiral selectors. The negatively charged CDs showed excellent resolution abilities with low concentrations at pH 2.5 due to the high electrophoretic mobility of these CDs in the opposite direction to fluoxetine, as well as due to the enhanced binding with positively charged fluoxetine. An increase of pH from 2.5 to 9.0 reduced the chiral resolution of fluoxetine when the other experimental conditions were kept constant. The faster EOF and the weaker ionic interaction with less positively charged fluoxetine at pH 9.0 would be two main reasons for the poorer resolution. The resolution improved as the concentration of the chiral selector increased at both pH 2.5 and 9.0 in compensation for the longer migration time. The optimized experimental conditions could be applied to the determination of the chiral impurity (*R*-fluoxetine) with the LOQ of 0.2%.

The analyses of the fluoxetine analogs showed that the alkyl substituents on the amine moiety had little effect on chiral recognition. The addition of a carboxyl group on the amine moiety altered the separation behavior depending on the type of CDs and pH. This functional group has ability to hydrogen bond and carries the negative charge at pH higher than 5, which greatly affected the chiral recognition mechanism, especially when the charged CDs were used.

In order to investigate the intermolecular interactions between the analyte and the selector more precisely, NMR and MS studies are currently in progress. In addition, chiral separation of fluoxetine analogs with different substituents at different positions are also under study to obtain more information about the correlation between the chemical structure and chiral recognition.

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