Chiral Separation of (±)-Higenamine by Capillary Electophoresis

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ABSTRACT

Higenamine [1-(4-hydroxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) is a cardiotonic constituent of Aconiti tuber, one of the most widely prescribed oriental medicines. *S*-(-)-higenamine was reported to have a stronger cardiotonic activity than *R*-(+)-higenamine and known as a central intermediate in the biosynthesis of various benzyl isoquionoline alkaloids in plants. The separation of higenamine enantiomers has been accomplished with capillary electrophoresis using cyclodextrins (CDs) as chiral selectors. Good resolution of this enantiomers was obtained using a 50 mM sodium phosphate buffer containing hydroxypropyl β-CDs using 27 cm fused silica capillary (50㎛ i.d., 20 cm to detector) at 25 ℃. With the electric field of 340 V/cm, the separation time of higenamine enantiomers was less than 6 min. Under this optimum conditions, the relative standard deviations of migration time and peak area were less than 1.6% and 3.2%. A 512-channel diode array detector was confirmed for the higenamine. The detection limits (*S/N* = 3) of these enantiomers are 1.5㎍/mL. We confirmed the chiral form of higenamine in medicinal plants.

Key words : Capillary electrophoresis, *Higenamine enantiomers*, cyclodextrin

INTRODUCTION

In the pharmaceutical industry, approximately 50% of pharmaceutical compounds are chiral, while only about 20% of the formulations use just one enantiomeric form of the drug (Roth et al, 1982). Different enantiomers often have different biological and pharmacological properties. Consequently, great differences in pharmacological behavior have been observed for the optical antipodes of drugs. This can be explained by the fact that the chiral compounds's interactions with receptors, plasma proteins, metabolizing enzymes, and carriers, for example, take place in an asymmertical environment, which results in different binding affinities for different enantiomers. Separation of enantiomeric forms drugs has become an increasingly important and challenging task in pharmaceutical analysis and new drug investigation (Stouter, 1985, Maris et al, 1991). The separation of racemates into enantiomers is also an essential procedure in the synthetic chemistry of pharmaceutical. There are some methods available to separate enantiomers, such as liquid chromatography(Venema et al, 1991), capillary gas chromatography and

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supercritical fluid chromatography (Konig, 1987, Peter et al, 1991). However, direct separation of enantiomers using capillary electrphoresis (CE) has been a fast developing field in recent years due to its high efficiency, short analysis time, and small sample requirement(Jorgenson, 1983).

(±)-Higenamine [1-(4-hydroxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline)] is a cardiotonic constituent of Aconiti tuber, one of the most widely prescribed oriental medicines. The positive inotropic effect of higenamine was found to be potentiated by extracelluar calcium suggesting that higenamine may accelerate calcium influx through the sarcoplasma which gives rise to an increasing intracellular calcium concentration, thus resulting in the positive inotropic action. From the plant, Kosuge et al.(1976) isolated higenamine as hydrochloride salt and identified the structure [1-(4-hydroxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline)], and its total synthesis was reported starting either from vanillin (4-hydroxy-3-methoxy-dimethoxyphenyl-ethylamine). *S*-(-)-higenamine was reported to have a stronger cardiotonic activity than *R*-(+)-higenamine(Kosuge et al, 1976). (*S*)-higenamine is known a very important intermediate in the biosynthesis of benzylisoquinoline alkaloids such as morphines, protoberberins and aporphine etc. in plant(Stadler et al, 1988, Stadler et al, 1989). These enantiomers have the strong possibility of its different role and effect in biological system(Koshiyama et al, 1970, Kosuge et al, 1976). Various methods such as HPLC(Park et al, 1987) and gas chromatography (GC)(Ryu et al, 1993) have been applied for the analysis of the higenamine enantiomers. The HPLC technique, however, usually takes a lot of run time and required a special expensive column for the analysis of these enantiomers and this chiral column has the limitation of usability. The technique based on N-methylbis-trifluoroacetamide has been introduced into the GC-MS spectrometry for higenamine enantiomeric separation in biofluid samples. This technique also has some disadvantages in regards to the cumbersome pretreatment procedure and their stability of the derivatized analytes. This technique also has some disadvantages in regards to the cumbersome pretreatment procedure and their stability of the derivatized analytes.

We reported on the use of capillary zone electrophoresis with cyclodextrins (CDs) added as a chiral selector combined with diode array detection for separation and identification of higenamine enantiomers. In addition, we examined the optimization of CD type, concentration, and buffer pH and identified the optical states of higenamine in plant samples.

MATERIALS AND METHODS

Chemicals

(±)-Higenamine synthesized according to Prof. Chang's reported method (Chang et al, 1984) and (R)-(+)- form higenamine standard were obtained from Natural Products Research Institute (Seoul National University, Seoul, Korea). This compound was identified by GC/Mass. Sodium phosphate and various CDs were purchased from Sigma (St. Louis, MO, USA). Distilled water was further treated by a ion exchange exchanger NANOpure II purification system (Barnsted, USA).

Stock solutions of 1,000㎍/mL higemamine enantiomer mixture in water were newly prepared every week. The 10㎍/mL solutions were prepared by diluting the stock solutions with water.

Sample preparation

We purchased the medicinal plant samples from Kyungdong-Market (Seoul, Korea). Ten gram of dried sample was extracted with 100 mL of methanol for 3 hr (three times at room temperature) on a reflux. This extract was filtered, and the filtrate was evaporated to

dryness under reduced pressure at 50℃. This filtrate was partitioned with chloroform and H_2O (1:1 v/v) and then the H_2O fraction was freeze-dried. After transferring the H_2O extract to a clear vial, a the running buffer were added to the extract. This solution was passed through a 0.45㎛ membrane filter and the filtrate was directly injected into the CE system.

Instrumentation and running conditions

We carried out the CE by using a P/ACE 5500 CE system (Beckman Inc., Fullerton, CA, USA) with a 512 channel diode array detector, an automatic injector, a fluid cooled column cartridge. The fused silica capillaries (20 cm to detector, 50 (m i.d.) was used at 25 ℃. The applied voltage was 5~20 kV, well within the linear region of the Ohm's plot. The pH of run buffer, an aqueous 50 mM sodium phosphate solution, was adjusted to be 2.5 ~ 4.5 with phosphoric acid. Samples were introduced by pressure upon applying 3.45×10^{-3} M Pa nitrogen for 1 s. All run buffers were filtered throughout a 0.45㎛ membrane filter prior to use. Every new fused silica capillary was flushed with the 100 mM NaOH for 1 h, the 0.1 M HCl for 30 min, the 100 mM phosphate (pH 2.5) for 10 min, and the run buffer for 10 min, successively. Resolution of selected ions was calculated by using the equation where t is the migration time of the enantiomers and w is the peak width(Nielen 1993).

$$R = 2 [(t_2 - t_1) / (w_2 + w_1)]$$

RESULT AND DISCUSSION

The CDs have been used extensively to effect chiral separations in CE(Terabe et al, 1985; Guttman et al, 1988; Fanali, 1989; Snopek et al, 1991). A variety of different CDs (α-, β-, γ-) and modified CDs are available for these experiments. Analyte molecules that have large hydrophobic groups and appropriate shapes and sizes can form inclusion complexes with CDs. Because inclusion complex formation is a spatial interaction, there are substantial differences in the strengths for the inclusion complexes formed from an enantiomeric pair of guests. The separation of enantiomers is based on differences in the electrophoretic mobility between the CD-enanatiomer complexes and the free molecules uncomplexed.

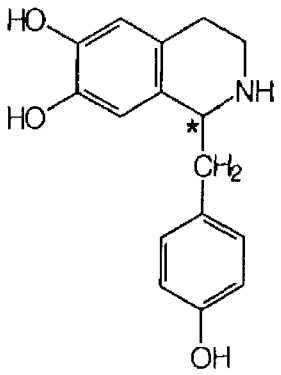

Fig. 1. Structures of higenamine enantiomers.

The structures of the higenamine enantiomers are shown in Fig. 1. The compound has one chiral center and, thus two enantiomeric forms. The large hydrophobic groups and the polar methoxy or hydroxy groups make these compounds relatively straightforward to separate by complexation with CDs in CE. Capillary zone electrophoresis without CDs of higenamine enantiomer results in a single peak.

Effect of cyclodextrin type and concentration on chiral separation

The separation of higenamine enantimers, the five most commonly used native and derivatized CDs (α-, β-, γ-, dimethyl-β-, hydroxypropyl β-) were tested as chiral selectors. Since the amount of chiral selcetor is a critical parameter in the optimization of an enantio-separation (Stephen et al, 1992; Stephen, 1993]. We studied the influence of its concentration on the resolution at various CDs. The effects of the choice of

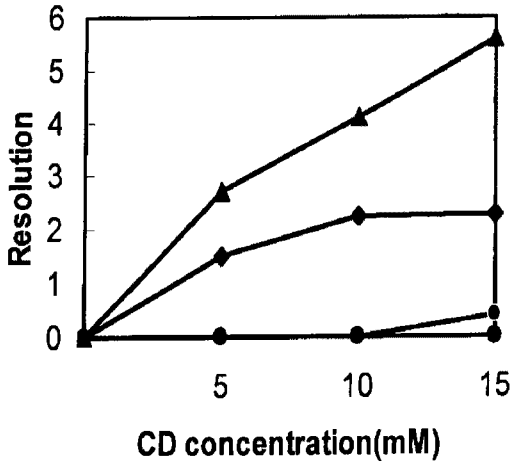

Fig. 2. Effect of cyclodextrin type and concentration on separation resolution for higenamine enantiomers: α-, γ-CD; : β-CD; : DMe-α-β-CD; : HP-β-CD. CE condition: Run buffer, 50 mM sodium phosphate (pH 3.0); capillary, 27cm × 50㎛ i.d. (20 cm to detector); hydrodynamic injection for 2 s at 3.45 × 10-3 M Pa; detection, 200 nm; applied voltage, 15 kV, sample concentration, 15 (g/mL).

CD and concentration on the separation higenamine enantiomers are summarized in Fig. 2. Experiments performed by adding different amount of α-, β-, γ-CD, and dimethyl-β-CD (DMe-β-CD), hydroxypropyl β-CD (HP-β-CD) result in different resolution of the higenamine enantiomers. When using α- and γ-CD, no resolution of recemic higenamine is obtained. Apparently, the size of the α-CD cavity is too small to form inclusion complexes with higenamine. Resolution of enantiomers of higenamine is improved at high concentration (up to 15 mM) of β-CD and modified β-CD. However, β-CD provided the poor resolution value (R = 0.4). When the concentration of DMe-β-CD or HP-β-CD consistently provides the best separation. When the concentration of DMe-β-CD is raised to 15 mM, the resolution higenamine enantiomers decreases gradually. This might be due to a shift in the equilibrium toward the higename-DMe-β-CD complex at higher DMe-β-CD concentrations, leading to an increased apparent capacity factor and a concomitant decrease in the resolution.

Effect of pH on migration time and chiral resolution

The effects of buffer pH on the migration time and resolution of higenamine enantiomers are summarized in Fig. 3. The electrophoretic mobility of higenamine and the complex formation constant with β-CD should be highly dependent on pH. An increased migration time is observed at low pH values. Interestingly, resolution of the enantiomers of higenamine is poor at high pH values. This can be explained by considering the effect of electroosmotic flow. The electoosmotic flow rate is a function of the pH. Generally, it is increased as the pH is increased (Lukacs et al, 1985; Lambert et al, 1990)

At low pH, electroosmotic flow is negligible and the separation mechanism is controlled only by the electrophoretic mobility of the higenamine enantiomer and cyclodextrin complexes. At higher pH values, the electroosmotic flow rate becomes more significant relative to electrophoretic mobility and overall migration time decreases. The shorter migration time does not permit adequate time for chiral resolutiom via differential electrophoretic mobility. CDs are generally used over a range of pH values from 2 to 12(McLaughlin et al, 1992); hence, the optimum pH for

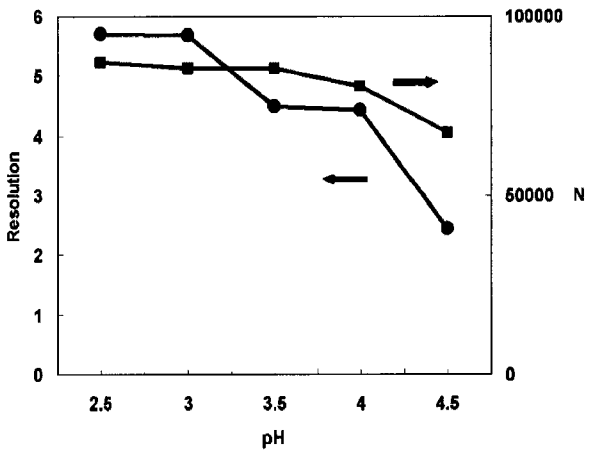

Fig. 3. Effect of pH on resolution and efficiency for separation of the higenamine Enantiomers. Run buffer, 50 mM sodium phosphate (pH 2.5 ~ 4.5) with 15 mM hydroxyprophyl β-CD. Other conditions as in Fig. 2.

separation selectivity of higenamine is around pH 2.5 with an obvious tradeoff with analysis time.

The effects of the applied voltage and injection time

The effects of the applied voltage on the separation efficiency of the higenamine enantiomers were thoroughly investigated. The increase of the electric field raises the temperature inside the capillary column (Janini et al, 1993). The temperature increase leads to the increase of the buffer conductivity and the solute diffusion coefficient, which is accompanied by the decrease of the buffer density and the viscosity. Attempts were made to optimize the separation conditions by using different applied voltages ranging 5~20 kV (data not shown). The values of theoretical plate number were then plotted against voltage for higenamine enantiomers. The result of this experiment clearly indicates that as the applied voltage increases up to 10 kV, the theoretical plate number increases, but decrease beyond 10 kV. For the 27cm × 50㎛ i.d. (20cm to detector) capillary, the optimal applied electric field was found to be 370 V/cm. In CE, resolution of the separation is dependent on the injection volume(Alvin et al, 1993, Terabe et al, 1994). We found the optimum injection volume was 2.5 nL, which was introduced for 1 s under pressure of 3.45×10^{-3}M Pa nitrogen. The optimum conditions which offered the best compromise in terms of resolution and peak efficiency was judged to be 50 mM sodium phosphate with 15 mM HP-β-CD and an injection time of 1 s and applied voltage of 10 kV. Fig. 4 shows the electropherogram of a racemic mixture under optimum conditions. Under these CE conditions, the higenamine enantiomers could be completely determined within 15 min with high resolution and high efficiency (N = 80,000 ~ 100,000).

Reproducibility, linearity and detection limit

The assay was evaluated in terms of reproducibility of the migraiton times, quantitation and efficiency of the electrophoretic peaks, and of the resolution by analyzing the enantiomers. We employed the set of conditions optimized using HP-β-CD as chiral selector; the results are summarized in Table 1. The assay features excellent reproducibility (<1.6%) of the migration times as well as good data for a quantitative determination of the relative amount of individual stereoisomers. Peak efficiency was 100,000 theoretical plates/m for both peaks. Also Note that the excellent linear responses were obtained in the sample

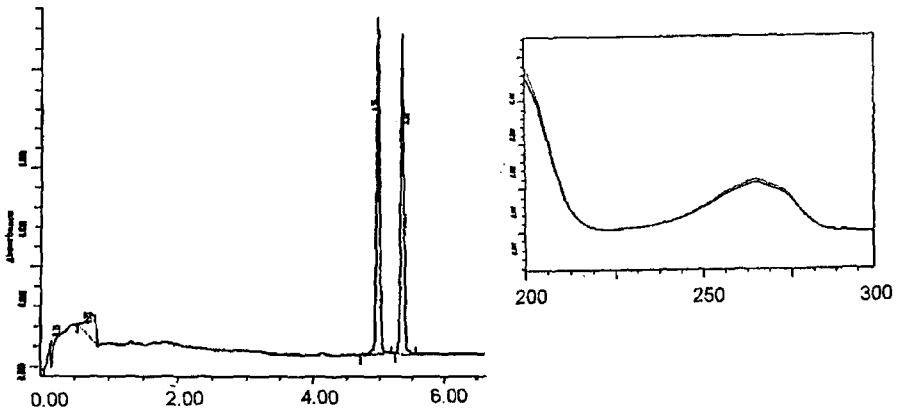

Fig. 4. Chiral separation of higenamine. CE condition: Run buffer, 50 mM sodium phosphate (pH 2.5) with 15 mM hydroxy propyl β-CD; capillary, 27cm × 50㎛ i.d. (20 cm to detector); hydrodynamic injection for 1 s at 3.45 × 10-3 M Pa; detection, 200 nm; applied voltage, 10 kV.

Table 1. Reproducibility and detection limit of (±)higenamine.

	Migration time (min)			Peak area	
Mean	S.D.	RSD(%)	Mean	S.D.	RSD(%)
4.96	0.08	1.61	184860	5180	2.80
5.33	0.06	1.13	188061	6018	3.20

S.D.: standard deviation

RSD (%): relative standard deviation (n=5)

concentration range of 5 ~ 400μg/mL, with the linear correlation coefficient ranging from 0.9956 to 0.9998. The detection limits (S/N = 3) of these enantiomers are 1.5μg/mL.

Applications

We applied the new CE method to the analysis of higenamine in medicinal plants (Aconitcum Japonicum and Nelumbo nucifera-embro) and confirmed (*R*)-(+)- and (*S*)-(-)-higenamine in sample by UV spectrum and spiking the sample with standard. The sample analyzed within 10 min as shown in Fig. 5 and 6, and the migration time of higenamine was 5.01 min. Although the sample contained unknown components, the

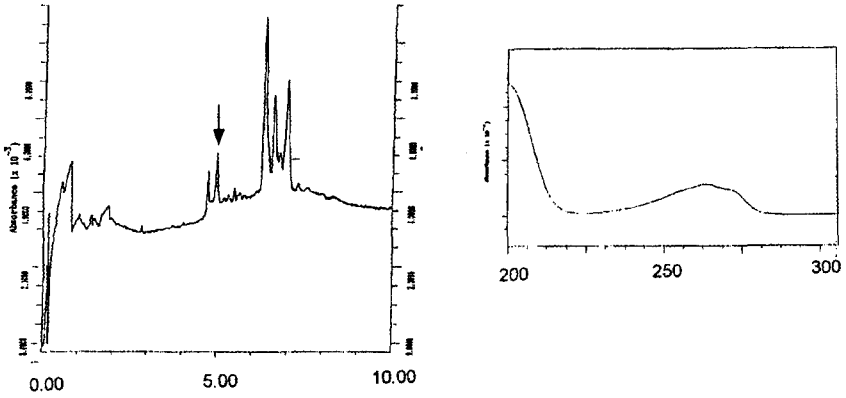

Fig. 5. Electropherogram of *Aconitcum Japonicum* analysis. CE conditions as in Fig. 4.

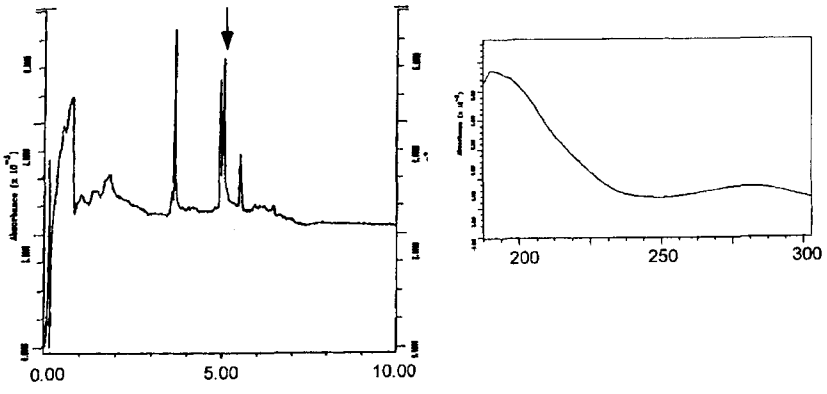

Fig. 6. Electropherogram of *Nelumbo nucifera* (embryo) analysis. CE conditions as in Fig. 4.

separation of (*R*)-(+)-higenamine showed a baseline resolution in appropriate CE conditions, so I can easily determine in the natural product. This result was in accord with Koshiyama's (1970) report that the (*R*)-(+)-higenamne was isolated from *Nelumbo nucifera*, and racemic occurs in *Aconitum japonicum* Thunb. These data mean that the determinatin of higenamine enantiomer in medicinal plant extracts, its standardization and quality control in pharmaceutical plants or bulky samples are possible by the CE method.

CONCLUSION

Chiral separation of higenamine has been achieved by capillary electrophoresis with added cyclodextrins. Diode array detection has been used to confirm the enantiomers of higenamine and impurities in the samples. In addition, the effect of pH, CD types and concentration; on the separation scheme have been examined. The best observed resolution was 5.17 using pH 2.5 phosphate buffer with 15 mM hydroxy prophyl-β-CD as a chiral selector. We recognized (*R*)-(+)-form of higenamine in medicinal plant by new CE method

LITERATURE CITED

Alvin, M., Grossman, P.D. and Moring, S.E. 1993. Anal. Chem. 65:489A.

Chang, K.C., Yun-Choi, H.S., Lim, J.K. and Park, C.W. 1984. Arch. Pharm. Res. 7:133~136.

Guttman, A., Paulus, A., Cohen, A. S., Grinberg, N. and Karger, B. L. 1988. Use of complexing agents for selective separation in high-performance capillary electrophoresis : Chiral resolution via cyclodextrins in incorporated within polyacrylamide gel columns. J. Chromatogr.448: 41~53.

Fanali, S. 1989. Separation of optical isomers by capillary zone electrophoresis based on host-guest complexation with cyclodextrins. J. Chromatogr. 474:441~446.

Janini, G.M. and Issaq, H.J. 1993. Capillary electrophoresis technology, New York; Marcel Dekker. p. 138.

Jorgenson, J.W. and Lukacs, K.D. 1983. Capillary zone electrophoresis. Science. 222(4621):266~272.

Konig, W. A. 1987. The practice of enantiomer separation by capillary gas chromatography, Heidelberg: Huthig Verlag.

Koshiyama, H., Ohkuma, H., Kawaguchi, H., Sur, H.Y. and Chen, Y.P. 1970. Chem. Pharm. Bull. 18:2564.

Kosuge, T.and Yokota, M. 1976. Chem. Pharn. Bull. 24:176.

Lukacs, K.D. and Jorgenson, J.W. 1985. Capillary zone electrohoresis: effect of physical parameters on separation efficiency and quantitation. J. High Res. Chromatogr. 8:407~411.

Lambert, W.J., Middleton, D.L. 1990. pH hysteresis effect with silica capillaries in capillary zone electrophoresis. Anal. Chem. 62:1585~1589.

Maris, F. A., Vervoort, R. J. M. and Hindriks, H. 1991. Applicability of new chiral stationary phases in the separation of racemic pharmaceutical compounds by high-performance liquid chromatography. J. Chromatogr. 547:45~78.

McLaughlin, G.M., Nolan, J. A. and Lindahl, J. L. 1992. Pharmaceutical drug separations by HPCE: practical guidelines. J. Liq. Chromatogr. 15: 961~1021.

Nielen, M.W.F. 1993. Anal. Chem. 65: 887~893.

Park, S. O., Hong, C. Y., Paik, S. W. and Yun-Choi, H. S. 1987. Arch. Pharm. Res. 10:60~66.

Peter, P., Markides, K.D., Johnson, D.F., Rossiter, B.E., Bradshaw, J.S. and Lee, M.L. 1991. J. Microcol. Sep. 4:155~62.

Roth, H. J. and Kleeman, A. 1982. Pharmazeutische chemie I,arzneistoffsynthese. Stuttgart: Thieme G.

Ryu, J.C., Song, Y.S., Kim, M.S., Cho, J.H. and Yun-

Choi, H.S. 1993. Arch. Pharn. Res. 16 (3): 213~218.

Snopek, J., Soini, H., Novotny, M., Smolkova-Keulemansova, E. and Jelinek, I. 1991. Selected applications of cyclodextrin selectors in capillary electrophoresis. J. Chromatogr. 559: 215~222.

Stadler, R., Loeffer, S., Cassels, B.C. and Zenk, M.H. 1988. Bisbenzylisoquinoline biosynthesis in Berberis stolonifera cell cultures. Phytochemistry. 27:2557~2565.

Stadler, R., Kutchan, T.M. and Zend, M.H. 1989. Phytochemistry. 28:1083~1086.

Stouter, R.W. 1985. Chromatogrphic separation of stereoisomers, Boca Raton: CRC Press.

Terabe, S., Ozaki, H., Otsuka, K. and Ando, T. 1985.Electrokinetic chromatography with 2-0-carbosymethyl-β-cyclodextrin as a moving stationary phase. J. Chromatogr. 322: 211~217.

Terabe, S., Spelsberg, T.C. and Landers, J.P. 1994. LC-GC.12:50.

Venema, A., Henderiks, H. and VanGeest, R.J. 1991. The enantioselectivity of modified cyclodextrins: studies on interaction mechanisms. J. High Res. Chromatogr. 14:676~80.

Stephen A.C. and Rowe, R.C. 1992. Theoretical aspects of chiral separation in capillary electrophoresis : I. Initial evaluation of a model. J. Chromatogr. 603:235~241.

Stephen A.C. 1993. Theory of chiral separation in capillary electrophoresis, J. Chromatogr. 636:57~62.

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